รายงานวิจัยฉบับสมบูรณ์

การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล

DEVELOPMENT OF MICRO-AND NANO-SCALE ANALYSIS BY FLOW-BASED TECHNIQUES

โดย ศาสตราจารย์ ดร. เกตุ กรุดพันธ์ และคณะ

สิงหาคม 2550

สัญญาเลขที่ RTA4780010

รายงานวิจัยฉบับสมบูรณ์

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คณะผู้วิจัย	สังกัด
1. ศ. ดร. เกตุ กรุดพันธ์	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
2. ดร. จรูญ จักร์มุณี	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
3. รศ. ดร. สุภาภรณ์ ครัดทัพ	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
4. ผศ. ดร. ดวงใจ นาคะปรีชา	คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
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6. ดร. รัตติกาล จันทิวาสน์	สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี
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8. รศ. ดร. ปรัชญา คงทวีเลิศ	คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
9. อ. ศุภชัย ชัยสวัสดิ์	คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
10. ผศ. ดร. สุนันทา วังกานต์	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
11. ดร. สมชัย ลาภอนันต์นพคุณ	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
12. ผศ. ดร. ศิริวรรณ องค์ไชย	คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
13. ดร. พลยุทธ ศุขสมิติ	สำนักงานอุตสาหกรรมพื้นฐานและการเหมืองแร่ เขต 3
14. อ. ดร. นิสา ชวพันธ์	คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
15. อ. ดร. วรรณจันทร์ แสงหิรัญ ลี	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
16. ดร. ปิยรัตน์ นิมมานพิภักดิ์	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
17. ผศ. ดร. จีรยุทธ ไชยจารุวณิช	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
18. ผศ. ดร. สุคนธ์ ประสิทธิ์วัฒนเสรี	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่สนับสนุนให้ทุน โครงการวิจัยเมธีวิจัยอาวุโส “การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล (Development of Micro-and Nano-scale Analysis by Flow-based Techniques)” โครงการวิจัยนี้สำเร็จลุล่วงไปด้วยการสนับสนุนและความร่วมมือจากหลายท่าน หลายหน่วยงาน อันได้แก่ สมาชิกเครือข่ายทั้งในและต่างประเทศทุกท่าน (อาจารย์ นักวิจัย และนักศึกษาที่มีส่วนร่วมอย่างมาก) ที่ทำให้เกิดผลงานวิจัยอันมีคุณภาพและคุณค่า ผู้ที่มีส่วนเสริมแต่เป็นกำลังสำคัญในการทำงาน (เลขานุการของกลุ่ม เจ้าหน้าที่ของ สกว. และ เจ้าหน้าที่ของสถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยีที่มีส่วนเกี่ยวข้องกับโครงการนี้)

ขอขอบคุณการสนับสนุนเพิ่มเติมโดยแหล่งทุนอื่นๆที่ช่วยเสริมการทำวิจัย ทำให้มีประสิทธิภาพดียิ่งขึ้น ได้แก่ The Royal Golden Jubilee (RGJ) Ph.D. Program (TRF), Center of Excellence for Innovation in Chemistry (PERCH-CIC), The Commission on Higher Education, The Alexander von Humboldt Foundation (AvH), Germany, Deutcher Akademischer Austausch Dienst (DAAD), Germany.

ขอขอบคุณผู้บริหารทุกท่าน ได้แก่ ผู้อำนวยการฝ่ายวิชาการ สกว. อธิการบดีมหาวิทยาลัยเชียงใหม่ ผู้อำนวยการสถาบันวิจัยวิทยาศาสตร์และเทคโนโลยี คณบดีคณะวิทยาศาสตร์ หัวหน้าภาควิชาเคมี ที่ให้การสนับสนุน

ขอขอบคุณทุกท่านที่มีส่วนร่วมในทางตรงและทางอ้อม แต่ไม่ได้เอ่ยนามในที่นี้

ในการพัฒนาซึ่งจะทำให้เกิดการเปลี่ยนแปลง มักจะมีขั้นตอนต่างๆ ซึ่งเป็นที่น่าสังเกตุเป็นไปตาม CLARK’S LAW OF REVOLUTIONARY IDEAS ซึ่งกล่าวดังนี้

Every revolutionary idea in Science, Politics, Art or Whatever evokes three stages of reaction. They may be summed up by the three phases:

1. “It is impossible, do not waste my time”
2. “It is possible, but it is not worth doing”
3. “I said it was a good idea all along”

ในฐานะหัวหน้าโครงการฯ มีความรู้สึกว่าโครงการฯ นี้ทำให้เกิดความภาคภูมิใจและคาดว่าผลลัพธ์ที่ได้รับจากโครงการฯ นี้ได้มีความคุ้มทุนจากเงินภาษีราษฎรซึ่งจะมีผลกระทบต่อประเทศในทางตรงและทางอ้อม

ศาสตราจารย์ ดร. เกตุ กรุดพันธ์
หัวหน้าโครงการ

บทคัดย่อ

การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล

โครงการเมธีวิจัยอาวุโส สกว. สัญญาเลขที่ RTA4780010

หัวหน้าโครงการ: ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

โครงการนี้ดำเนินการวิจัยต่อเนื่องจากโครงการเมธีวิจัยอาวุโส สกว. “การพัฒนาการวิเคราะห์ที่ใช้การไหล” ในการวิจัยเกี่ยวกับการพัฒนาเครื่องมือและวิธีการวิเคราะห์ทางเคมีที่ใช้เทคนิคการไหลสำหรับการวิเคราะห์ระดับไมโครและนาโน โดยเน้นการคำนึงถึงค่าใช้จ่ายที่ถูก แต่มีนวัตกรรม ประกอบด้วย 4 โครงการย่อย

โครงการย่อยที่ 1: Development of instrumentation ดำเนินการวิจัยอย่างต่อเนื่องในการพัฒนาระบบเครื่องมือต่าง ๆ รวมถึง micro-total analysis system (micro-TAS) เป็นการพัฒนาต่อเนื่องในเทคนิค Sequential Injection Analysis with Lab-at-Valve (SIA-LAV) ซึ่งเป็นการนำเสนอเทคนิคนี้ครั้งแรกโดยเป็นผลงานวิจัยจากโครงการนี้ นอกจากนี้ยังได้พัฒนาต่อเนื่องในระบบการวิเคราะห์ที่มีราคาถูก (cost-effective) และพัฒนาระบบตรวจวัดแบบต่าง ๆ รวมถึง spectrophotometric detection systems และ electrochemical detection systems.

โครงการย่อยที่ 2: Novel approaches for flow based analysis รวมถึงได้ดำเนินการวิจัยเพื่อลดขนาดระบบการวิเคราะห์โดยเน้น sequential injection analysis (SIA) และ flow injection analysis (FIA) รวมถึงแนวทางใหม่ในเทคนิค stopped flow injection systems (stopped FI) ได้พัฒนาระบบ Lab-on-Valve (LOV) systems และ Bead injection (BI) ได้เสนอแนวทางใหม่ที่เรียกว่า Lab-at-Valve (LAV) systems ซึ่งได้ทำการวิจัยพัฒนาควบคู่ไปกับการพัฒนาเครื่องมือ (ในโครงการย่อยที่ 1 ข้างต้น) ได้นำมาใช้ในการศึกษาทาง Chemical kinetics ด้วย ยังมีการศึกษาเกี่ยวกับการเตรียมตัวอย่างเพื่อใช้ใน Chromatography ด้วยนอกจากนี้ ยังได้ศึกษาแนวทางใหม่ทาง chemometrics ด้วย

โครงการย่อยที่ 3: New materials employed in chemical analysis ได้วิจัยเพื่อการเตรียมตัวอย่างโดยพัฒนาถึงการแยก (sample separation) โดยใช้ on-line mini-column separation โดยใช้ Packing material ชนิดต่าง ๆ รวมถึง monolithic column ด้วย ได้ทำการวิจัยต่อเนื่องเพื่อหา reagent ใหม่ ๆ เช่น สาร WF6 เพื่อการหาปริมาณ chondroitin sulfate proteoglycans ได้ศึกษาพัฒนาต่อเนื่องในการวิเคราะห์แอมโมเนีย/แอมโมเนียม โดยใช้ปฏิกิริยาใหม่แต่เป็นแนวทางของ Berthelot reaction

โครงการย่อยที่ 4: Application of flow-based analysis ได้ดำเนินการต่อเนื่องในการมุ่งเน้นเพื่อการวิเคราะห์ตัวอย่างทางการแพทย์โดยสนใจศึกษาในการทำคัดกรองของโรคบางชนิด เช่น

โรคมะเร็ง โรคตับ โรคข้อ เป็นต้น ทางสิ่งแวดล้อม ทางการเกษตรและอุตสาหกรรมการเกษตร การวิเคราะห์อาหาร

งานวิจัยดังกล่าวเป็นผลงานของนักวิจัยรุ่นเก่าและรุ่นใหม่ที่ร่วมกันดำเนินการโดยเป็นนักวิจัยมาจากสถาบันต่างๆ ทั้งในประเทศและต่างประเทศ ซึ่งทำให้เกิดความเข้มแข็งในระดับบัณฑิตศึกษาและสร้างการทำงานเป็นทีม

Keywords: Microaanalysis, Nanoanalysis, flow-based analysis, instrumentation, analytical procedure development

ABSTRACT

DEVELOPMENT OF MICRO-AND NANO-SCALE ANALYSIS BY FLOW-BASED TECHNIQUES

TRF SENIOR RESEARCH SCHOLAR GRANT CONTRACT NO. RTA4780010

Principal investigator: Professor Dr. Kate Grudpan

In this project a continued investigation from the previous TRF Seniors scholor Project on "Development of Flow-Based Analysis", instrumentation, chemistry and procedures involving flow-based techniques for micro-and nano-analysis, with emphasis on cost effective benefits but novel approaches have been investigated. It was composed of 4 subprojects.

Subproject 1: Development of instrumentation: Various instrument systems/ components have been continuously developed, including micro-total analysis system (micro-TAS). It has been continuously developed as Sequential Injection Analysis with lab-at-Valve (SIA-LAV) which has been introduced under this project for the first time. Also cost-effective analytical instrumentation and detection devices, including spectrophotometric, electrochemical, have been investigated.

Subproject 2: Novel approacheds for flow based analysis: Down-scaling analytical instrumentation with emphasis on novelty in sequential injection analysis (SIA) and flow injection analysis (FIA) has been explored. This included stopped flow injection systems (stopped FI), Lab-on-Valve (LOV) and Bead injection (BI). A novel approach so-called "lab-at-Valve (LAV)" system has been developed together with instrumentation development (in subproject 1). This has been employed for chemical kinetics study. Chemometrics has also been studied.

Subproject 3: New materials employed in chemical analysis: Sample preparation with on-line mini-column, employing some packing materials, including monolithic column has been investigated. New reagents have been explored for chemical analysis such as WF6 for the assay of chondroitin sulfate proteoglycans. Some Berthelot reactions were investigated for ammonia/ammonium determination.

Subproject 4: Application of flow-based analysis: Applications on flow-based techniques to medical/clinical analysis were aimed to deceases screening such as liver cancer, liver decease, bone & joint decease. Applications to environmental, agricultural, agroindustrial and food analysis have been studied.

The research works were conducted by various generations of researchers in partnership network of various institutions in Thailand and overseas.

Keywords: Microaanalysis, Nanoanalysis, flow-based analysis, instrumentation, analytical procedure development

Executive Summary

TRF Senior Research Scholar Grant (No. RTA4780010)

1. Title: การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล

Development of Micro-and Nano-scale Analysis by Flow-based Techniques

2. Principal investigator:

ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

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3. Research Field: Analytical Chemistry

5. Research period: 3 years (3 August 2004 - 2 August 2007)

6. Supports from other agencies:

1. The Royal Golden Jubilee (RGJ) Ph.D. Program, TRF
2. The Commission for Higher Education
3. Postgraduate Education and Research Program in Chemistry (PERCH)
4. The Alexander von Humboldt Foundation (AvH), Germany
5. Deutcher Akademischer Austausch Dienst (DAAD), Germany

7. Research Work

This project was conducted in continuation from the TRF senior Research Scholar Grant on “Development of Flow-Based Analysis” The research was aimed to develop novel approaches

on flow-based techniques for micro-and nano-analysis with emphasis on cost effective benefits. Both instrumentation and chemistry were investigated. The project consisted of 4 subprojects:

1. Development of instrumentation
2. Novel approaches for flow-based analysis
3. New materials employed in chemical analysis
4. Applications of flow-based analysis

8. Out-puts

The research work has resulted in 68 Publications in international journals (39 papers from this project and 29 papers from relevant works), 57 presentations in international conferences/meetings while 23 presentations in national meetings. It was the work by professors, researchers and students from institutions of various geographical parts of Thailand as well as from overseas: Chiang Mai University, Mahidol University, Chulalongkorn University, Khon Kean University, The Office of Fundamental Industry and Mining Region 3 in Thailand, and University of Washington, Seattle, (USA) (Professor Dr. Gary D. Christian, Professor Dr. Jaromir Ruzicka, Professor Dr. Robert E. Synovec), Monash University, Melbourne, (AUSTRALIA) (Dr. Ron Beckett, Assoc. Prof. Dr. Ian D. McKelvie) , Karlsruhe Research Centre, Karlsruhe, (GERMANY) (Professor Dr. Thomas Fanghaenel, Dr. Horst Geckeis), University of Plymouth, (ENGLAND) (Professor Dr. Paul J. Worsfold), Turku University, Okayama University, JAPAN (Professor Dr. Shoji Motomizu, President of the Japanese Association for Flow Injection Analysis), Aichi Institute of Technology, JAPAN (Professor Dr. Tadao Sakai), The University of Texas at Arlington, Arlington, Texas, USA (Professor Dr. Pernendu K. Dasgupta)

During the period of this project, continuous series of annual symposia has been organized since 2002 (the first year of TRF Senior Research Scholarship to the Group), in 2005 and 2006 The 4th and 5th Annual Symposia on TRF Senior Research Scholar on Development of Micro-and Nano-Scale Analysis by Flow-based Techniques I and II" were organized respectively. In 2007, the 6th Annual Symposium on TRF Senior Research Scholar and Research on Innovation on Analytical Instrumentation CHE: "Development of Flow-based Analysis for Life Quality" was organized. In the Symposia, internationally recognized scientists in the field, were invited to give planetary/ invited lectures. The Symposia activities were reported in international journals.

It was for the first time that US-NSF Co-funded with TRF and CHE for a workshop in Thailand "Interfaces of Analytical Sciences: Workshop of US and Thai Analytical Scientists", in January 2006. More than 10 top US analytical scientists attended the workshop, to exchange information and create and/or strengthen collaboration between US-Thai Scientists. The event was reported in Analytical Chemistry, the top in analytical chemistry international journals.

The above Symposia/workshop have created networking.

Members of the research team have received various awards nationally and internationally. The team has engaged in various national and international activities such as serving in committees, e.g. editorial advisory boards of international journals, organizing committees for international conferences.

การวิจัย “การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล”

1. ความสำคัญและที่มาของปัญหา

การวิเคราะห์ที่มีประสิทธิภาพแต่มีค่าใช้จ่ายไม่สูง เป็นสิ่งสำคัญที่ต้องคำนึงถึงเสมอ ไม่เฉพาะสำหรับในสถานที่ห่างไกลความเจริญซึ่งมีงบประมาณไม่มาก แต่รวมถึงการใช้งานทั่วๆ ไปด้วยในการเลือกรูปแบบการวิเคราะห์ นอกจากจะต้องคำนึงถึง ความถูกต้อง ความแม่นยำ ความไว และความจำเพาะ ซึ่งเป็นลักษณะทั่วไปที่ดีของเทคนิคการวิเคราะห์แล้ว ความสามารถในการวิเคราะห์แบบ real time และค่าใช้จ่ายของการวิเคราะห์ก็เป็นปัจจัยที่สำคัญด้วยเช่นกัน

เมื่อพิจารณาถึงค่าใช้จ่ายในการวิเคราะห์ จะหมายรวมถึงเวลาที่ต้องใช้ในการวิเคราะห์สารเคมีที่ต้องใช้ในปฏิกิริยา และราคาของเครื่องมือที่เกี่ยวข้อง ซึ่งราคาของเครื่องมือนี้คือราคาของระบบรวมถึงค่าใช้จ่ายในการทำงานของเครื่อง และค่าใช้จ่ายในการดูแลรักษาด้วย

เทคนิคการวิเคราะห์ที่ใช้การไหล (Flow-based analytical techniques) มีข้อดีหลายประการและข้อเด่นของเทคนิคนี้คือ ความสามารถในการวิเคราะห์สารได้หลายตัวอย่างในเวลาอันสั้น โดยใช้สารตัวอย่าง และสารเคมีปริมาณน้อย อีกทั้งใช้เครื่องมือที่ง่าย ไม่ซับซ้อน การวิเคราะห์ที่ใช้การไหลจึงเป็นเทคนิคการวิเคราะห์ที่เหมาะสมในการวิเคราะห์ ตามข้อที่ควรพิจารณาดังที่กล่าวถึงข้างต้น [2-28]

ได้มีการใช้เทคนิคโฟลอินเจคชันอะนาไลซิส (Flow injection analysis (FIA)) ในการวิเคราะห์แบบ routine แล้วมากมาย ซึ่งบ่งบอกถึงความคุ้มค่า และ ประสิทธิภาพของระบบนี้ได้เป็นอย่างดี

ระบบซีเควนเชียลอินเจคชันอะนาไลซิส (Sequential injection analysis (SIA)) เป็นระบบมีความเป็นอัตโนมัติ แต่ไม่ซับซ้อนมากนัก เมื่อเทียบกับเทคนิคอื่นๆ

มีความจำเป็นต้องพัฒนาระบบการวิเคราะห์ในระดับไมโคและนาโน เพื่อใช้ในการศึกษาด้านต่าง ๆ จึงสนใจที่จะทำการศึกษาต่อยอดงานที่ได้ทำมาโดยใช้ประสบการณ์ที่ได้รับโดยเฉพาะจากงานที่ทำในโครงการเมธีวิจัยอาวุโสที่ผ่านมา (เมธีวิจัยอาวุโส สกว.(ในโครงการที่ได้รับครั้งแรก), วุฒิเมธีวิจัย สกว., เมธีวิจัย สกว., ทุนวิจัยหลังปริญญาเอกและ ทุนปริญญาเอก คปก.)

โครงการนี้มีจุดมุ่งหมายที่จะพัฒนาการวิเคราะห์ในระดับไมโครและนาโน โดยการใช้พื้นฐานเทคนิคการวิเคราะห์ที่ใช้การไหล โดยจะทำการศึกษาทั้งในด้านเครื่องมือและด้านเคมี โครงการนี้ประกอบด้วยโครงการย่อย ดังนี้

1. Development of instrumentation
2. Novel approaches for flow-based analysis

3. New materials employed in chemical analysis
4. Applications of flow-based analysis

ทั้งนี้ ทั้ง 4 โครงการย่อยจะมีความสัมพันธ์ซึ่งกันและกันด้วย

2. การพัฒนาระบบเครื่องมือ (Development of instrumentation)

ได้พัฒนาระบบเครื่องมือที่เกี่ยวข้องกับ flow-based analysis หลายชนิด โดยมุ่งเน้นให้มีราคาไม่สูง (cost-effective)(ดังรายละเอียดใน reprint ในภาคผนวก ก1) ซึ่งรวมถึงการพัฒนาระบบ field-flow fractionation เพื่อการศึกษา size-based speciation (ดังรายละเอียดใน reprint ในภาคผนวก ก12 และ ก31) การพัฒนาระบบตรวจวัด (detection device/ system) ที่เป็น electrochemical detector (ดังรายละเอียดใน reprint ในภาคผนวก ก22, ก29, ก30, ก38) ระบบการตรวจวัดแบบ dynamic surface tension detector (ดังรายละเอียดใน reprint ในภาคผนวก ก8 และ ก26) การลดขนาดของระบบวิเคราะห์ (ดัง reprint ในภาคผนวก ก28)

3. การพัฒนาแนวใหม่ๆ ในการวิเคราะห์ที่อาศัยหลักการไหล (Novel approaches for flow-based analysis)

ได้พัฒนาระบบ bead injection เข้ากับ flow injection (ดังรายละเอียดใน reprint ในภาคผนวก ก2, ก11) การไตเตรดในระดับไมโครโดยใช้ Lab-on-Valve (ดังรายละเอียดใน reprint ในภาคผนวก ก 13) การเสนอเทคนิค Lab-at-Valve โดยใช้ poteutiometric detection (ดังรายละเอียดใน reprint ในภาคผนวก ก7) และใช้ spectrometry ในการตรวจวัด (ดังรายละเอียดใน reprint ในภาคผนวก ก17 และ ก27) ทั้งนี้ เทคนิค Lab-at-Valve(LAV) เป็นเทคนิคที่เสนอขึ้นโดยกลุ่มวิจัยนี้

ได้มีการพัฒนาระบบ Stopped flow injection analysis เพื่อใช้กับเทคนิค amperometry (ดังรายละเอียดใน reprint ในภาคผนวก ก16) ได้ศึกษาการทำ sample pretreatment ซึ่งเป็นแบบ on-line (ดังรายละเอียดใน reprint ในภาคผนวก ก23, ก25,ก31) ได้มีการพัฒนาการใช้คณิตศาสตร์และสถิติพร้อมด้วยโปรแกรมคอมพิวเตอร์อย่างง่าย (package program) เพื่อช่วยให้ประสิทธิภาพการวิเคราะห์ทางเคมีดีขึ้น (ดังรายละเอียดใน reprint ในภาคผนวก ก30)

4. วัสดุใหม่ๆ เพื่อใช้ในการวิเคราะห์ทางเคมี (New materials employed in chemical analysis)

ได้ศึกษาถึง on-line sample pretreatment ซึ่งได้ศึกษาการใช้ monolith ด้วย (ดังรายละเอียดที่ได้เสนอในการประชุมวิชาการ (ภาคผนวก 9) นอกเหนือไปจากการใช้ resin (ดังรายละเอียดใน reprint ในภาคผนวก ก4) ได้เสนอการใช้รีเอเจนต์ที่หาได้ง่ายจากธรรมชาติ (เช่น natural regent) เช่น ใช้ใน

ฝรั่งเพื่อหาปริมาณเหล็กโดย FIA (รายละเอียดใน reprint ในภาคผนวก ก18) การใช้ยาแก้ปวดศีรษะ (Salicylate reagent) สำหรับการหาประมาณเหล็กโดย FIA (reprint ในภาคผนวก ก3) การศึกษา Berthelot reaction ในการศึกษาเพื่อการวิเคราะห์หาปริมาณในแอมโมเนีย/ แอมโมเนีย ด้วย flow system. การใช้ WF6 เป็นรีเอเจนต์ในการปริมาณ chondroitin sulfate (รายละเอียด reprint ในภาคผนวก ก35)

5. การประยุกต์ flow-based analysis ในด้านต่างๆ

ได้พัฒนาอย่างต่อเนื่องในการวิเคราะห์ตัวอย่างทางการแพทย์เพื่อศึกษาในการคัดกรองโรคบางชนิด เช่น ธาลัสซีเมีย (ดัง reprint ในภาคผนวก ก10) โรคมะเร็งและโรคตับ (reprint ในภาคผนวก ก9) โรคข้อ (reprint ในภาคผนวก ก32) โรคเบาหวาน (reprint ในภาคผนวก ก33, ก36) การวิเคราะห์ทางสิ่งแวดล้อม (reprint ในภาคผนวก ก20, ก21, ก15, ก2) การวิเคราะห์ยา (reprint ในภาคผนวก ก5, ก6, ก18) การวิเคราะห์ทางอุตสาหกรรมเกษตร (reprint ในภาคผนวก ก24) การวิเคราะห์ในอาหารและเครื่องดื่ม (reprint ในภาคผนวก ก34 และ ก39)

Out-put ที่ได้รับจากโครงการ

การตีพิมพ์ในวารสารวิชาการนานาชาติ (ที่มี impact factor) จำนวน 68 เรื่อง (เป็นผลงาน 39 เรื่องจากทุนโครงการวิจัยฯ นี้) มี 3 papers ได้ปรากฏใน 25 hottest articles on Science Direct.com โดยที่ 2 papers อยู่ในวารสาร Talanta และ 1 paper ในวารสาร Analytica Chimica Acta

ได้รับเกียรติในการจัด "Interfaces of analytical Sciences: Workshop of US and Thai analytical scientists" โดยการสนับสนุนของ NSF (สหรัฐอเมริกา), สกว., สกอ. และ PERCH มีนักวิทยาศาสตร์ชั้นนำ 11 คน จากสหรัฐฯ มาประชุมร่วมกับนักวิทยาศาสตร์ไทย (ประมาณ 60 คน) เพื่อหาแนวทางความร่วมกัน (มีบทความในวารสาร Analytical Chemistry (มี impact factor 5.635) Government and Society: Thailand and US strengthen analytical ties, vol.79 No.3, Feb 1, 2007 การประชุมดังกล่าวนอกเหนือจะจัดที่มหาวิทยาลัยเชียงใหม่แล้วยังได้จัดที่สำนักงาน สกว.ด้วย (มีผู้ร่วมประชุมประมาณ 100 คน)

การพัฒนานักวิจัย

(ก) ในประเทศ

มีคณาจารย์และนิสิต/ นักศึกษาจาก 4 มหาวิทยาลัยต่างๆ เข้าร่วมในโครงการ (รวม อาจารย์ 19 คน นักศึกษาปริญญาเอก 25 คน ปริญญาโท 28 คน ปริญญาตรี 18 คน และปริญญาโทจากประเทศพม่า 1 คนและเนปาล 1 คน) ดังนี้

- **มหาวิทยาลัยเชียงใหม่**

มีอาจารย์ 15 คน (ภาควิชาเคมี 8 คน, ภาควิชาสถิติ 1 คน, ภาควิชาวิทยาการคอมพิวเตอร์ 1 คน, ภาควิชาชีวเคมี (คณะแพทย์ฯ 2 คน), ภาควิชารังสีรักษา (คณะแพทย์ฯ 2 คน))

มีนักศึกษาปริญญาเอก 18 คน ปริญญาโท 16 คน (และปริญญาโทจากประเทศพม่า 1 คน และเนปาล 1 คน) ปริญญาตรี 18 คน

- **มหาวิทยาลัยมหิดล**

มีอาจารย์ 1 คน* นักศึกษาปริญญาเอก 2 คน ปริญญาโท 3 คน

(*อนึ่งในช่วงที่ผ่านมา ผศ.ดร.ดวงใจ นาคะปรีชาได้เริ่มฟอร์มกลุ่มวิจัยเริ่มเป็นกลุ่มใหม่ (FIRST labs) โดยการรวบรวมศิษย์เก่าที่ไปเป็นอาจารย์ในมหาวิทยาลัยต่างๆ (ศรีนครินทรวิโรฒ, อุบลราชธานี, บูรพา, สถาบันเทคโนโลยีพระจอมเกล้า พระนครเหนือ) ซึ่งบางคนเคยได้รับการสนับสนุนในจากโครงการเมธีวิจัยอาวุโสฯ นี้)

- **จุฬาลงกรณ์มหาวิทยาลัย**

  มีอาจารย์ 1 คน มีนิสิตปริญญาเอก 2 คน ปริญญาโท 1 คน
- **มหาวิทยาลัยขอนแก่น**

  มีอาจารย์ 2 คน มีนักศึกษาปริญญาเอก 1 คน ปริญญาโท 7 คน

(ข) ต่างประเทศ

มีนักศึกษาปริญญาโทจาก Okayama University ประเทศญี่ปุ่น 2 คน มาทำวิจัยที่เชียงใหม่ 4 เดือน

มี Postdoc จากประเทศสหรัฐอเมริกา มาทำวิจัยที่เชียงใหม่ 2 เดือน (สืบเนื่องจากโครงการในข้อ 2 ข้างต้น (สนับสนุนจาก NSF))

Assoc. Prof. Dr. Norio Teshima จาก Aichi Institute of Technology ประเทศญี่ปุ่น มาทำวิจัยที่เชียงใหม่ 2 เดือน

ความร่วมมือกับต่างประเทศ

- มีความร่วมมือกับ 6 มหาวิทยาลัย/ สถาบัน ในต่างประเทศ (5 ประเทศ)
- จากความร่วมมือในข้อ 4.1 ซึ่งเริ่มที่มหาวิทยาลัยเชียงใหม่ได้ขยายไปยังมหาวิทยาลัยอื่นๆ (มหิดล, จุฬา, ขอนแก่น)
- มีความร่วมมือกับ Aichi Institute of Technology (AITech) (ผ่านโครงการ Frontier Research Project ของ AITech ที่ได้รับการสนับสนุนจากรัฐบาลญี่ปุ่น) มีอาจารย์ในกลุ่ม 1 คนไปทำ postdoc และอาจารย์จาก AITech ได้มาทำวิจัยที่เชียงใหม่ 2 เดือน (ดังกล่าวข้างต้น)

รางวัล/ เกียรติยศที่ได้รับ

- เป็นครั้งแรกในการจัดการประชุมที่สนับสนุนโดย NSF ในประเทศไทย (ต้องเขียน proposal โดย Professor Gary D. Christian (University of Washington, Seattle) ร่วมเป็น co-organizer กับเกตุ กรุดพันธ์) (ดังกล่าวในข้อ 2)
- การทำงานให้กับวารสารนานาชาติ

(1) Associate Editor ของวารสาร Water Research (Elsevier) (มี impact factor = 3.019) (เกตุ กรุดพันธ์ ตั้งแต่ 2003-ปัจจุบัน)

(2) Advisory Editorial Board ของวารสาร Talanta (Elsevier) (มี impact factor = 2.391) (เกตุ กรุดพันธ์ (2000-2006) และดวงใจ นาคะปรีชา (2007-2009))

- **รางวัลที่ได้รับโดยอาจารย์และนักศึกษา**

(1) ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

บุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์) จากสำนักงานเสริมสร้างเอกลักษณ์ของชาติ สำนักนายกรัฐมนตรี (พ.ศ. 2547)

(2) ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

รางวัลเมธีวิจัยอาวุโส สกว. 2547-2548 สาขาเคมีวิเคราะห์ ประจำปี 2547 จากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

(3) รองศาสตราจารย์ ดร. สุภาภรณ์ ครัดทัพ

รางวัลนักวิจัยรุ่นใหม่ที่มีผลงานตีพิมพ์จากสกว.และสกอ. สำหรับผู้รับทุนพัฒนาศักยภาพการทำงานของอาจารย์รุ่นใหม่ (พ.ศ. 2548)

(4) ดร. จรูญ จักรมุณี

รางวัล "นักวิจัยรุ่นใหม่ดีเด่น" สาขาวิทยาศาสตร์และเทคโนโลยี รางวัลมหาวิทยาลัยเชียงใหม่ "ช้างทองคำ" (พ.ศ. 2549)

(5) ผู้ช่วยศาสตราจารย์ ดร. ดวงใจ นาคะปรีชา

Model Lecturer of the Year 2005 คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

(6) น.ส.จันทร์เพ็ญ ครุวรรณ์ (นักศึกษาปริญญาโท)

The Award of Excellence for great poster presentation (12th International Symposium on Advanced Technology and Application, Korea (พฤศจิกายน 2549))

(7) น.ส. กาญจนา อุไรสิน (นักศึกษาปริญญาเอกไทย เรียนที่ Okayama University)

The Best Poster Award (13 ICFIA, Las Vegas, USA)

(8) ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

รางวัล "นักวิจัยดีเด่น" สาขาวิทยาศาสตร์และเทคโนโลยี รางวัลมหาวิทยาลัยเชียงใหม่ "ช้างทองคำ" ประจำปี 2550

- **การมีส่วนร่วมในงานประชุมนานาชาติทาง flow analysis**

(1) Co-organizer and invited speaker, , PACIFICHEM 2005 -Symposium #16: Advances in flow-based analytical techniques, Honolulu, USA (เกตุ กรุดพันธ์)

(2) Steering committee and invited speaker, 13th International Conference on Flow Injection Analysis, April 2005, Las Vegas, USA (เกตุ กรุดพันธ์)

(3) Internation advisory committee and invited speaker, 10th International Flow Analysis Conference, September 2006, Porto, Portugal (เกตุ กรุดพันธ์)

(4) Steering committee and invited speaker, 14th International Conference on Flow Injection Analysis, September 2007, Berlin, Germany (เกตุ กรุดพันธ์)

นอกเหนือจากการสนับสนุนของทุนเมธีวิจัยอาวุโส ของ สกว. นี้แล้ว ยังได้รับการสนับสนุนจากองค์กรต่างๆ ทั้งในประเทศและต่างประเทศได้แก่

1. The Royal Golden Jubilee (RGJ) Ph.D. Program, TRF
2. Postgraduate Education and Research Program in Chemistry (PERCH)
3. The collaborative Ph.D. Program (The Commission for Higher Education)
4. The Alexander von Humboldt Foundation (AvH), Germany
5. Deutcher Akademischer Austausch Dienst (DAAD), Germany
6. The Post-graduate Education Development (PED) Program (The Commision on Higher Education)
7. The Enhancement on the Country's Performance and Competitiveness Program (The Commission on Higher Education)

ได้มีความร่วมมือกับภาคเอกชนทั้งในและนอกประเทศ ได้แก่

1. Metrohm Siam/Metrohm AG, Switzerland
2. FIA Instrument, Co. Ltd., Japan

ในการจัดประชุมวิชาการประจำปีของกลุ่มวิจัยได้ดำเนินการจัดเป็น

ครั้งที่ 1 The 4th Annual Symposium on TRF Senior Research Scholar on "Development of Micro-and Nano-scale Analysis by Flow-based Techniques I" ณ ห้องสัมมนา คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ วันที่ 19 กันยายน 2548

ครั้งที่ 2 The 5th Annual Symposium on TRF Senior Research Scholar on "Development of Micro-and Nano-scale Analysis by Flow-based Techniques II" ณ ห้องสัมมนา คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ วันที่ 12 สิงหาคม 2549

ครั้งที่ 3 The 6th Annual Symposium: TRF Senior Research Scholar and Research Group on Innovation on Analytical Instrumentation CHE: "Development of Flow-based

Analysis for better life quality" ณ ห้องสัมมนา คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ วันที่ 16 สิงหาคม 2550

และ

ครั้งที่ 4 Interfaces of Analytical Sciences: Workshop of U.S. and Thai Analytical Scientists ณ โรงแรมอมารีรินคำ เชียงใหม่ จ.เชียงใหม่ วันที่ 4-8 มกราคม 2549

ในแต่ละครั้งได้รับการสนับสนุนจากหน่วยงานอื่นๆ ด้วย จึงทำให้มีผู้เข้าร่วมประชุมจากต่างประเทศด้วย และมีผู้ประชุมมากกว่า 70 – 80 คนในแต่ละครั้ง

Selected Presentations

เสนอผลงานวิจัยในการประชุมวิชาการนานาชาติ จำนวน 57 เรื่อง

(ทั้งที่ได้รับเชิญเป็นวิทยากรบรรยายนำ และการเสนอผลงานตามปกติ)

1. Amatotongchai M, Chailapakul O, Nacapricha D, Wilairat P and Grudpan K (2004) NEW APPROCH FOR CHROMATOGRAPHEIC DETECTION OF IODIDE IN PHARMACEUTICAL PRODUCTS USING THE BORON DOPED-DIAMOND THIN FILM ELECTRODE. PBA 2004 Symposium, Florence, Italy.
2. Chantiwas R, Ngangrungreung S, Kongtawelert P and Grudpan K (2004) A SIMPLE FLOW SYSTEM FOR REPID BRADFORD PROTEIN ASSAY IN MILKS SAMPLES. PBA 2004 Symposium, Florence, Italy.
3. Chantiwas R, Kongtawelert P, Kradtap S, Jakmunee J and Grudpan K (2004) THE FLOW-MICROPARTICLES BASED IMMUNOASSAY SYSTEM. PBA 2004 Symposium, Florence, Italy.
4. Grudpan K (2004) NOVEL APPROACHES IN ANALYTICAL INSTRUMENTATION AND PROCEDURES FOR WATER MONITORING
5. Burakham R, Oshima M, Grudpan K and Motomizu S (2004) MONITORING WATER SAMPLES FOR NITRITE AND NITRATE USING FLOW INJECTION WITH A NOVEL SPECTROPHOTOMETRIC REACTION SYSTEM. The 2 rd Asian International Conference on Ecotxiclogy and Environmrnt Safety, Songkla, Thailand
6. Jakmunee J , Somnam S, and Grudpan (2004) NOVEL STOPPED FLOW INJECTION IODOMETRY FOR DETERMINATION OF CHLORATE IN SOIL. The 2 rd Asian International Conference on Ecotxiclogy and Environmrnt Safety, Songkla, Thailand
7. Burakham R, Oshima M, Grudpan K and Motomizu S (2004) MONTORING WATER SAMPLES FOR NITRITE AND NITRATE USING FLOW INJECTION WITH A NOVEL SPECTROPHOTOMETRIC RECTION SYSTEM. The 2 rd Asian International Conference on Ecotxiclogy and Environmrnt Safety, Songkla, Thailand
8. Lapanantnoppakhun S, Jitmanee K, Jakmunee J, Jayasvasti S, Motomizu S, Sakai T and Kate Grudpan (2004) DETERMINATION OF TRACEAMOUNT OF

ALUMINIUM IN WATER BY FLOW INJECTION WITH SEMI-AUTOMATIC IN – VALVE PRECONCENTRATOR SYSTEM. *The 2 rd Asian International Conference on Ecotxiclogy and Environmrnt Safety*, Songkla, Thailand

9. Tue –Ngeun Orawan, Ellis P, Ian D. McKelvie, Faul Worsfold, Jakmunee J and Grudpan K (2004) FLOW INJECTION WITH ON – LINE UV PHOTO-OXIDATION FOR SPECTROPHOTOMETRIC DETERMINATION OF DISSOLVED REACTIVE PHOSPHORUS(DOP) IN NATURAL WATER. *The 2 rd Asian International Conference on Ecotxiclogy and Environmrnt Safety*, Songkla, Thailand
10. Dungthong S, Hans Mosbaek and Grudpan K. (2004) Cr(III)/Cr(VI) SPECIATION BY USING AN AUTOMATER ON – LINE SOLVENT EXTRACTION COUPLING WITH FLAME ATOMIC ABSORPTION SPECTROMATRY. *The 2 rd Asian International Conference on Ecotxiclogy and Environmrnt Safety*, Songkla, Thailand.
11. Burakham R, Jakmunee J and Grudpan K (2005) Sequential Injection Lab-at-Valve System for Solvent Extraction. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
12. Jakmunee J, Somnam S and Grudpan K (2005) New Approach Cost Effective Sequential Injection (SI) System: Speciation of Iron(II) and Iron(III) by Manual SI. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
13. Grudpan K and Jakmunee J (2005) Miniaturization for cost-effective analysis: Some aspects in flow-based analysis. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
14. Uraisin K, Nacapricha D, Lapanantnoppakhun S, Grudpan K and Motomizu S (2005) Determination of Trace Amounts of Bromide by Flow Injection/Stopped-Flow Detection Technique Using Kinetic-Spectrophotometric method. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
15. Preechaworapun A, Chuanuwatanakul S, Einaga Y, Grudpan K, Motomizu S and Chailapakul O (2005) Electroanalysis of sulfanomides by flow injection system/high-performance liquid chromatography coupled with amperometric

detection using boron-doped diamond electrode. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.

16. Setheevorawit T, Hartwell S.K, Christian G.D and Grudpan K (2005) Exploiting of Guava Leaf Extract as an Alternative Natural Reagent for Flow Injection Determination of Iron. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
17. Chantiwas R, Kongtawelert P, Kradtap S and Grudpan K (2005) Development of Sequential Injection Lab-at-Valve-Bead immunoassay System for Chondroitin 6-Sulfate. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
18. Synovec R, Christian G, Maneejamnong P, Khamphachua J, Chaijaruwanich J, Prasitwattanaseree S, Lenghor N, Jakmunee J, Nimmanpipug P, Sanghiran V and Grudpan K (2005) Flow injection system with dynamic interfacial pressure detection for unleaded gasoline. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
19. Lapanantnoppakhun S, Kasiwad S, Ganranoo L, Jakmunee J and Grudpan K (2005) Flow Injection Determination of Chromium(III) using EDTA after On-line Preconcentration. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
20. Ganranoo L, Lapanantnoppakhun S, Jakmunee J and Grudpan K (2005) Flow Injection and Sequential Injection Systems for the Determination of Tetracyclines by Employing Diazotization Using Sulfanilic Acid and Nitrite. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
21. Tue-Ngeun O, Jakmunee J and Grudpan K (2005) A Novel Stopped Flow Injection-Amperometric Procedure for Chlorate Determination. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
22. Jakmunee J, Somnam S, Jayasvati S and Grudpan K (2005) Determination of Dissolved Phosphate in Fertilizer and Soil Samples by Electronically Controlled Stopped-Flow Injection System. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.

23. Jakmunee J, Tue-ngeun P, Nimmanpipug P, Sanghiran V, Chijaruwanich J and Grudpan K (2005) Flow injection differential pulse voltammetry with principal component regression technique for simultaneous of chlorate and chlorite. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
24. Jitmanee K, Jakmunee J, Lapanantnoppakhun S, Wangkan S and Grudpan K (2005) Application of derivatization employing a commercial simple package software program to batch and flow analyses: A technical note. *13th International Conference on Flow Injection Analysis, Including Related Techniques,* Las Vegas, Nevada USA.
25. Jakmunee J, Siriangkhawat W and Grudpan K (2005) Sequential injection with UV digestion and anodic stripping voltammetry for speciation of some metals in water. International chemical congress of pacific basin societies, Honolulu, Hawaii.
26. Jakmunee J, Wongwilai W, Jayasvasit S and Grudpan K (2005) Determination of trace orthophosphate in natural water by flow injection amperometry with electronic control pretreatment column. International chemical congress of pacific basin societies, Honolulu, Hawaii.
27. Grudpan K, Hartwell SK, Somprayoon D, Kongtawelert P and Jakmunee J (2005) On-line bone-alkaline phosphatase assay system based on a simple bead injection-flow injection technique. International chemical congress of pacific basin societies, Honolulu, Hawaii.
28. Lapanantnoppakhun S, Jakmunee J, Poouthree K and Grudpan K (2005) On-line sample pre-separation for flow injection spectrophotometric assays of ascorbic acid. International chemical congress of pacific basin societies, Honolulu, Hawaii.
29. Ohno S, Teshima N, Sakai T, Grupan K and Polasek M (2005) Simultaneous spectrophotometric determination of trace amounts of copper and iron by sequential injection lab-on-valve technique. International chemical congress of pacific basin societies, Honolulu, Hawaii.
30. Chailapakul O, Dungchai W, Wangfuengkanagul N, Chuanuwatanakul S, Einaga Y, Motomizu S and Grudpan K (2005) FIA-electrocatalytic determination of hydrogen peroxide at chromium (III) hexacyanoferrate (II) modifies boron-doped

diamond electrodes. International chemical congress of pacific basin societies, Honolulu, Hawaii.

31. Jakmunee J, Somnam S and Grudpan K (2005) Novel approach for simple sequential injection (SI) system: Development of an electronically controlled SI for speciation of iron(II) and iron(III). International chemical congress of pacific basin societies, Honolulu, Hawaii.
32. Grudpan K, Hartwell S.K, Jakmunee J and Christian G.D (2005) Titration using flow-based techniques. International chemical congress of pacific basin societies, Honolulu, Hawaii.
33. Burakham R, Jakmunee and Grudpan K (2005) Micro-total analysis system using sequential injection analysis with lab-at-valve (SIA-LAV) for on-line micro extraction determination of anionic surfactants. International chemical congress of pacific basin societies, Honolulu, Hawaii.
34. Rodjana, Supalax Srijaranai, Kate Grudpan (2006) ON-LINE DERIVATIZATION AND DETERMINATION OF SOME HEAVY METALS BY COUPLING OF SEQUENTIAL INJECTION WITH ION-PAIR REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY . 10th International Conference on Flow analysis, Porto, Portugal
35. Tinakorn Kanyanee, Walter L. Borst, Jaroon Jakmunee, kate Grudpan, Jianzhong Li ,Purnendu K. Dasgupta (2006) A FLOW SYSTEM WITH SOAP BUBBLE FOR GAS SAMPLING DEVICE. 10th International Conference on Flow analysis, Porto, Portugal
36. Jaroon Jakmunee,Somchai Lapanantnoppakhun,Gary D. Christian, Kate Grudpan (2006) FLOW INJECTION AND SEQUENTIAL INJECTION EMPLOYING STOPPED FLOW. 10th International Conference on Flow analysis, Porto, Portugal
37. Tadao Sakai, Yoshikazu Kito, Norio Teshima, Shuji Kato, and Kate Grudpan (2006) FLOW INJECTION ANALYSIS FOR THE DETERMINATION OF PROTEIN IN BIOLOGICAL SAMPLES USING NONIONIC SURFACTANT. 10th International Conference on Flow analysis, Porto, Portugal
38. Kate Grudpan,Jaroon Jakmunee, Supaporn Kraptap, Somchai Lapanantnoppakhun, Rodjana Burakham EXPLOITING FLOW ANALYSIS WITH LAB-AT-VALVE

(LAV) APPROACH. 10th International Conference on Flow analysis, Porto, Portugal

39. Jaroon Jakmunee, Sarawut Somnam, Somchai Lapanantnoppakhun, Narong Lenghor, Shoji Motomizu, Kate Grudpan (2006) DEVELOPMENT OF HYDRODYNAMIC FLOW ANALYTICAL SYSTEMS. 10th International Conference on Flow analysis, Porto, Portugal
40. Wasin Wongwilai,Jaroon Jakmunee, Somchai Lapanantnoppakhun, Shoji Motomizu, Tadao Sakai ,Kate Grudpan (2006) FLOW INJECTION AMPEROMETRIC SYSTEM WITH SAMPLE PRETREATMENT COLUMN FOR DETERMINATION OF TRACE ORTHOPHOSPHATE. 10th International Conference on Flow analysis, Porto, Portugal
41. Jaroon Jakmunee, Natsuki Miyoshi, Watsaka Siriangkhawut,Somchai Lapanantnoppakhun, Shoji Motomizu, Kate Grudpan (2006) DEVELOPMENT OF FLOW INJECTION VOLTAMMETRIC SYSTEM WITH SAMPLE PRETREATMENT COLUMN FOR THE DETERMINATION OF CADMIUM , LEAD, COPPER AND ZINC. 10th International Conference on Flow analysis, Porto, Portugal
42. Kate Grudpan, Kanchana Watlaiad, Takashi Suekane, Narong Lenghor, Somchai Lapanantnop pakhun, Jaroon Jakmunee,Tadao Sakai, Shoji Motomizu (2006) DEVELOPMENT OF FLOW SYSTEMS FOR THE DETERMINATION OF AMMONIA. 10th International Conference on Flow analysis, Porto, Portugal
43. Duangjai Nacapricha, Chanpen Karuwan, Benjamapawn Promthong, Thitirat Mantim, Orawon Chailapakul, Kate Grudpan , Einaga Yasuaki (2006) USE OF BORON-DOPED DIAMOND THIN FILM AS SENSOR FOR FLOW ANALYSIS OF BETA-AGONISTS. 10th International Conference on Flow analysis, Porto, Portugal
44. Kate Grudpan, Supaporn Karadtap Hartwel, Duangporn Somprayoon, Somchai Lapanantnop pakhun, Siriwan Ongchai, Prachya Kongtawelert (2006) FLOW INJECTION-BEAD INJECTION SYSTEM FOR THE ASSAY OF BONE ALKALINE PHOSPHATASE. 10th International Conference on Flow analysis, Porto, Portugal

45. Duangjai Nacapricha, Benjamapawn Promthong Prapin Wilairat, Piyada Jittangprasert, Orawon Chailapakul,Kate Grudpan (2006) APPLICATION OF BORON-DOPED DIAMOND THIN FILM AS SENSOR FOR ANALYSIS OF INORGANIC ANIONS IN FLOW INJECTION AND ION CHROMATOGRAPHY. 10th International Conference on Flow analysis, Porto, Portugal

46. Kate Grudpan (2006) Some Approaches in Cost-effective (Flow) Analysis for Development in Environment. Symposium on Frontier Research Project: Materials for the 21st Century-Materials Development for Environment, Energy and Information. Aichi Institute of Technology, Toyota, Japan, 11-13 October 2006.

47. Christian, Gary D., Grudpan, Kate (2006) Analytical chemistry in Thailand: Opportunities for U.S. electrochemists and analytical scientists. Abstracts of Papers, 232nd ACS National Meeting, San Francisco, CA, United States, Sept. 10-14, 2006.

48. Kate Grudpan, Kritsana Jitmanee, Somchai Lapanantnoppakhun, Jaroon Jakmunee, Tadao Sakai and Norio Teshima (2007), "Flow-based Analytical Techniques: Some Developments for Materials and Environment", In "Frontier Research Project : Materials for the 21st Century-Materials Development for Environment,Energy and Information" ,Aichi Institute of Technology,Japan,pp 104-107.

49. Kate Grudpan, Jaroon Jakmunee, Somchai Lapanantnoppakhun, Supaporn Kradtap Hartwell, Kritsana Jitmanee, Wasin Wongwilai and Gary D Christian (2007) Flow Injection Analysis and Related Techniques: How Related are They? (Keynote Lectures). Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007

50. Kancha Watla-iad, Kate Grudpan and Shoji Motomizu (2007) A simple flow injection simultaneous determination of nitrate and nitrite in real water samples using 1-naphthol. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007

51. Sarawut Somnam, Jaroon jakmunee, Kate Grudpan and Shoji Motomizu (2007) An automated hydrodynamic sequential sequential injection system for

determination of phosphate and silicate in waste water. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007

52. Watsaka Siriangkhawut, Muriel Bouby, Horst Geckeis, Jaroon Jakmunee and Kate Grudpan (2007) A flow-based analytical method to study kinetics of metal-humic acid complexes. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007
53. Wasin Wongwilai, Jaroon Jakmunee, Somchai Lapanantnoppakhun and Kate Grudpan (2007) Flow Injection Spectrometric determination of Ascorbic Acid using Methylene Blue. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007
54. Rodjana Burakham and Kate Grudpan (2007) Miniaturized spectrophotometric determination of amoxicillin using sequential injection lab-om-valve. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007
55. Kuruna Jainontee, Vannajan Sanghiran Lee, Sukon Prasitwattanaseree, Jeerayut Chaijaruwanich, Jaroon Jakmunee and Kate Grudpan (2007) Spectrophotometric analysis of component colors in colorant with the aids of principal component regression and partial least squares. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007
56. Somchai Lapanantnoppakhun, Lucksagoon Ganranoo, Wasin Wongwilai, Jaroon Jakmunee, Prasak Thavornyutikarn and Kate Grudpan (2007) Sequential Injection Chromatography for Some Quinine Alkaloids. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007
57. Lucksagoon Ganranoo, Somchai Lapanantnoppakhun, Wasin Wongwilai, Jaroon Jakmunee and Kate Grudpan (2007) Sequential Injection Chromatography of Calcium and Magnesium using Murexide Reagent. Fourteenth International Conference on Flow Injection Analysis (ICFIA 2007) ,Berlin, Germany September 3-7,2007

การเสนอผลงานวิจัยในการประชุมวิชาการภายในประเทศ จำนวน 23 เรื่อง
(ทั้งที่ได้รับเชิญเป็นวิทยากรบรรยายนำ และการเสนอผลงานตามปกติ)

1. Watla-iad K, Chantiwas R, Jakmunee J and Grudpan K (2005) Determination of Milk Protein by Using a Developed Sequential Injection System with Bradford Method. *RGJ-Ph.D. Congress VI*, Jomtien Palm Beach Resort, Pattaya, Chonburi, 215.
2. Grudpan K, Jakmunee J, Lapanantnoppakhun S, Kradtap S, Jitmanee K, Chantiwas R, Nacapricha D, Srijaranai S, Burakham R and Chailapakul O (2005) Some Aspects for Miniaturization in Chemical Analysis. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-L4.
3. Siriangkhawut W, Suteerapataranon S, Jakmunee J and Grudpan K (2005) Development of On-line Dilution Procedure Using Sequential Injection-Monosegmented Fiow for Voltammetric Determination of Some Heavy Metals. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-O2.
4. Watla-iad K, Chantiwas R, Jakmunee J and Grudpan K (2005) Development of Sequential Injection Analysis for Protein Assay Using Bradford Method. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-O4.
5. Somnam S, Jakmunee J and Grudpan K (2005) Determination of Fe(II) and Fe(III) by a Novel Hydrodynamic Flow Injection System. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-O6.
6. Burakham R, Motomizu S and Grudpan K (2005) Flow Injection with a Novel Spectrophotometric Reaction System for water Monitoring of Nitrite and Nitrate. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-P20.
7. Somprayoon D, Hartwell S.K and Grudpan K (2005) Flow Injection-Bead Injection System for Determination of Bone Alkaline Phosphatase. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-P24.
8. Boomalai A, Hartwell S.K and Grudpan K (2005) Development of Flow Injection-Capillary Immunoassay System. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-P30.

9. Ganranoo L, Lapanantnoppakhun S and Grudpan K (2005) Development of Flow Injection Spectrophotometric for Determination of Tetracyclines. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-P31.

10. Wongwilai W, Jakmunee J and Grudpan K (2005) Investigation on Electrochemical Reactions for Determination of Phosphate. *PERCH congress IV*, Jomtien Palm Beach Resort, Pattaya, Chonburi, AC-P44.

11 Wasin Wongwilai, Jaroon Jakmunee, Somchai Lapanantnopakhun and Kate Grudpan (2006) Sequential Injection Analysis for Fluorometric Determination of Aluminium. 32nd congress on Science and Technology of Thailand, Queen Sirikit National Convention Center (QSNCC), Bangkok, C1_C0180

12. Lucksagoon , Somchai Lapanantnopakhun ,Jaroon Jakmunee and Kate Grudpan (2006) Sequential Injection Spectrophotometry for The Determination of Tetracyclines. 32nd congress on Science and Technology of Thailand, Queen Sirikit National Convention Center (QSNCC), Bangkok, C1_C0154

13. Rodjana Burakham, Supalax Sijaranai and Kate Grudpan (2006) On- Line Derivatization Determiantion of Metal Ions Using Sequential Injection High Performance Liquid Chromatography. 32nd congress on Science and Technology of Thailand, Queen Sirikit National Convention Center (QSNCC), Bangkok, C1_C0073

14. Lapanantnoppakhun S, Ganranoo L, Kasiwad S, Jakmunee J and Grudpan K (2006) Development of Simple Systems with Usage of Simple Reagent. *การประชุมนักวิจัยรุ่นใหม่ พบเมธีวิจัยอาวุโส สกว.*, Regent Hotel, Cha Am, Phetchaburi, O-S1F-16.

15. Jakmunee J and Grudpan K (2006) Development of Instrument and Methods for Coulometric and Amperometric Analysis of Some Foods and Beverages. *การประชุมนักวิจัยรุ่นใหม่ พบเมธีวิจัยอาวุโส สกว.* , Regent Hotel, Cha Am, Phetchaburi, P-S1E-12.

16. Kradtap-Hartwell S, Pathanon K, Fongmoon D, Grudpan K and Kongtawelert P (2006) Simple Assay of Chondroitin Sulfate Proteoglycans Using a Flow Injection System with Mini-Immunoaffinity Chromatographic Column. *การประชุมนักวิจัยรุ่นใหม่ พบเมธีวิจัยอาวุโส สกว.*, Regent Hotel, Cha Am, Phetchaburi, P-S1E-17.

17. Jakmunee J, Kradtap-Hartwell S, Lapanantnoppakhun S, Jitmanee, K and Grudpan K (2006) **Plenary Lecture:** Development of Flow-Based Analysis in Thailand, PRECH-CIC Congress V, Jomtien Palm Beach Resort Pattaya, PL7
18. Sarawut Somnam, Jaroon Jakmunee, Kate Grudpan and Shoji Motomizu (2007) Development of an Automated Hydrodynamic Sequential Injection System for determination of Phosphate and Silicate. 33rd Congress on Science and Technology of Thailand.(STT.33) , Walailak University ,Nakhon Si Thammarat, Thailand. October 18-20, 2007
19. Napaporn Wannaprom, Supaporn Kradtap Hartwell, Prachya Kongtawelert, Jaroon Jakmunee and Kate Grudpan (2007) Preliminary Studies on Development of Capillary Immunoassay for Biomarker of cancer. 33rd Congress on Science and Technology of Thailand.(STT.33) , Walailak University ,Nakhon Si Thammarat, Thailand. October 18-20, 2007
20. Supada Khonyoung, Supaporn Kradtap Hartwell, Prachya Kongtawelert, Somchai Lapanantnopakhun, Jaroon jakmunee and Kate Grudpan (2007) Immobilization of Chondroitin Sulfate Proteoglycan on Glass Capillary for its use in Immunoassay. 33rd Congress on Science and Technology of Thailand.(STT.33) , Walailak University ,Nakhon Si Thammarat, Thailand. October 18-20, 2007
21. Pipoon Bunpeng, Jaroon jakmunee and Kate Grudpan (2007) Development of Flow Injection-Amperometric method for Determination of Ascorbic Acid in Fruit Juice Samples. 33rd Congress on Science and Technology of Thailand.(STT.33) , Walailak University ,Nakhon Si Thammarat, Thailand. October 18-20, 2007
22. Kraingkrai Ponhong, Jaroon Jukmunee and kate Grudpan (2007) Cost-Effective Amperometric-Iodometric Titration for Determination of Some Reducing Agents. 33rd Congress on Science and Technology of Thailand.(STT.33) , Walailak University ,Nakhon Si Thammarat, Thailand. October 18-20, 2007
23. Nuanlaor Ratanawimarnwong, Saowapak Teerasong Kate Grudpan and Duangjai Nacapricha (2007) Determanation of Formaldehyed contaminated in food using Hybrid-Flow Analysis. 33rd Congress on Science and Technology of Thailand.(STT.33) , Walailak University ,Nakhon Si Thammarat, Thailand. October 18-20, 2007

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
1	Hartwell S Kradtap, Grudpan K and Christian G D	Bead injection with a simple flow-injection system: an economical alternative for trace analysis.	Trends in Analytical Chemistry (2004), 23(9) , 619-623.	5.068
2	Grudpan K	Some recent developments on cost-effective flow-based analysis.	Talanta (2004), 64(5), 1084-1090.	2.810
3	Udnan Y, Jakmunee J, Jayasavati S, Christian GD, Synovec RE, Grudpan K	Cost-effective flow injection spectrophotometric assay of iron content in pharmaceutical preparations using salicylate reagent.	Talanta (2004) , 64(5), 1237-1240.	2.810
4	Tanikkul S, Jakmunee J, Lapanantnoppakhun S, Rayanakorn M, Sooksamiti P, Synovec RE, Christian GD, Grudpan K	Flow injection in-valve-mini-column pretreatment combined with ion chromatography for cadmium, lead and zinc determination.	Talanta (2004), 64(5), 1241-1246.	2.810
5	Charoenraks T, Palaharn S, Grudpan K, Siangproh W, Chailapakul O	Flow injection analysis of doxycycline or chlortetracycline in pharmaceutical formulations with pulsed amperometric detection.	Talanta (2004), 64(5), 1247-1252.	2.810

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
6	Chailapakul O, Amatatongchai M, Wilairat P, Grudpan K, Nacapricha D	Flow-injection determination of iodide ion in nuclear emergency tablets, using boron-doped diamond thin film electrode.	Talanta (2004), 64(5), 1253-1258.	2.810
7	Jakmunee, J, Patimapornlert L, Suteerapataranon S, Lenghor N, Grudpan K	Sequential injection with lab-at-valve (LAV) approach for potentiometric determination of chloride.	Talanta (2005), 65(3), 789-793.	2.810
8	Lenghor N, Staggemeier BA, Hamad ML, Yuthapong U, Tanikkul S, Jakmunee, J, Grudpan K, Prazen BJ and Synovec RE	A dynamic liquid-liquid interfacial pressure detector for the rapid analysis of surfactants in a flowing organic liquid.	Talanta (2005), 65(3), 722-729.	2.810
9	Hartwell S Kradtap, Srisawang B, Kongtawelert P, Jakmunee Jand Grudpan K	Sequential injection-ELISA based system for online determination of hyaluronan.	Talanta (2005), 66(2), 521-527.	2.810

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
10	Hartwell S Kradtap, Srisawang B, Kongtawelert P, Christian GD and Grudpan K	Review on screening and analysis techniques for hemoglobin variants and thalassemia.	Talanta (2005), 65(5), 1149-1161.	2.810
11	Hartwell S Kradtap, Boonmalai A, Jayasvati S, Lapanantnoppakhun S, Jakmunee J and Grudpan K	Simple Bead Injection – Flow Injection System for Determination of Copper.	Anal. Science (2005) , 21(4), 437-439.	1.589
12	Chantiwas R, Beckett R, Grudpan K	Size-based speciation of iron in clay mineral particles by gravitational field-flow fractionation with electrothermal atomic absorption spectrometry.	Spectrochimica Acta Part B (2005), 60(1), 109-116.	3.092
13	Jakmunee J, Pathimapornlert L, Hartwell SK and Grudpan K	Novel approach for mono-segmented flow micro-titration with sequential injection using a lab-on-valve system: a model study for the assay of acidity in fruit juices.	Analyst (2005), 130(3), 299-303.	3.198
14	Udnan Y, McKelvie ID, Grace MR, Jakmunee J and Grudpan K	Evaluation of on-line preconcentration and flow-injection amperometry for phosphate determination in fresh and marine waters.	Talanta (2005), 66(2), 461-466.	2.810

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
15	Wangkarn S, Soisungnoen P, Rayanakorn M and Grudpan K	Determination of linear alkylbenzene sulfonates in water samples by liquid chromatography-UV detection and confirmation by liquid chromatography-mass spectrometry.	Talanta (2005), 67(4), 686-695.	2.810
16	Tue-Ngeun O, Jakmunee J, Grudpan K	A novel stopped flow injection-amperometric procedure for the determination of chlorate.	Talanta (2005), 68(2), 459-464.	2.810
17	Burakham R, Lapanantnoppakhun S, Jakmunee J and Grudpan K	Exploiting sequential injection analysis with lab-at-valve (LAV) approach for on-line liquid-liquid micro-extraction spectrophotometry.	Talanta (2005) , 68(2), 416-421.	2.810
18	Settheeworrarit T, Hartwell SK, Lapanatnoppakhun S, Jakmunee J, Christian GD and Grudpan K	Exploiting guava leaf extract as an alternative natural reagent for flow injection determination of iron.	Talanta (2005), 68(2), 262-267.	2.810

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
19	Manee-on K, Lapanantnoppakhun S, Jakmunee J and Grudpan K	A simple flow injection assay of Ca(II) in mineral supplement using Mg(II)-EDTA.	Journal of Flow Injection Analysis (2005), 22(1), 9-10.	
20	Suteerapataranon, Siripat; Bouby, Muriel; Geckeis, Horst; Fanghaenel, Thomas; Grudpan, Kate.	Interaction of trace elements in acid mine drainage solution with humic acid.	Water Research (2006), 40(10), 2044-2054.	2.459
21	Grace, Michael; Udnan, Yuthapong; McKelvie, Ian; Jakmunee, Jaroon; Grudpan, Kate.	On-line Removal of Sulfide Interference in Phosphate Determination by Flow Injection Analysis.	Environmental Chemistry (2006), 3(1), 19-25.	0.814
22	Preechaworapun, Anchana; Chuanuwatanakul, Suchada; Einaga, Yasuaki; Grudpan, Kate; Motomizu, Shoji; Chailapakul, Orawon.	Electroanalysis of sulfonamides by flow injection system/high-performance liquid chromatography coupled with amperometric detection using boron-doped diamond electrode.	Talanta (2006), 68(5), 1726-1731.	2.810
23	Srijaranai, Supalax; Chanpaka, Saiphon; Kukusamude, Chutima; Grudpan, Kate.	Flow-injection in-line complexation for ion-pair reversed phase high performance liquid chromatography of some metal-4-(2-pyridylazo) resorcinol chelates.	Talanta (2006), 68(5), 1720-1725.	2.810

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
24	Jakmunee, Jaroon; Rujiralai, Thitima; Grudpan, Kate.	Sequential injection titration with spectrophotometric detection for the assay of acidity in fruit juices.	Analytical Sciences (2006), 22(1), 157-160.	1.589
25	Lapanantnoppakhun, Somchai; Kasuwas, Supharphorn; Ganranoo, Lucksagoon; Jakmunee, Jaroon; Grudpan, Kate.	Simple spectrophotometric flow injection system with an in-valve minicolumn for enhancement during the determination of chromium(III) using EDTA.	Analytical Sciences (2006), 22(1), 153-155.	1.589
26	Lenghor, Narong; Jakmunee, Jaroon; Prazen, Bryan J.; Synovec, Robert E.; Christian, Gary D.; Grudpan, Kate.	Simple sequential injection analysis systems with a dynamic surface tension detector.	Analytical Sciences (2006), 22(1), 147-151.	1.589
27	Burakham, Rodjana; Jakmunee, Jaroon; Grudpan, Kate.	Development of sequential injection-lab-at-valve (SI-LAV) micro-extraction instrumentation for the spectrophotometric determination of an anionic surfactant.	Analytical Sciences (2006), 22(1), 137-140.	1.589

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
28	Kate Grudpan, Supada Khonyoung, Supaporn Kradtap Hartwell, Somchai Lapanantnoppakhun and Jaroon Jakmunee .	Down scaling : From operation on lab bench space to manipulation at a valve.	Flow Injection Anal (2006), 23(2), 94 – 101	
29	Chanpen Karuwan, Thitirat Mantim, Patcharin Chaisuwan, Prapin Wilairat, kate Grudpan, Piyada Jittangprasert, Yasuaki Einaga, Orawon Chailapakul, Leena Suntornsuk, Oraphan Anurukvorakun and Duangjai Nacapricha.	Pulsed Amperometry for Anti-fouling of Boron-doped Diamond in Electroanalysis of β-Agonists : Application to Flow Injection for Pharmaceutical Analysis.	Sensors (2006), 6, 1837-1850.	1.373
30	Kritsana Jitmanee, Jaroon Jakmunee, Somchai Lapanantnoppakhun, Sunanta Wangkarn, Norio Teshima, Tadao Sakai, Gary D. Christian and Kate	Enhancing chemical analysis with signal derivatization using simple available software packages.	Microchemical Journal (2007), 8(2), 195-203	1.558
31	Apichai Santalad, Rodjana Burakham, Supalax Srijaranai and Kate Grudpan	Field-amplified sample injection and in-capillary derivatization for capillary electrophoretic analysis of metal ions in local wines.	Microchemical Journal (2007), 86(2), 209-215	1.558

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
32	Supaporn Kradtap Hartwell, Duangporn Somprayoon, Prachya Kongtawelert, Siriwan Ongchai, Olarn Arppornchayanon, Lucksagoon Ganranoo, Somchai Lapanantnoppakhun and Kate Grudpan	Online assay of bone specific alkaline phosphatase with a flow injection-bead injection system.	Analytica Chimica Acta (2007), 600(1-2), 188-193	2.894
33	Tadao Sakai, Yoshikazu Kito, Norio Teshima, Shuji Katoh, Kanchana Watla-lad and Grudpan	Spectrophotometric Flow Injection Analysis of Protein in Urine Using Tetrabromophenolphthalein Ethyl Ester and Triton X-100.	Flow Injection Anal (2007), 24(1), 23-26	
34	Rodjana Burakham, Supalax Srijaranai and Kate Grudpan	High-Performance Liquid Chromatography with Sequential Injection for On-line Pre-column Derivatization of Some Heavy Metals.	Journal of Separation Science (2007), 30 (16), 2614-2619	2.535
35	Supaporn Kradtap Hartwell, Kanokphan Pathanon, Duriya Fongmoon, Prachya Kongtawelert and Kate Grudpan	Exploiting flow injection system with mini-immunoaffinity chromatographic column for chondroitin sulfate proteoglycans assay.	Analytical and Bioanalytical Chemistry (2007), 388 (8), 1839-1846	2.591
36	Watla-iad Kanchana, Tadao Sakai, Norio Teshima, Shuji Katoh and Kate Grudpan	Successive determination of urinary protein and glucose using spectrophotometric sequential injection method.	Analytica Chimica Acta (2007) , 604(2) 139-146	2.894

ตาราง 1 output จากกลุ่มวิจัยจากโครงการวิจัยฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
37	Beckett, R., Sharma, R., Andric, G., Chantiwas, R., Jakmunee, J., Grudpan, K.	Illustrating some principles of separation science through gravitational field-flow fractionation.	Journal of Chemical Education (2007), 84 (12), 1955-1962	0.439
38	Chaisuksant, R., Pattanarat, L., Grudpan, K.	Naphthazarin modified carbon paste electrode for determination of copper(II).	Microchimica Acta Article (2007), 162, 181-188	1.237
39	Orawon Chailapakul, Sarawadee Korsrisakul, Weena Siangproh and Kate Grudpan	Fast and simultaneous detection of heavy metals using a simple and reliable microchip-electrochemistry route: An alternative approach to food analysis.	Talanta (2008), 74(4), 683-689	2.810

ตาราง 2 output จากกลุ่มวิจัยที่ไม่ได้ร่วมในโครงการฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
1	Burakham R, Higuchi K, Oshima M, Grudpan K, Motomizu S	Flow injection spectrophotometry coupled with a crushed barium sulfate reactor column for the determination of sulfate ion in water samples.	Talanta (2004) , 64(5), 1147-1150.	2.810
2	Burakham R, Oshima M, Grudpan K, Motomizu S	Simple flow- injection system for the simultaneous determination of nitrite and nitrate in water samples.	Talanta (2004), 64(5), 1259-1265.	2.810
3	Tue-Ngeun O, Ellis P, McKelvie ID, Worsfold P, Jakmunee J and Grudpan K	Determination of dissolved reactive phosphorus (DRP) and dissolved organic phosphorus (DOP) in natural waters by the use of rapid sequenced reagent injection flow analysis.	Talanta (2005), 66(2), 453-460.	2.810
4	Uraisin K, Nacapricha D, Lapanantnoppakhun S, Grudpan K. and Motomizu S.	Determination of trace amounts of bromide by flow injection/stopped-flow detection technique using kinetic-spectrophotometric method.	Talanta (2005), 68(2), 274-280.	2.810
5	Rujiwatra A, Phueadpho M and Grudpan Kate	Selective synthesis of zeolitic phillipsite and hibschite hydrogarnet from lignite ash employing calcium hydroxide under mild conditions.	Journal of Physics and Chemistry of Solids (2005), 66(6), 1085-1090	1.164

ตาราง 2 output จากกลุ่มวิจัยที่ไม่ได้ร่วมในโครงการฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
6	Orawan Tue-Ngeun, Richard C. Sandford, Jaroon Jakmunee, Kate Grudpan, Ian D. McKelvie, Paul J. Worsfold.	Determination of dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) in freshwaters by sequential injection spectrophotometry with on-line UV photo-oxidation,	Analytica Chimica Acta (2005), 554, 17-24.	2.894
7	Kanyanee, Tinakorn; Borst, Walter L.; Jakmunee, Jaroon; Grudpan, Kate; Li, Jianzhong; Dasgupta, Purnendu K.	Soap Bubbles in Analytical Chemistry. Conductometric Determination of Sub-Parts Per Million Levels of Sulfur Dioxide with a Soap Bubble.	Analytical Chemistry (2006), 78(8), 2786-2793.	5.646
8	Ohno, Shinsuke; Teshima, Norio; Sakai, Tadao; Grudpan, Kate; Polasek, Miroslav.	Sequential injection lab-on-valve simultaneous spectrophotometric determination of trace amounts of copper and iron.	Talanta (2006), 68(3), 527-534.	2.810
9	Natchanon Amornthammarong, Jaroon Jakmunee, Jianzhong Li, and Purnendu K. Dasgupta	Hybrid Fluorometric Flow Analyzer for Ammonia.	Anal. Chem (2006) , 78(6), 1890 -1896.	5.646
10	McBrady, Adam D.; Chantiwas, Rattikan; Torgerson, Ana Kristine; Grudpan, Kate; Synovec, Robert E.	An absorbance-based micro-fluidic sensor for diffusion coefficient and molar mass determinations.	Analytica Chimica Acta (2006), 575(2), 151-158.	2.894

ตาราง 2 output จากกลุ่มวิจัยที่ไม่ได้ร่วมในโครงการฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
11	Kritsana Jitmanee, Norio Teshima, Tadao Sakai and Kate Grudpan	DRC™ ICP-MS coupled with automated flow injection system with anion exchange minicolumns for determination of selenium compounds in water samples.	Talanta (2007), 73(2),352-357	2.810
12	Pothacharoen P, Siriaunkgul S, Ong-chai S, Supabandhu J, Kumja P, Wanaphirak C, Sugahara K,	Raised Serum Chondroitin Sulfate Epitope Level in Ovarian Epithelial Cancer	J. Biochem. 140, 517–524. (2006)	2.160
13	Srijaranai S, Sririangkhawut W, Srijaranai S, Ruksakulpiwat C, Deming RL	Effect of bound copper(II) on the LC separation of selected phenols using an aminophosphonic acid silica stationary phase	Anal. Lett 37 (2004) 2577-2594	1.036
14	Santalad A, Teerapornchaisit P, Burakham R, Srijaranai S	Capillary zone electrophoresis of organic acids in beverages	LWT Food Sci. Technol ' (2007), 40(10),1741-1746	1.155
15	Amornthammarong N., Anujaravat P.,Sereenonchai K., Chaisuwan P., Sastranurak P., Wilairat P. and Nacapricha D.	Method development based on all injection analysis for determination of phosphorus in soil and sediment extracts.	Talanta 2005: **68**: 480-487	2.391

ตาราง 2 output จากกลุ่มวิจัยที่ไม่ได้ร่วมในโครงการฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
16	Choengchan N., Mantim T., Wilairat P., Dasgupta P. K., Motomizu S., Nacapricha D.	A membraneless gas diffusion unit: design and its application to determination of ethanol in liquors by spectrophotometric flow injection.	Anal. Chim. Acta 2006: 579: 33-37	2.760
17	**Nacapricha D.**, Amornthammarong N., Sereenonchai K., Anujaravat P., Wilairat P.	Low cost telemetry with PC sound card for chemical analysis applications	Talanta 2007: **71**: 605–609	2.391
18	Amatatongchai M., Hofmann O., Nacapricha D., Chailapakul O., and J. deMello A.	A microfluidic system for evaluation of antioxidant capacity based on a peroxyoxalate chemiluminescence assay.	Anal. Bioanal. Chem. 2007: 387: 277–285	2.695
19	Nimmanpipug, P.; Jitonnom, J.; Ngaojampa, C.; Lee, V. S.	H5N1 Neuraminidase Implicit Molecular Dynamics Model and Its Binding Interactions with Commercial Drugs.	*Molecular Simulations* 2007, *in revision*.	1.345
20	Yeap, B.; Othman, A. S.; Lee, V. S.; Lee, C.	Molecular phylogenetics of the Asian subterranean termite, *Coptotermes gestroi* (Wasmann) and the Philippines milk termite, *Coptotermes vastator* Light (Isoptera: Rhinotermitidae)	*Journal of Economic Entomology* (Household and Structural Insects) 2007, *in press* .	1.205

ตาราง 2 output จากกลุ่มวิจัยที่ไม่ได้ร่วมในโครงการฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
21	Junkaew, A.; Jitonnom, J.; Nimmanpipug, P.; Lee, V. S.	Polymerizability of lactones calculated by molecular mechanics, semiempirical and density functional theory methods.	Chiang Mai Journal of Science 2007, 34(1), 1-9.	
22	Aruksakunwong, O.; Wittayanarakul, K.; Sompornpisut, P.; Sanghiran, V.; Parasuk, V.; Hannongbua, S.	"Structural and Dynamical Properties of Different Protonated States of Mutant HIV-1 Protease Complexed with the Saquinavir Inhibitor Studied by Molecular Dynamics Simulations"	Journal of Molecular Graphic and Modelling 2006, 25, 324-332.	2.333
23	Wittayanarakul, K.; Aruksakunwong, O.; Sompornpisut, P.; Lee, V. S.; Parasuk, V.; Pinitglang, S.; Hannongbua, S.	Structure, Dynamics and Solvation of HIV-1 Protease/Saquinavir Complex in Aqueous Solution and their Contributions to Drug Resistance: Molecular Dynamic Simulations.	Journal of Chemical Information and Modeling 2005, 45, 300-308.	2.923
24	Promsri, S.; Chuichay, P.; Lee, V. S.; Parasuk, V.; Hannongbua, S.	Molecular and Electronic Properties of HIV-1 Protease Inhibitor C60 derivatives as Studies by the ONIOM Method.	Journal of Molecular Structure: THEOCHEM 2004, 715, 47-53.	1.045
25	Nimmanpipug, P.; Tashiro, K; Rangsiman, O.	Factors Governing the Three-Dimensional Hydrogen-Bond Network Structure of Poly(*m*-Phenylene Isophthalamide) and a Series of Its Model Compounds (4): Similarity in Local Conformation and Packing Structure between a Complicated Three-Arm Model Compound	J. Phys. Chem. 2006; 110B; 20858-20864	4.033

ตาราง 2 output จากกลุ่มวิจัยที่ไม่ได้ร่วมในโครงการฯ : ผลงานตีพิมพ์

ลำดับ	ผู้ร่วมวิจัย	ผลงานตีพิมพ์	วารสารวิชาการ	Impact Factor
26	Metawee Srikummool, Jeerayut Chaijaruwanich, Jatupol Kampuansai, Daoroong Kangwanpong	Minimal Y-chromosomal Haplotype Selection for Phylogenetic Study using the Bootstrapped DTI Method	Science Asia , 2006, Vol.32, 361-364	
27	Traisathit P, Le Cœur S, Mary JY, Kanjanasing A, Lamlertkittikul S, Lallemant M.	Gestational age determination and prevention of human immunodeficiency virus perinatal transmission.	IJGO 2006; 92:176-180.	1.147
28	Briand N, Le Cœur S, Traisathit P	Growth of HIV-uninfected children exposed to perinatal zidovudine for the prevention of mother-to-child HIV transmission.	PIDJ. 2006; 25:325–332.	2.262
29	Briand N, Lallemant M, Jourdain G, Traisathit P	Haematological Parameters in HIV-1 Pregnant Women Exposed to Perinatal Zidovudine in Thailand: Secondary Analysis of a Randomized Trial.	PLoS Clinical Trials (2007) Vol. 2, No. 4, e11	

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

1	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	บุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์)
	ชื่อโครงการ	จากสำนักงานเสริมสร้างเอกลักษณ์ของชาติ
	ปีที่ได้รับ	2547
2	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	เมธีวิจัยอาวุโส สกว.
	ชื่อโครงการ	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ปีที่ได้รับ	2547-2550
3	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	เป็นผู้ประสานงานฝ่ายไทยในการจัด Joint Workshop on Modern Analytical Science ระหว่างสหรัฐ-ไทย
	ชื่อโครงการ	ได้รับทุนของ NSF, สกอ.และ สกว.
	ปีที่ได้รับ	
4	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	นักวิจัยดีเด่น
	ชื่อโครงการ	รางวัลมหาวิทยาลัยเชียงใหม่ ‘ ช้างทองคำ ’
	ปีที่ได้รับ	2550

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

5	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	กรรมการสภาวิจัยแห่งชาติ สาขาวิทยาศาสตร์เคมีและเภสัช
	ชื่อโครงการ	สำนักงานคณะกรรมการวิจัยแห่งชาติ
	ปีที่ได้รับ	(2545-ปัจจุบัน)
6	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะอนุกรรมการสำนักงานคณะกรรมการอุดมศึกษาในการจัดทำ software เกี่ยวกับข้อมูลในการวิจัย
	ชื่อโครงการ	สำนักงานคณะกรรมการการอุดมศึกษา
	ปีที่ได้รับ	(2547-ปัจจุบัน)
7	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะอนุกรรมการสำนักงานคณะกรรมการอุดมศึกษาเกี่ยวกับการจัดการทุนการศึกษาชั้นสูงเชิงกลยุทธ์ เพื่อสร้างเครือข่ายวิจัยระดับแนวหน้า
	ชื่อโครงการ	สำนักงานคณะกรรมการการอุดมศึกษา
	ปีที่ได้รับ	(2547-ปัจจุบัน)
8	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ผู้ประสานงานศูนย์ความเป็นเลิศทางวิชาการโครงการพัฒนาการลดขนาดเพื่อการวิเคราะห์ระดับไมโครและนาโน
	ชื่อโครงการ	คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่
	ปีที่ได้รับ	

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

9	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ผู้ประสานงานห้องปฏิบัติการวิจัยเพื่อพัฒนาเครื่องมือวิเคราะห์
	ชื่อโครงการ	สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยเชียงใหม่
	ปีที่ได้รับ	
10	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะกรรมการผู้ทรงคุณวุฒิพิจารณาตำแหน่งทางวิชาการสภามหาวิทยาลัยแม่โจ้
	ชื่อโครงการ	มหาวิทยาลัยแม่โจ้
	ปีที่ได้รับ	(2549-ปัจจุบัน)
11	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ผู้ประสานงานโครงการวิจัยร่วมมือกับสถาบันในต่างประเทศ เช่น (Karlsruhe Research Center (เยอรมนี); University of Washington, Seattle
	ชื่อโครงการ	
	ปีที่ได้รับ	
12	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ที่ปรึกษาเครือข่าย สวทช. ภาคเหนือ
	ชื่อโครงการ	สวทช.
	ปีที่ได้รับ	(2545-ปัจจุบัน)

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

13	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ที่ปรึกษาการวิจัยคณะวิทยาศาสตร์ มหาวิทยาลัยอุบลราชธานี
	ชื่อโครงการ	มหาวิทยาลัยอุบลราชธานี
	ปีที่ได้รับ	
14	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะกรรมการพิจารณาทุนมหาวิทยาลัยเชียงใหม่ เพื่อสนับสนุนการไปศึกษาต่อระดับปริญญาเอก ณ ต่างประเทศ
	ชื่อโครงการ	มหาวิทยาลัยเชียงใหม่
	ปีที่ได้รับ	
15	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะทำงานเพื่อพัฒนารูปแบบและระบบการจัดสรรทุนการผลิตและพัฒนาอาจารย์ในสถาบันอุดมศึกษา
	ชื่อโครงการ	สำนักงานคณะกรรมการการอุดมศึกษา
	ปีที่ได้รับ	2549 – ปัจจุบัน
16	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะทำงานสรรหาโครงการวิจัยด้านวิทยาศาสตร์เคมีและเภสัช
	ชื่อโครงการ	สำนักงานคณะกรรมการวิจัยแห่งชาติ (วช.)
	ปีที่ได้รับ	

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

17	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะกรรมการผู้ทรงคุณวุฒิพิจารณาตำแหน่งทางวิชาการสภามหาวิทยาลัยนเรศวร
	ชื่อโครงการ	มหาวิทยาลัยนเศวร
	ปีที่ได้รับ	(2549-ปัจจุบัน)
18	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ผู้ประสานงานโครงการเพิ่มสมรรถนะความแข่งขันกับต่างประเทศของ สกอ.
	ชื่อโครงการ	ในโครงการ “การพัฒนาเครื่องมือและวิธีสำหรับวิทยาศาสตร์การวิเคราะห์ในระดับไมโครและนาโนเพื่อใช้ในทางสุขภาพและสิ่งแวดล้อม”
	ปีที่ได้รับ	(2546 – 2549)
19	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	Editorial Advisory board
	ชื่อโครงการ	ของวารสาร Talanta (Elsevier Publish)
	ปีที่ได้รับ	(2543 - ปัจจุบัน)
20	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	Associate Editor
	ชื่อโครงการ	ของวารสาร Water Research (Elsevier Publish)
	ปีที่ได้รับ	(2546 - ปัจจุบัน)

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

21	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	Editorial board
	ชื่อโครงการ	ของวารสาร Science Asia
	ปีที่ได้รับ	2544 – 2547
22	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	Referee พิจารณา manuscript เพื่อตีพิมพ์
	ชื่อโครงการ	ในวารสาร เช่น Analytica Chimica Acta, Talanta, Journal of Pharmaceutical and Biomedical Analysisและ Analytical Chemistry ซึ่ง
	ปีที่ได้รับ	
23	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	Member, Steering Committee
	ชื่อโครงการ	13th International Conference on Flow Injection Analysis
	ปีที่ได้รับ	24 – 29 เมษายน 2548.
24	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	Co-organizer,
	ชื่อโครงการ	Symposium on Flow based analysis, Pacifichem 2005, Honolulu, USA.
	ปีที่ได้รับ	ธันวาคม 2005,

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

25	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	ได้รับเชิญเป็นคณะกรรมการในการจัดงานประชุม รวมทั้งเป็นผู้บรรยายรับเชิญ และนำเสนอผลงาน
	ชื่อโครงการ	การประชุมวิชาการนานาชาติ The 14th International Conference on flow Injection Analysis(ICFIA 2007) and including related
	ปีที่ได้รับ	ระหว่างวันที่ 3 – 7 กันยายน 2550
26	ชื่อนักวิจัย	ศ. ดร. เกตุ กรุดพันธ์
	ได้รับรางวัล/เกียรติยศ	คณะกรรมการและวิทยากรบรรยายนำ
	ชื่อโครงการ	ในการ ประชุม 10th International Conference on Flow analysis, Porto, Portuga
	ปีที่ได้รับ	
27	ชื่อนักวิจัย	ดร.จรูญ จักร์มุณี
	ได้รับรางวัล/เกียรติยศ	นักวิจัยรุ่นใหม่ดีเด่นสาขาวิทยาศาสตร์และเทคโนโลยี
	ชื่อโครงการ	มหาวิทยาลัยเชียงใหม่
	ปีที่ได้รับ	2549
28	ชื่อนักวิจัย	ผศ.ดร.ศิริวรรณ องค์ไชย
	ได้รับรางวัล/เกียรติยศ	เป็นที่ปรึกษาวิทยานิพนธ์ได้อย่างมีคุณภาพ ทำให้นักศึกษาได้รับวิทยานิพนธ์ดีมาก ของ มหาวิทยาลัยเชียงใหม่ ปี 2549
	ชื่อโครงการ	Investigation of the epitope in chondroitir-6-sulfate recognized by monoclonal antibody W-F-6
	ปีที่ได้รับ	15 มกราคม 2550
	นักศึกษา	นางสาวพีรพรรณ โปธาเจริญ

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

29	ชื่อนักวิจัย	น.ส.รจนา บุระคำ (นักศึกษาปริญญาเอก)
	ได้รับรางวัล/เกียรติยศ	Best Presentation Award (Oral presentation)
	ชื่อโครงการ	RERCH Congress III, Chonbari Thailand.
	ปีที่ได้รับ	2547
30	ชื่อนักวิจัย	ผู้ช่วยศาสตราจารย์ ดร.สุภาภรณ์ ครัดทัพ
	ได้รับรางวัล/เกียรติยศ	รางวัลนักวิจัยรุ่นใหม่ที่มีผลงานตีพิมพ์ดี จาก สกว.และสกอ.
	ชื่อโครงการ	ทุนพัฒนาศักยภาพการทำงานวิจัยของอาจารย์รุ่นใหม่
	ปีที่ได้รับ	2548
31	ชื่อนักวิจัย	ผู้ช่วยศาสตราจารย์ ดร.สุภาภรณ์ ครัดทัพ
	ได้รับรางวัล/เกียรติยศ	นักศึกษาในความดูแลได้รับรางวัลชนะเลิศการเสนอผลงานแบบโปสเตอร์
	ชื่อโครงการ	ในงานวันเสนอผลงานทางวิชาการของนักศึกษาปี 4
	ปีที่ได้รับ	2548
32	ชื่อนักวิจัย	รศ.ดร. อรวรรณ ชัยลภากุล (หัวหน้าโครงการ)
	ได้รับรางวัล/เกียรติยศ	Best article Award สำหรับ"Cost-effective flow cell for the determination of malachite green and leucomalachite green at a
		diamond thin-film electrode"
	ชื่อโครงการ	Development of Boron-doped Diamond Thin film by Anodization
	ปีที่ได้รับ	พฤศจิกายน /2006

ตาราง 3 นักวิจัยในโครงการที่ได้รับรางวัลและเกียรติยศในระหว่างรับทุนส่งเสริมกลุ่มวิจัย

33	ชื่อนักวิจัย	ผศ. ดร. ดวงใจ นาคะปรีชา
	ได้รับรางวัล/เกียรติยศ	Exampler Lecturer of the Year 2005
	ชื่อโครงการ	Faculty of Science, Mahidol University
	ปีที่ได้รับ	2548
34	ชื่อนักวิจัย	นางสาวจันทร์เพ็ญ ครุวรรณ์ (นักศึกษาปริญญาโท)
	ได้รับรางวัล/เกียรติยศ	"The Award of Excellence" for great poster presentatior
	ชื่อโครงการ	the 12th International Symposium on Advanced Technology and Application, Masan, Korea
	ปีที่ได้รับ	2-11 พฤศจิกายน 2549

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
1	ชื่อนักวิจัย	ศ.ดร.เกตุ กรุดพันธ์ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	ได้รับทุน คปก. รุ่นที่ 7
	ปีที่ได้รับ	2548
2	ชื่อนักวิจัย	ศ.ดร.เกตุ กรุดพันธ์ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	ได้รับทุน คปก. รุ่นที่ 8
	ปีที่ได้รับ	2549
3	ชื่อนักวิจัย	ศ.ดร.เกตุ กรุดพันธ์ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	ได้รับทุน คปก. รุ่นที่ 9
	ปีที่ได้รับ	2550
4	ชื่อนักวิจัย	ดร. จรูญ จักร์มุณี (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	การพัฒนาเครื่องมือและวิธีทางเคมีไฟฟ้าสำหรับการวิเคราะห์อาหารและเครื่องดื่ม
	ปีที่ได้รับ	2546-2549
5	ชื่อนักวิจัย	ดร. จรูญ จักร์มุณี (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	คณะวิทยาศาสตร์, มหาวิทยาลัยเชียงใหม่
	ชื่อโครงการ	การพัฒนาขั้วไฟฟ้าอ้างอิงและขั้วจำเพาะคลอไรด์สำหรับประยุกต์ในอุตสาหกรรม
	ปีที่ได้รับ	2547

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
6	ชื่อนักวิจัย	ดร. จรูญ จักร์มุณี (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	คณะวิทยาศาสตร์, มหาวิทยาลัยเชียงใหม่
	ชื่อโครงการ	ทุนสนับสนุนการวิจัยจากเงินรายได้คณะวิทยาศาสตร์ เรื่อง การสร้างและทดสอบประสิทธิภาพของระบบย่อยสลายสารด้วยคลื่นอัลตราไวโอเลต
	ปีที่ได้รับ	2547
7	ชื่อนักวิจัย	ดร. จรูญ จักร์มุณี (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สวทช.
	ชื่อโครงการ	ทุนโครงการนำร่องเพื่อส่งเสริมการวิจัยด้านเทคโนโลยีสะอาด
	ปีที่ได้รับ	2549
8	ชื่อนักวิจัย	ดร. จรูญ จักร์มุณี (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา ประจำปี พ.ศ. 2549
	ปีที่ได้รับ	2549-2552
9	ชื่อนักวิจัย	ดร. จรูญ จักร์มุณี (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	ได้รับทุน คปก. รุ่นที่ 9
	ปีที่ได้รับ	2549

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
10	ชื่อนักวิจัย	ดร. สมชัย ลาภอนันต์นพคุณ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	The Postgraduate Education and Research Program in Chemistry (PERCH)
	ชื่อโครงการ	Determination of Flow Injection Procedure for Determination of Amylose in Rice
	ปีที่ได้รับ	2546-2547
11	ชื่อนักวิจัย	ดร. สมชัย ลาภอนันต์นพคุณ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	คณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	Development of Simple System with simple Reagent
	ปีที่ได้รับ	2547-2549
12	ชื่อนักวิจัย	ดร. สมชัย ลาภอนันต์นพคุณ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (สวทช.ภาคเหนือ)
	ชื่อโครงการ	Development of test kit for trans fatty acid in edible oi
	ปีที่ได้รับ	
13	ชื่อนักวิจัย	ผู้ช่วยศาสตราจารย์ ดร.สุภาภรณ์ ครัดทัพ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	
	ชื่อโครงการ	การพัฒนาระบบ FIA/SIA สำหรับบีดอิมมูโนแอสเสย์
	ปีที่ได้รับ	2545-2547
14	ชื่อนักวิจัย	ผู้ช่วยศาสตราจารย์ ดร.สุภาภรณ์ ครัดทัพ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
	ชื่อโครงการ	การพัฒนาระบบวิเคราะห์ขนาดเล็กสำหรับสารบ่งชี้ทางชีวภาพ
	ปีที่ได้รับ	2548-2550

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
15	ชื่อนักวิจัย	ผู้ช่วยศาสตราจารย์ ดร.สุภาภรณ์ ครัดทัพ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สกว.และสกอ.
	ชื่อโครงการ	ทุนพัฒนาศักยภาพการทำงานวิจัยของอาจารย์รุ่นใหม่
	ปีที่ได้รับ	2548
16	ชื่อนักวิจัย	อ. ศุภชัย ชัยสวัสดิ์ (ผู้ร่วมวิจัย)
	ได้รับรางวัล/ทุนวิจัย	
	ชื่อโครงการ	การสร้างเครื่องมือควบคุมคุณภาพเครื่องล้างฟิล์มอัตโนมัติ
	ปีที่ได้รับ	2546-2547
17	ชื่อนักวิจัย	อ. ศุภชัย ชัยสวัสดิ์ (ผู้ร่วมวิจัย)
	ได้รับรางวัล/ทุนวิจัย	
	ชื่อโครงการ	การออกแบบและสร้างเครื่องมือตรวจสอบคุณภาพลำรังสีประจำวันของเครื่องเร่งอนุภาค
	ปีที่ได้รับ	2550
18	ชื่อนักวิจัย	อ.ดร.นิสา ชวพันธุ์ (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	
	ชื่อโครงการ	การออกแบบและสร้างเครื่องมือตรวจสอบคุณภาพลำรังสีประจำวันของเครื่องเร่งอนุภาค
	ปีที่ได้รับ	2550

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
19	ชื่อนักวิจัย	ดร. รจนา บุระคำ (ผู้ร่วมวิจัย)
	ได้รับรางวัล/ทุนวิจัย	ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ (สกว.)
	ชื่อโครงการ	การพัฒนาระบบการแยกโดยอาศัยหลักการไหลที่ทำให้ขนาดเล็กลงเพื่อการเตรียมตัวอย่างแบบออนไลน์
	ปีที่ได้รับ	มิถุนายน 2548 - พฤษภาคม 2550
20	ชื่อนักวิจัย	ผศ.ดร.ศิริวรรณ องค์ไชย (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	The Postgraduate Education and Research Program in Chemistry (PERCH)
	ชื่อโครงการ	ผลของไพลต่อเซลล์กระดูกอ่อน
	ปีที่ได้รับ	ตุลาคม 2548 - กันยายน 2550
21	ชื่อนักวิจัย	อ. ดร. วรรณจันทร์ แสงหิรัญ ลี, อ. ดร.ปิยรัตน์ นิมมานพิภักดิ์, อ. ดร. จีรยุทธ ไชยจารุวณิช,อ. ดร. สุคนธ์ ประสิทธิ์วัฒนเสรี,อ. ภัทรินี ไตรสถิตย์ (นักวิจัย)
	ได้รับรางวัล/ทุนวิจัย	ทุนวิจัยพื้นฐานแบบกำหนดทิศทางเคมีทางยา ทุนสกว.
	ชื่อโครงการ	การใช้เทคนิคใหม่ทางสถิติเพื่อหาการยึดจับระหว่างลิแกนด์ และตัวรับ เพื่อการประยุกต์สำหรับการคัดกรองข้อมูลเพื่อค้นหายาอย่างรวดเร็ว
	ปีที่ได้รับ	2548-2551
22	ชื่อนักวิจัย	รักสกุล แก่นเรณู (นักศึกษาปริญญาเอก)
	ได้รับรางวัล/ทุนวิจัย	Partial support from the Postgraduate Education and Research in Chemistry Program (PERCH)
	ชื่อโครงการ	Development of Flow Injection and Seqvential Injection System for the Determination of Tetracycline, Oxytetracycline and Chlortetraoycline
	ปีที่ได้รับ	2546-2548
23	ชื่อนักวิจัย	รักสกุล แก่นเรณู (นักศึกษาปริญญาเอก)
	ได้รับรางวัล/ทุนวิจัย	Royal Golden Jubilee Ph.D. Program (RGJ)
	ชื่อโครงการ	Development of a Coat Effective Micro-Total Analysis System using Lab-at-Valve Approach
	ปีที่ได้รับ	2548-2550

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
24	ชื่อนักวิจัย	ทินกร กันยานี (นักศึกษาปริญญาเอก)
	ได้รับรางวัล/ทุนวิจัย	Full support from The Postgraduate Education and Research in Chemistry Program (PERCH) and Development and Promotion of
		science and Technology Talent (DPST)
	ชื่อโครงการ	Scholarship from the Institute for Promotion of Teaching Science and Technology
	ปีที่ได้รับ	2546-2548
25	ชื่อนักวิจัย	น.ส.กรุณา ใจนนถีย์ (นักศึกษาปริญญาเอก)
	ได้รับรางวัล/ทุนวิจัย	โครงการพัฒนาและส่งเสริมผู้มีความสามารถพิเศษ ทางวิทยาศาสตร์ และเทคโนโลยี: พสวท
	ชื่อโครงการ	การใช้ Chemometrics กับงานทางเคมีวิเคราะห์
	ปีที่ได้รับ	2547-2552
26	ชื่อนักวิจัย	น.ส.สุภาดา คนยัง (นักศึกษาปริญญาโท)
	ได้รับรางวัล/ทุนวิจัย	Full support from the Postgraduate Education and Research in Chemistry Program (PERCH)
	ชื่อโครงการ	Development of Stopped-Flow Injection System for Screening of Thalassemia
	ปีที่ได้รับ	2546-2549
27	ชื่อนักวิจัย	น.ส.นภาพร วรรณาพรม (นักศึกษาปริญญาโท)
	ได้รับรางวัล/ทุนวิจัย	Full support from the Postgraduate Education and Research in Chemistry Program (PERCH)
	ชื่อโครงการ	
	ปีที่ได้รับ	2548-2550

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
28	ชื่อนักวิจัย	รศ.ดร. อรวรรณ ชัยลภากุล (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	ทุนเมธีวิจัย สกว.
	ชื่อโครงการ	Development of Boron-doped Diamond Thin film by Anodization
	ปีที่ได้รับ	2547-2550
29	ชื่อนักวิจัย	รศ.ดร. ศุภลักษณ์ ศรีจารนัย (หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานคณะกรรมการวิจัยแห่งชาติ หมวดเงินอุดหนุนทั่วไป มหาวิทยาลัยขอนแก่น
	ชื่อโครงการ	การศึกษาการเปลี่ยนแปลงทางธรณีเคมีของน้ำใต้ดินด้วยการจัดการการแพร่กระจายตัวของดินเค็ม-น้ำเค็มบริเวณลุ่มน้ำพอง-น้ำชี พื้นที่ผลกระทบ
		ท้ายเขื่อนอุบลรัตน์ จ.ขอนแก่น
	ปีที่ได้รับ	2547- 2548
30	ชื่อนักวิจัย	รศ.ดร. ศุภลักษณ์ ศรีจารนัย (ผู้ร่วมวิจัย)
	ได้รับรางวัล/ทุนวิจัย	สำนักงานคณะกรรมการวิจัยแห่งชาติ หมวดเงินอุดหนุนทั่วไป มหาวิทยาลัยขอนแก่น
	ชื่อโครงการ	การศึกษาผลกระทบของการชะล้างเกลือตามธรรมชาติและการผลิตเกลือต่อคุณภาพน้ำในแม่น้ำมูล-น้ำชี
	ปีที่ได้รับ	2546 - 2547
31	ชื่อนักวิจัย	ดร. ดวงใจ นาคะปรีชา (**หัวหน้าโครงการ)
	ได้รับรางวัล/ทุนวิจัย	ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา
	ชื่อโครงการ	การทำไอระเหยเพื่อความจำเพาะเจาะจงในการวิเคราะห์และการออกแบบอุปกรณ์
	ปีที่ได้รับ	กรกฎาคม 2548 ถึง กรกฎาคม 2551

ตาราง 4 นักวิจัยในโครงการที่ได้รับทุนวิจัยอื่นในระหว่างรับทุนส่งเสริมกลุ่มวิจัย		
32	ชื่อนักวิจัย	ดร.วรรณจันทร์ แสงหิรัญ ลี, ดร.ปิยรัตน์ นิมมานพิภักดิ์, ดร.จีรยุทธ ไชยจารุวณิช, ดร.สุคนธ์ ประสิทธิ์วัฒนเสรี, ดร.ภัทรินี ไตรสถิตย์
	ได้รับรางวัล/ทุนวิจัย	ทุนวิจัยพื้นฐานแบบกำหนดทิศทางเคมีทางยา ทุนสกว.
	ชื่อโครงการ	การใช้เทคนิคใหม่ทางสถิติเพื่อหาการยึดจับระหว่างลิแกนด์ และตัวรับ เพื่อการประยุกต์สำหรับการคัดกรองข้อมูลเพื่อค้นหายาอย่างรวดเร็ว
	ปีที่ได้รับ	30 สิงหาคม 2548-28 สิงหาคม2551
33	ชื่อนักวิจัย	ดร.ปิยรัตน์ นิมมานพิภักดิ์
	ได้รับรางวัล/ทุนวิจัย	ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ ทุนสกว.
	ชื่อโครงการ	การศึกษาการจับระหว่างสาร สกัดจากเมล็ดลิ้นจี่กับเอช ไอ วี-1 โพรทีเอส
	ปีที่ได้รับ	31 กรกฎาคม 2547-1 สิงหาคม2549

ตาราง 5 รายชื่อคณะผู้ร่วมวิจัยในโครงการวิจัย "การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล" หัวหน้าโครงการ: ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
1 เกตุ กรุดพันธ์	รศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มช.	หัวหน้าโครงการ	ศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	หัวหน้าโครงการ
2 จรูญ จักร์มุณี	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช.	นักวิจัย	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ
3 ปรัชญา คงทวีเลิศ	รศ.	ภาควิชาชีวเคมี คณะแพทยศาสตร์ มช.	นักวิจัย	รศ.	ภาควิชาชีวเคมี คณะแพทยศาสตร์ มช.	นักวิจัยในโครงการ
4 อรวรรณ ชัยลภากุล	ผศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักวิจัย	รศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักวิจัยในโครงการ
5 ดวงใจ นาคะปรีชา	ผศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักวิจัย	รศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักวิจัยในโครงการ
6 พลยุทธ ศุขสมิติ	นักวิทยาศาสตร์	สำนักงานอุตสาหกรรมพื้นฐานและการเหมืองแร่ เขต 3	นักวิจัย	นักวิทยาศาสตร์	สำนักงานอุตสาหกรรมพื้นฐานและการเหมืองแร่ เขต 3	นักวิจัยในโครงการ
7 ศุภลักษณ์ ศรีจารนัย	ผศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักวิจัย	รศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักวิจัยในโครงการ
8 สุนันทา วังกานต์	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัย	ผศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ
9 สมชัย ลาภอนันต์นพคุณ	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช.	นักวิจัย	ผศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มช.	นักวิจัยในโครงการ
10 สุภาภรณ์ ครัตทัพ	ผศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัย	รศ.	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ
11 รัตติกาล จันทิวาสน์	นักวิจัย	สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี มช.	นักวิจัย		Postdoc Louisina State University	
12 นิสา ชวพันธุ์	อาจารย์	ภาควิชารังสีวิทยา คณะแพทยศาสตร์ มช.	นักวิจัย	อาจารย์	ภาควิชารังสีวิทยา คณะแพทยศาสตร์ มช.	นักวิจัยในโครงการ
13 ศุภชัย ชัยสวัสดิ์	อาจารย์	ภาควิชารังสีวิทยา คณะแพทยศาสตร์ มช.	นักวิจัย	อาจารย์	ภาควิชารังสีวิทยา คณะแพทยศาสตร์ มช.	นักวิจัยในโครงการ
14 ศิริวรรณ องค์ไชย	ผศ.	ภาควิชาเคมี คณะแพทย์ศาสตร์ มช	นักวิจัย	ผศ.	ภาควิชาเคมี คณะแพทย์ศาสตร์ มช	นักวิจัยในโครงการ
15 วรรณจันทร์ แสงหิรัญลี	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัย	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ
16 จิรยุทธ ไชยจารุวาณิช	อาจารย์	ภาควิชาวิทยาการคอมพิวเตอร์ คณะวิทยาศาสตร์ มช	นักวิจัย	ผศ.	ภาควิชาวิทยาการคอมพิวเตอร์ คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ
17 ปิยรัตน์ นิมมานพิภักดิ์	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัย	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ

ตาราง 5 รายชื่อคณะผู้ร่วมวิจัยในโครงการวิจัย "การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล" หัวหน้าโครงการ: ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
18 สุคนธ์ ประสิทธิ์วัฒนเสรี	อาจารย์	ภาควิชาสถิติ คณะวิทยาศาสตร์ มช	นักวิจัย	ผศ.	ภาควิชาสถิติ คณะวิทยาศาสตร์ มช	นักวิจัยในโครงการ
19 ทินกร กันยานี *		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	
20 รจนา บุระคำ *		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	-
21 ขวัญจิต มณีอ่อน *		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยทักษิณ	-
22 อรวรรณ ถือเงิน *		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยนเรศวร	-
23 สิริพัชร์ สุธีรภัทรานนท์		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยแม่ฟ้าหลวง	
24 สุมาลี ธนิกุล		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี	
25 เสาวภา เมืองแก้ว		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์	
26 สุพรรณี ดวงทอง		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์	
27 ณรงค์ เล่งฮ้อ		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	ผช.กรรมการผู้จัดการ	บริษัท ไทยยูนีค จำกัด	
28 กาญจนา วัดละเอียด*(1,2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยแม่ฟ้าหลวง	
29 สราวุฒิ สมนาม*(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยราชภัฏเชียงใหม่	
30 วศิน วงศ์วิไล*		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท	นักวิจัย	สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี มช.	นักวิจัยในโครงการ
31 วัสกา ศิริอังคาวุธ (2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
32 รักสกุล แก่นเรณู (1)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
33 จรูญ จันทร์สมบูรณ์ (2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
34 เกรียงไกร พลหงษ์ (1)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก

ตาราง 5 รายชื่อคณะผู้ร่วมวิจัยในโครงการวิจัย "การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล" หัวหน้าโครงการ: ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
35 สำอาง ศุภฤกษ์ (1)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
36 สายฝน จันผกา (2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
37 กรุณา ใจนนถีย์ (4)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
38 สุภาดา คนยัง **(2,3)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาเอก
39 พิพูน บุญเป็ง* (2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		-	สำเร็จการศึกษาแล้ว
40 ศิลา กิตติวัชนะ **		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		Bristol University, UK	นักศึกษาปริญญาเอก
41 อภิชาต บุญมาลัย*(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		-	สำเร็จการศึกษาแล้ว
42 ดวงพร โสมประยูร*(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		-	สำเร็จการศึกษาแล้ว
43 สุภาภรณ์ กสิวัฒน์*		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		-	สำเร็จการศึกษาแล้ว
44 นภาพร วรรณาพรม**(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาเอก
45 วริศรา คชสิทธิ์*(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		-	สำเร็จการศึกษาแล้ว
46 โสภา ตนตรง(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท
47 วิษณุ แจ้งใบ(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท
48 วรทิพย์ ศรีเปารยะ(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท
49 พรทิวา นันตาบุญ(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	นักศึกษาปริญญาโท
50 ฐิติรัตน์ มานทิม*		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	สำเร็จการศึกษาแล้ว
51 จันทร์เพ็ญ ครุวรรณ์ *(2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	สำเร็จการศึกษาแล้ว

ตาราง 5 รายชื่อคณะผู้ร่วมวิจัยในโครงการวิจัย "การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล" หัวหน้าโครงการ: ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
52 ณัฐวุฒิ เชิงชั้น*(1,2,5)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดก	สำเร็จการศึกษาแล้ว
53 เบญจมาภรณ์ พรหมทอง* (2)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักศึกษาปริญญาโท	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยอุบลราชธานี	สำเร็จการศึกษาแล้ว
54 มะลิวรรณ อมตธงไชย*(1,2,5)		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	นักศึกษาปริญญาเอก	อาจารย์	ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล	สำเร็จการศึกษาแล้ว
55 วีณา เสียงเพราะ		ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาเอก		ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	สำเร็จการศึกษาแล้ว
56 วนิดา วอนสวัสดิ์		ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาเอก
57 สราวดี กอศรีสกุล		ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาโท
58 อภิชัย แสนตลาด		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาเอก		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาเอก
59 จิราภรณ์ โฮมวงศ์		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท
60 วรางคณา สุขสม		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท
61 ทรงพร จึงมั่นคง		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท
62 ณัฐพร บุตรวงศ์		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท
63 สุธาสินี บุญเชียงมา		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท
64 ปฑิตตา ราชครุฑ		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท
65 จิลาดา วิชาผง		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท		ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น	นักศึกษาปริญญาโท

* นักศึกษาสำเร็จการศึกษาแล้ว

** นักศึกษาที่ศึกษาต่อ

ตาราง 5 รายชื่อคณะผู้ร่วมวิจัยในโครงการวิจัย "การพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยเทคนิคที่ใช้การไหล" หัวหน้าโครงการ: ศาสตราจารย์ ดร. เกตุ กรุดพันธ์

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน

1 = นักศึกษาทุน คปก.

2 = นักศึกษาทุนโครงการพัฒนาบัณฑิตศึกษาและวิจัยทางเคมี (PERCH-CIC)

4 = นักศึกษาทุนโครงการ พสวท.

หมายเหตุ

สังกัด หมายถึง ภาควิชา/คณะ/มหาวิทยาลัย ในกรณีที่อยู่หน่วยงานอื่น ให้ใส่รายละเอียดที่อยู่ต้นสังกัด

สถานภาพปัจจุบัน หมายถึง ยังอยู่ในโครงการหรือไม่ หรือหากออกจากโครงการแล้ว ขณะนี้สังกัดหน่วยงานใด หรือย้ายไปทำงานที่ใด

ภาคผนวก ก

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Some recent developments on cost-effective flow-based analysis☆

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Received 19 April 2004; received in revised form 2 July 2004; accepted 2 July 2004
Available online 18 September 2004

Abstract

This paper reviews some recent developments on cost-effective flow-based analysis. They include the newly developed Lab-at-Valve (LAV), concepts in using the stopped-flow injection approach, on-line sample pretreatment systems, including bead injection–flow injection and flow injection–ion-chromatography, systems for size-based speciation, and cost-effective reagents. Applications and advantages of such techniques are discussed.
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Keywords: Lab-at-Valve (LAV); Stopped-flow injection; Sample pretreatment; Bead injection; Sequential injection; Flow injection; Size-based speciation; Cost-effective reagent

1. Introduction

This paper describes some recent developments of techniques aiming for cost-effective analysis related to flow injection analysis (FIA). It is based on a presentation in the 12th International Conference on Flow Injection Analysis (12th ICFIA) including related techniques, which was dedicated to Professor Gary and Mrs. Sue Christian and also for the celebration of the 20th Anniversary of the Japanese Association for Flow Injection Analysis (JAFIA). Some of the developments were initiated and/or resulted from discussion during the previous ICFIAs. Some are from Talanta, which provides good geographical distribution in contributed papers.

Cost-effectiveness is always a consideration in performing an analysis, not only in remote places where only limited budget is available, but also in general practice [1,2]. To select a scheme for an analysis, apart from analytical characteristics of accuracy, precision, sensitivity and selectivity, and real-time analysis, the cost of analysis should also be taken into account (Fig. 1).

When considering cost of an analysis, this should include analysis time, chemical(s)/reagent(s) consumption, and instrument. The last one includes cost of the system itself, operating cost, and cost dealing with maintenance.

Flow-based analytical techniques, which offer various advantages, with the main features being high sample throughputs and much less samples/reagents consumption, and simpler instrumentation, should be suitable to serve the above considerations [2–28].

Flow injection analysis introduced in the early stage by Ruzicka and Hansen, already demonstrated well the cost-effectiveness along with high efficiency in various routine analyses in Brazil [3,4].

Sequential injection analysis (SIA) provides automation, being possible with relatively simple instrumentation, compared to other techniques [4,13,14,29–38].

Systems with cheap components in a remote place may be very valuable in research training, in investigating and demonstrating novel concepts for further development [39,40]. Examples are an overhead projector FIA set-up for demonstrating concepts [41], using an aquarium pump in various simple flow injection (FI) systems [42]: offering practical advantages in sample handling [43] for those with FI–AAS (e.g., the determination of Pb [44,45]), and for in-valve mini-column for determination of ppb-levels of ura-

☆ Presented at the 12th International on Flow Injection Analysis, December 7–13, 2003, Venezuela.

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doi:10.1016/j.talanta.2004.07.046

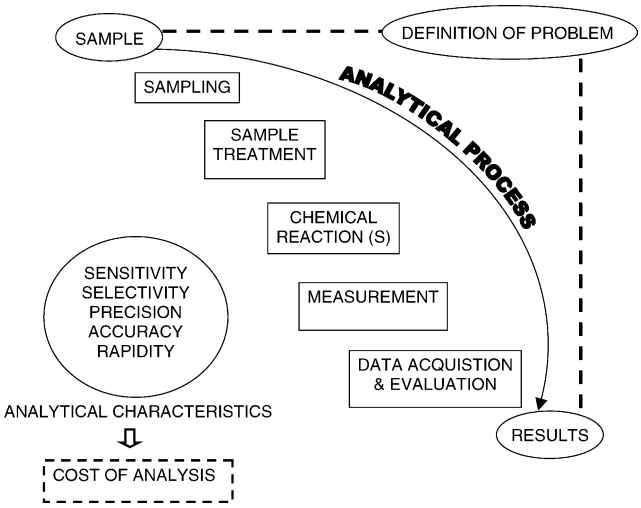

Fig. 1. Analytical process.

nium [46]. Also, such simple systems make single standard calibration possible [44–46]. By applying even simple systems, procedures become much easier, compared to the batch ones, for example, the dissolved oxygen determination by Winkler's method [47,48] and FI with on-line column system for hemoglobin typing leading to thalassemia screening [49].

Simple flow systems with some detection units offer various degrees for automation, depending on the various kinds of components of instruments involved [4,40,50–56], for example, the determination of acid concentration or acidity can be performed colorimetrically using visual detection [41], a simple peak-hold colorimeter [57], or using a very simple conductivity detector [58,59] or a computer-controlled detector [60–63]. Simple FI manifolds with radioactivity detection systems [64,65] offer basic automation using flow-based techniques. Higher degrees in automation and other advantages by employing sequential injection (SI) systems have proven to be useful to real applications for radioactivity monitoring [66–68]. Relatively simple FI and SI set-ups with dynamic surface tension detectors have proven to be very useful and fast for the economical study of some interfacial properties [69,70]. A very simple manifold with a light scattering detector using a laser pointer has been applied for nephelometric determination of sulfate [71]. Various simple FI systems for iodide determination have been reported [72–76].

2. Novel concepts for cost-effective flow-based systems

Some novel concepts have been investigated.

2.1. Sequential injection with "Lab-at-Valve" (LAV) concept

In the past decade, the field of miniaturization in chemical analysis has gained increased interest [77]. The miniaturization toward Lab-on-Chip (LOC) or micro total analysis system (μTAS) concepts includes assembly as an in integrated device, consisting of sample collection, sample pretreatment, and separation and/or chemical reaction taking place for the analytical detection, and the sensor.

Ruzicka [78] introduced a SI with "Lab-on-Valve" (LOV) system to perform analyses in micro- or nanoliter volumes by integrating sample processing, chemical reaction, and monitoring, in a conduit at a multiposition valve. A LOV system for such the above mentioned operation is a precisely fabricated monolithic structure mounted atop a conventional multiposition selection valve, by using computer-aided design (CAD). There have been various applications of LOV with various advantages [4,14,38,53,79–84], for example environmental monitoring [38,82,83], pharmaceutical applications [22], and bio- and clinical analysis [78,84].

A "Lab-at-Valve" concept has been introduced as an alternative cost-effective μTAS device. Instead of replacing a stator plate of a multiposition selection valve by a perfectly machined piece, as that of the "LOV", sample processing and detection unit(s) are attached or plugged onto port(s) of a commercial conventional multiposition selection valve without taking apart any component out of such a purchased valve.

This simpler approach has been demonstrated by SI–LAV potentiometric determination of chloride. A simple LAV flow-through electrode system, consisting of two simple laboratory-made Ag/AgCl electrodes, is plugged onto a port of a purchased multiposition selection valve (Fig. 2). Based on concentration cell behavior, chloride in a sample can be assayed [85].

SI–LAV solvent extraction has been proposed. Such a simple system was demonstrated for spectrophotometric determination of diphenhydramine hydrochloride and anionic surfactant (Fig. 3). Sample, reagents, and organic solvent are sequentially aspirated into a holding coil. By flow reversal, good extraction efficiency can be achieved. After that, the aqueous and organic phases are separated in a conical extraction chamber attached at one port of a conventional multiposition selection valve ("Lab-at-Valve" concept). The organic solution is then pushed into a flow cell of the spectrophotometer for detection of the extracted colored product.

2.2. New criteria for using a stopped-flow injection system

Stopped-flow injection analysis was proposed by Ruzicka and Hansen, for two different purposes for operating a stopped-FIA system: (1) to increase the sensitivity of measurement by increasing the residence time and thus the yield of measured species or (2) to measure a reaction rate serving a base for the analytical read-out. By stopping a flow so that the analyte and reagent(s) stay for a period at a flow-cell [86], progress of a reaction involved is continuously monitored, during the period of the stopped-flow. Various advantages can be obtained by the involvement of kinetic considerations

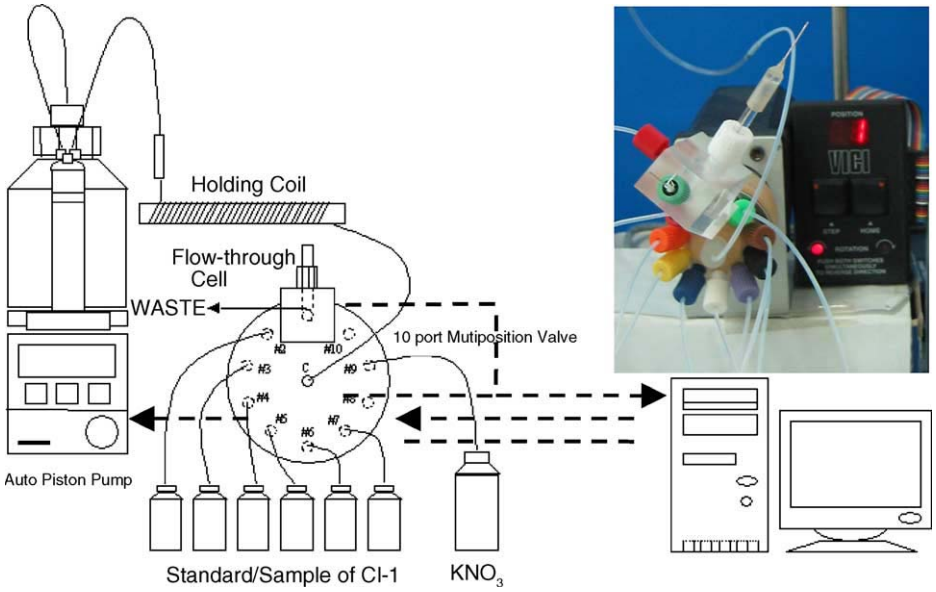

Fig. 2. SI–Lab-at-Valve (LAV) system for potentiometric determination of chloride (the inserted photo shows a close-up of the potentiometric cell (attached to the multiposition valve)).

such as kinetic information, increase in sensitivity, and kinetic separation [87].

A simple semi-automatic stopped-FI analyzer was developed (Fig. 4) [88]. One application example proposed by our group was for the simultaneous determination of phosphate and silicate by employing their differences in kinetic behaviors.

Optimization of such a stopped-FI system involves various parameters, both hardware and non-hardware, similar to the optimization in usual conventional FI systems. Hardware parameters normally are: tubing i.d., sample (loop) volume, mixing coil length and i.d., and flow through cell volume; while non-hardware ones involve: flow-rate, traveling time, stopping time, washing time, and concentration of reagents.

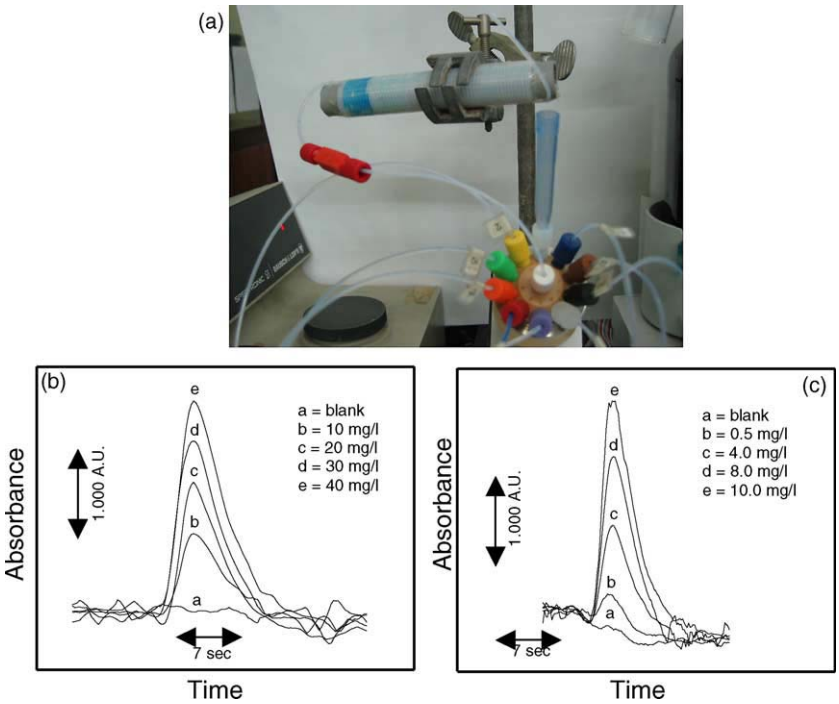

Fig. 3. SI–LAV solvent extraction spectrophotometric determination: (a) the LAV extraction system, (b) signal profiles obtained for the determination of diphenhydramine hydrochloride using bromocresol green [100], (c) SI grams for anionic surfactant determination (the methylene blue method using sodium dodecylsulfate as the standard).

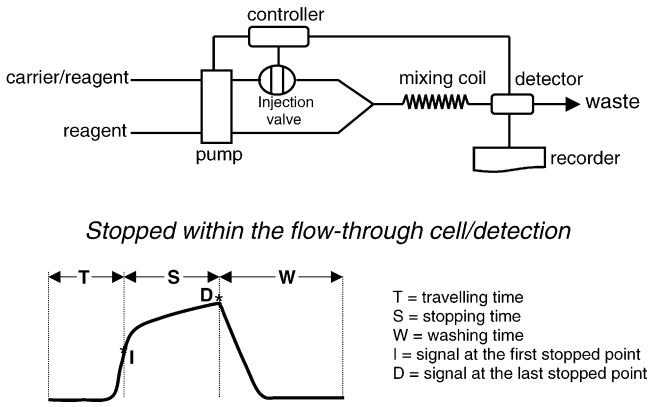

Fig. 4. Conventional stopped-FIA [88]; *T*, traveling time: a period from the injection till the flow stops; *S*, stopping time: a period of flow-stopping; *W*, washing time: a period after the flow is re-started until the next injection.

The optimization is concerned with slope in association with sensitivity (see Fig. 4).

For a conventional FI system, optimization is usually aimed for maximum sensitivity and high sample throughputs. Conventional optimization would involve the hardware parameters: tubing i.d., sample (loop) volume, mixing coil length and i.d., flow through cell volume; and the non-hardware ones: flow-rate and reagent concentration.

Now a concept for stopped-flow injection system has been investigated. By using a semi-automated stopped-flow injection analyzer being able to control pump (on–off) and switching an injection valve, after an analyte solution is injected into a carrier stream of reagent, the flow is stopped at a mixing coil for a period before allowing to flow further to the flow-through cell in a simple colorimeter as a detector. Fig. 5 illustrates the proposed concept of FIA using stopped-flow outside a detection cell. Optimization can be made by varying some parameters such as traveling time, stopping period, flow-rate, and reagent concentrations, without changing any hardware component such as injection loop (injection volume), and mixing coil length and i.d.

Ruzicka and Hansen suggested [86] to stop the flow outside the detector in order to gain reaction time and thereby generation of product to be measured. By our investigation, this would help promotion the mixing, but minimize dispersion of the product. Such a concept was demonstrated for chloride determination (Fig. 6) using mercury (II) thiocyanate and Fe(III) reagents, which were used in the previous conventional FI procedure [89]. Without changing the hardware parameters, but varying only non-hardware parameters, determination of nitrite (using well-known coupling of diazotized sulfanilamide with *N*-(1-naphtyl)ethylenediamine dihydrochloride) and determination of phosphate (using phosphomolybdovanadic acid method) can be performed. This

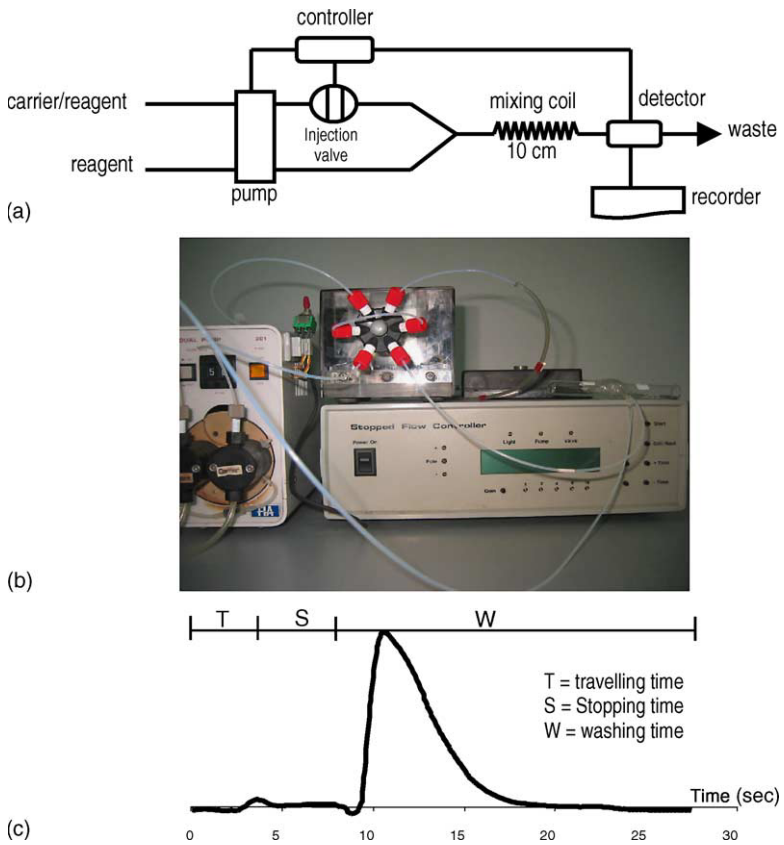

Fig. 5. A new concept stopped-FI: the flow manifold; (a) schematic diagram, (b) the picture, and (c) FI gram profile obtained, when stopping the flow at the mixing coil outside the flow through cell.

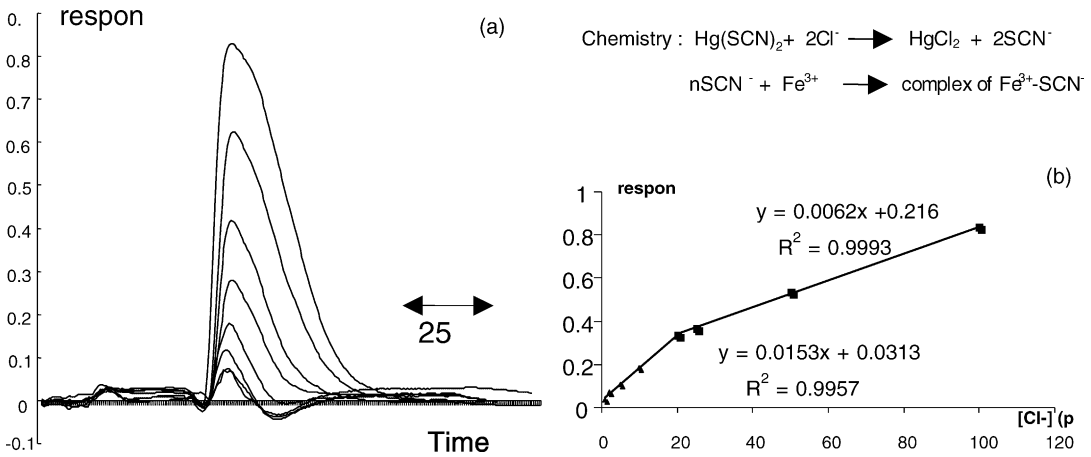

Fig. 6. The determination of chloride using the new proposed stopped-FI concept, by stopping the flow at the mixing coil (T = 1.9 s, S = 1 s, W = 15 s; a throughput of 120 injection/h): (a) FI gram profiles and (b) calibration graph (note: two breaks of the linear calibration ranges may be due to formation of different complexes for higher concentrations of chlorides).

stopped-FI approach offers various advantages, even for a determination involving fast reaction. The advantages include possibility of using only on FI analyzer with fixed components (hardware parameters) for various analytes without changing any hardware component, better sensitivity than conventional FIA, and less amounts of reagents consumption. All lead to economical aspects. This stopped-FI can be used for both slow and fast reactions.

3. On-line sample pretreatment

Sample pretreatment including preconcentration and preseparation, is an important step in analytical process (Fig. 1).

Flow-based techniques offer on-line sample pretreatment to gain advantages in less sample and reagent consumption, and possibility of automation with less sophisticate instrumentation.

Bead injection (BI) was introduced as a technique that utilizes beads as a solid surface to trap species of interest, to accommodate a chemical reaction and to transport them through the flow line or to a detection unit [33]. The renewal beads overcome the difficulty in finding suitable eluent to elute sorbed metal ions from resins, as the used beads will be discarded after each run [90].

3.1. BI with SI

This has proven to be very useful for sample preconcentration and/or preseparation for the determination of heavy metal ions in complicated matrix samples. The BI–SI can be useful to automate sample pretreatment for various types of detectors, including ET–AAS [33,79–84,91].

3.2. BI with FI

This offers an alternative for cost-effective operation for trace levels determinations, such as iron in water samples [92] and in beer samples [90]. Simple instrumentation (without a computer control) and single standard calibration are advantageous.

3.3. FI–ion-chromatograph (IC)

FI system with in-valve mini-column for sample pretreatment coupled to a simple ion-chromatograph with conductivity detector without suppressor for simultaneous determination of some cations (cadmium, lead, and zinc) in zinc ore samples, which have high amounts of matrix interference, has been proposed. The on-line sample matrix interference removals offer advantages including elimination of costly and time-consuming off-line operations, reducing IC column damage (i.e., prolong the column life-time) in addition to the simple instrumentation employed, and the normal advantages usually offered by FIA [93].

3.4. Size-based speciation

Speciation of a substance depending on its size, which has been termed as “size-based speciation”, has gained much of interest in various studies, especially in environmental aspects. Studies on combining field-flow fractionation (FFF) with analytical detection systems have been made. Some cost-effective flow-based systems have been investigated such as gravitational FFF (GrFFF)–FI chemiluminescence for Fe size-based speciation, and GrFFF–ET–AAS for size-based speciation applied to soil sample [94]. Flow FFF (FIFFF)–ICP–MS has been used for size-based speciation of some heavy ions in colloid formation of metal ions released from contaminated soil [95].

4. Cost-effective reagents

Additional advantage that flow-based systems are operated in a closed system (e.g., FIA, SIA), is that an unstable

reagent, especially one being air- or light-sensitive, can be employed.

Also, the chemicals/reagents which are not necessary to be in very high purity grade can be utilized in a flow-based technique since an analyte to be determined is to be run in the exactly the same conditions with that of the standard.

Murexide is a very common chemical in a laboratory but it is unstable in basic solution. However, it can be used in a spectrophotometric determination for Ca by a simple FI set-up [96].

$KMnO_4$, a very cheap and easily available chemical, can be used as an oxidant for an analyte with reducing species properties. Its color intensity change in the redox reaction involved can be used by employing FI [58] or SI [60] systems, whereas a batch procedure is not possible due to that it is sensitive to air and light. Pharmaceutical applications such as ascorbic acid assay in Vitamin C tablets have been reported.

Recently, aspirin, an antipyretic powder, has been proposed as a salicylate reagent, which is cheap and easily available, for the determination of Fe(III). A red Fe(III)–salicylate complex product can be monitored. An application to assay iron contents in pharmaceutical preparations has been made [97].

Solid reactants have been employed as different types of reaction columns for sulfate and sulfur dioxide [98]. Phloroglucinol has been proposed as alternative reagent, which would provide better cost-effective procedures for nitrite and nitrate determinations [99].

5. Conclusion

Sequential injection systems with Lab-at-Valve is an alternative cost-effective micro-total analysis system approach. A stopped-FI analyzer with a new concept in optimization offers the possibility for different analytes to be determined using only one fixed set-up of hardware parameters (i.e., only one instrument), while only non-hardware parameters are to be optimized. On-line sample pretreatment with flow-based techniques, such as FI–IC, BI–FI, and FFF–AAS, offers various advantages. Utilization of cost-effective reagents can be possible by using flow-based techniques, especially FI and SI.

Acknowledgements and proclaims

Some of the reported developments resulted from collaboration formed in connection with the International Conference on Flow Injection Analysis (ICFIA). The 12th ICFIA was dedicated to Professor Gary and Mrs. Sue Christian and also for the celebration of the 20th Anniversary of the Japanese Association for Flow Injection Analysis (JAFIA). I thank especially the above-mentioned persons, including Professors S. Motomizu and T. Sakai, for their contributions and generous support. Also thanks are due to those who share contributions to such developments and the networking throughout various geographical areas. Thanks also go to Professors Jose Luis Burguera and Koos van Staden for the excellent organizing of the 12th ICFIA. The Thailand Research Fund (TRF), the Postgraduate Education and Research Program in Chemistry (PERCH), the Post-graduate Education Development Program and the Project on Enhancement of Performance and Competitiveness of the Country (by the Commission for Higher Education of Thailand) are acknowledged for support.

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Bead injection with a simple flow-injection system: an economical alternative for trace analysis

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The use of beads for sample pretreatment, such as preconcentration, isolation, and separation, and for accommodation of chemical reactions has attracted increasing interest. Beads can be used as a removable solid surface in many analytical applications. It is very practical to use beads in a flow-based system, where beads can easily be moved in and out of the system using a flowing stream of reagent(s). This is called the bead-injection (BI) technique, which was first introduced to be used with a sophisticated computer-controlled sequential injection analysis (SIA) system. In many laboratories, a relatively high-cost SIA system is not available, whereas a simple, low-cost, first-generation flow-injection (FI) analysis system is easy to assemble. The development and applications of the BI technique coupled with a lower cost first-generation FI technique is then an alternative. This simple BI–FI system can be a useful tool for pretreatment of samples that are abundant and when there is no need for micro volume control.
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1. Introduction

Sample pretreatment is often a necessary step in analysis of real samples. Depending on the matrices and the sensitivity of the detection system, the type and the degree of sample pretreatment required will be different. In the flow-injection (FI) technique, continuous flow of solutions in a closed system enables on line sample pretreatment. Even though digestion, unlike other sample pretreatment methods, normally requires heating or harsh conditions, on-line sample digestion is possible with different techniques, such as microwave assisted digestion [1,2], stopped flow digestion [3,4] and on-line destruction of samples using radiation [5], oxidizing agents [6,7] and heat [8]. In some cases (e.g., when using a detection technique that has a relatively narrow dynamic range such as atomic absorption spectroscopy (AAS)), dilution of sample is actually needed prior to passing it to the detector. Since concentrations of samples are unknown, batch-wise dilution by trial and error before performing the analysis becomes very inconvenient. On-line dilution using the FI technique, either by sample dispersion [9] or by flow manipulation [10], helps avoid some of the tedious steps encountered in batch processes. In some cases, dilution can also be useful in extending the calibration working range, such as in the FI dialysis-ion chromatographic system (FID-IC) [11]. More commonly, preconcentration and preseparation are needed for samples with trace amounts of analyte, especially when the detector does not have extremely high sensitivity. Among the many preconcentration techniques available (e.g., column [12–17], precipitation [18,19] and dialysis [20,21]), the use of a column packed with materials that can selectively adsorb or absorb the analyte is probably the most widely employed [22]. The column technique allows the use of a single standard for construction of a calibration graph by varying the loading time [16,17]. This single standard calibration technique provides various flexibilities in operation, including eliminating or reducing prearation of a set of standard solutions, use of glassware and time consumption. Isolation and separation are other processes needed for samples that comprise complex matrices that can interfere in the analysis. Conventional solvent extraction requires large amounts of solvent and produces a lot of waste, and flow-based-column solid phase extraction systems are more efficient [23,24]. Extraction of sample can be carried out in a packed column of sorbant material [25], similar to the preconcentration column.

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However, there is a drawback of the packed column in that the surfaces of the sorbant material need to be regenerated after each analysis. It is very important to get rid of memory effects to ensure accuracy and precision. Even though it is useful to reuse the same column over again and again, the washing step is normally extensive (i.e. time consuming) and may require a strong washing solution [26,27], or, in many cases, a suitable washing solution is simply not available, so a replaceable solid surface becomes interesting in many studies [28]. Microbead particles have been used in drug discovery for a long time. Now that there are many different functionalized beads commercially available together with various detection techniques, the applications of beads for preconcentration and isolation of analyte arise [29–37]. For example, the use of beads in bead-based immunoassays can be accomplished using very small diameter beads (i.e. less than 10 μm) and in different forms, such as in a small-size droplet with microelectrodes [38,39] and in a flow system, such as flow cytometry [40] and spectrophotometry [41]. Size, material of beads and modification of functional groups on the bead surface can be selected according to the applications. In systems where direct measurement of analyte is done directly on the bead surface, the properties of the bead material become very important. Beads should not interfere in the analysis in any way (e.g., beads should not scatter or absorb light in the region where analyte is detected, and they should not be decomposed or have their properties changed in the solvent).

2. Bead injection

The bead-injection (BI) technique is the combination of the use of beads with a flowing stream of solution in a FI system. Beads are utilized as solid surfaces to preconcentrate or extract the analyte or to accommodate a chemical reaction. The flowing stream of solution is used to carry beads through the system. There is no need to regenerate the bead surfaces because they are discarded after each use and are replaced by fresh ones. This helps to reduce the risk of contamination, denaturation, and system fouling, and also makes it possible to operate BI in the continuous flow system even if no suitable eluent for bead cleaning is found possible [42].

Early BI techniques were coupled with the sequential injection analysis (SIA) technique [43]. Beads were retained in a specially designed jet ring cell that let only the solution pass through. SIA was introduced in 1990 by Ruzicka and Marshall [44]. The main features of the system are that it could precisely manipulate forward and reverse flow with micro-volumes of samples and reagents, so the sample and reagent flow path could be minimized between the injector and the detector with appropriate mixing. This is possible by using a multi-port valve and a bi-directional pump with computer control. Sample and reagent zones are drawn and stacked into a holding coil. Zones are penetrated and dispersed into each other by reverse flow on the way to the detector. SIA has an increasingly wide range of applications, such as trace chemical determinations, pharmaceutical studies and food chemistry [45–49]. There are two different forms normally employed. Micro-beads may first be trapped in a flow cell or column and then physically or chemically retain the analyte of interest. Upon eluting with a suitable solvent, the solution of the analyte is detected downstream. There are examples of applications with this form of detection using ETAAS [50,51]. Another form is on-bead detection, where the change on the surface of the beads after interacting with the analyte is monitored directly using the detector located at the flow cell, or by transporting the beads into the detector. There are a number of examples of this form of detection using fiber-optic spectrofluorometry [52], fluorescence microscopy [49], ETAAS [53] and Raman spectroscopy [54].

The SIA system has also been further developed into the lab-on-valve (LOV) system that comprises specially designed micro-channels on a piece of plastic integrated as a part of the multi-channel valve [55–57]. The BI technique can be coupled to the SIA–LOV system and can be used in various applications, such as environmental studies [58], immunoassays [59], and biological studies [60]. Beads can be trapped in an adjustable volume channel on the LOV. Now, a commercially available LOV can be adjusted into two different volumes or path-lengths by moving the optical fiber [61]. Detection can be done directly on the beads with the optical fibers that are made to fit the channel or can be done downstream via the eluted sample. The main advantage of the SIA–LOV–BI system is that the micro-miniaturization of on-line sample pretreatment and detection can be done automatically with various types of detectors [62–64].

3. Bead-injection–flow-injection system

In spite of the outstanding advantages of the SIA system, some laboratories cannot afford such relatively high-cost instrumentation, so a simpler FI technique is desirable. The development of a BI–FI system can be very useful for both general and trace analysis where samples and reagents are abundant and when there is no need of micro-volume control. Assays of some contents of environmental samples, foods and beverages, of which high quantities of samples are available, can be made using a simple FI system.

The BI–FI system can be constructed using common materials that can be found in the laboratory. A simple, cost-effective configuration of the cell and simple,

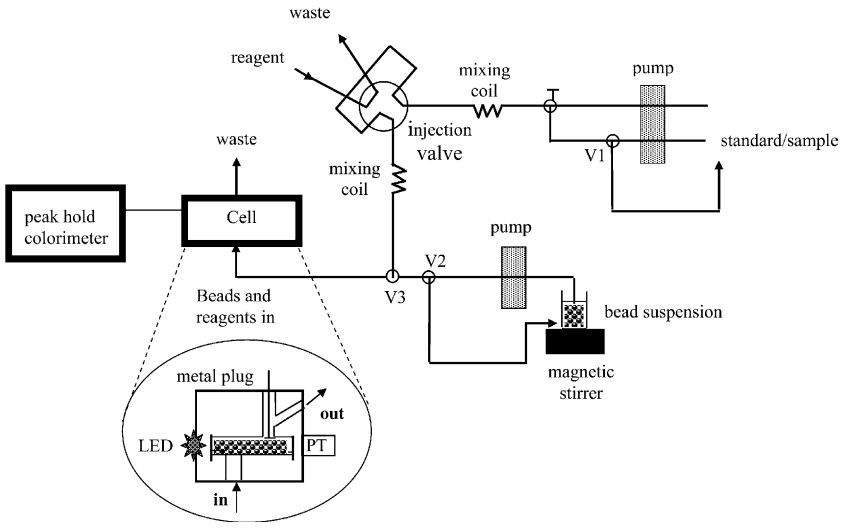

Figure 1. Bead injection with flow-injection system, adapted from Jitmanee *et al.* [65] (V1, V2 and V3 are three-way valves; and, T is a three-way connector).

unidirectional pumps can be used. The cell has the multi-purposes of bead retention, accommodation of chemical reaction (sorption for on-line preconcentration and/or separation and color development) and detection.

Our group has developed an FI–BI system [65], which is depicted in Fig. 1. The bead suspension is pumped into the cell and beads are retained while solution goes through the small space around the metal plug at the end of the cell. When a sample is passed through the cell, an analyte of interest, followed by a color reagent, is loaded onto the surface of the beads, and a color complex is formed. A suitable-wavelength LED and a phototransistor located opposite each other can monitor the intensity of the color complex directly from the surface of the beads. After each run, the used beads can be flushed out by moving the metal plug backward to open the outlet for the beads. The new analytical cycle can then be started. A time-based, single calibration can also be used with this system. There is no need for computer software control. It is also simple to operate and not expensive. The system design and the detector unit can be varied, depending on what components are available.

4. Examples of applications

To demonstrate the application of the BI–FI system, iron was first chosen as a model trace analyte to be used with the system shown in Fig. 1. Chelex-100 resin was used as solid phase and 1,10-phenanthroline was the color reagent used to form a red complex with Fe^{2+} that can be detected at 520 nm. The iron content of various types of water samples (tap, pond and drinking) were successfully determined with the proposed system. The lowest detectable concentrations of Fe^{2+} were 0.90 and 0.45 μmol/L for 3 and 5 min sample loading times, respectively. Both Fe^{2+} and Fe^{3+} were spiked for recovery studies. Fe^{3+} was reduced to Fe^{2+} with ascorbic acid before analysis. Total iron was found as 100–110% recoveries [65].

The application of the system was also extended to determine iron in a complicated sample, such as beer [42]. Even though an interference effect from tannic acid in beer was found, the results from the proposed BI–FI system agreed in all cases with the results obtained from the AOAC standard batch FAAS method and the FI–AAS within the tolerance limit of beer analysis when standard addition was performed.

The sampling rate was 10–20 analyses per hour, depending on sample loading time. The detection limit can be lowered by increasing sample loading time with, however, sacrifice of sample throughput. A precision of 4% RSD was achieved.

Another model trace element, chosen to express sensitivity and selectivity of the system, was copper. In the presence of other elements and matrices, determination of copper in swimming pool water and mineral supplement tablets was achieved with this system [66].

The FI–BI technique can be applied to analyze other ions in different samples by selecting suitable surface modified beads, color reagents and buffers [65–67].

Currently, the number of works published on FI–BI is far fewer than on SIA–BI. The main reason is possibly because SIA–BI was developed with high degree of automation and many applications were demonstrated by the developers to confirm the extended use of a commercial SIA system. However, the FI–BI system also

has potential as an alternative for versatile use in sample pretreatment and as a form of detection, similar to the SIA–BI system. The simpler, lower cost FI–BI system, having no fully automated computer control system, can be advantageous for many laboratories that do not have an SIA system. FI–BI should therefore be used more in the future, and many BI applications described above should be readily adapted to this simple system.

5. Important criteria

In the BI technique, the uniformity and the homogeneity of beads is very important for good precision. Beads should be sieved for suitable sizes and the bead suspension should be kept stirred at a fixed rate at all times of the analysis. The quantity of beads loaded into the cell or the bead loading time need to be optimized in line with the dimension of the cell. Excess beads outside the detection window will adsorb sample and reagent but cannot be detected, and that can yield false negative results, but too few beads cause space in the cell where light scattering may occur or color intensity measurement may not be effective. The concentration of the color reagent also needs to be sufficient for the range of sample concentrations. Bead- and sample-loading times for each analysis should be constant. Other important general guidelines for system design and operation involving the use of column sorption should be followed [22] (e.g., delivering sample by pumping it into the column located down stream is recommended instead of suction of the sample through the column). Tubing connecting from the pumps should be as short as possible to save reagents and to minimize the possibility of sample and reagent sorption on the tubing wall. Flow reversal should be considered to avoid tightening the column packing that may cause back pressure.

6. Conclusion

The BI technique can be used with the first-generation FI technique for successful trace-ion determination. The system is simple and economical without the need of computer technology. Contamination of samples from outside environment and from a previous run is very minute because it is a closed system, with a bead-discarding step done before a new analysis. It is suitable for the analysis of samples that are abundant and do not require micro volume control, such as environmental, food and beverage samples. The applications of the system can be extended using the suitable combination of surface-functionalized beads, buffer solutions and color reagents.

Acknowledgements

Thanks are due to the Thailand Research Fund (TRF), the Commission for Higher Education (CHE) and the Postgraduate Education and Research Program in Chemistry (PERCH) for support.

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Cost-effective flow injection spectrophotometric assay of iron content in pharmaceutical preparations using salicylate reagent☆

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Received 12 February 2004; received in revised form 12 March 2004; accepted 12 March 2004
Available online 27 July 2004

Abstract

A new flow injection procedure for an assay of Fe(III) by using salicylate obtained from antipyretic powder, which is a cheap and easily available reagent, is proposed. A red complex was continuously monitored by a laboratory-made green LED colorimeter. A linear calibration was obtained in the range of 1–20 mg Fe l^{-1} with a detection limit of 0.5 mg Fe l^{-1} and R.S.D.s of 1.4–5.4% ($n = 3$, for 1–20 mg Fe l^{-1}). The new procedure was applied to assay iron contents in pharmaceutical preparations. The results were in good agreement with those of the USP standard method.
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Keywords: Drugs; Flow injection; Iron; Salicylate; Aspirin

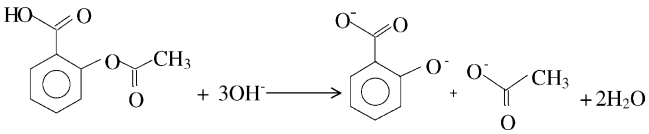
1. Introduction

Iron is a mineral essential to the human body. The average adult has 4–5 g of iron, of which 60–70% is present as heme in the circulating haemoglobin. The human body loses 0.5–1.5 mg iron per day, and sufficient amounts should be ingested (12 mg for adults, 15 mg during pregnancy and lactation and for adolescents, and 7.5–10.5 mg for children, rising to 13.5 mg in 11–14 years old group) [1]. Iron deficiencies are particularly common in premenopausal women, and even college-age women should pay particular attention to the amount of iron in their diets [2].

There are many methods for iron determination in pharmaceutical samples. Of these methods, USP and BP have served as the standard methods for a long time. However, both of the methods are based on a titration technique, which is tedious and involves many steps with consumption of large amounts of chemicals.

Spectrophotometric FI systems have been reported by using different color agents such as 1,10-phenanthroline [3,4], thiocyanate [5], Tiron [6] and 2,2-dipyridyl-2-pyridylhydrazone [7]. The last one was applied for the determination of iron in pharmaceutical samples.

Iron(III) was proposed to be a color agent for a batch spectrophotometric determination of acetylsalicylic acid in aspirin [8,9]. Acetylsalicylic acid in aspirin is hydrolyzed in an alkaline solution to yield salicylate dianion, as represented in Eq. (1).



(1)

Acidification, followed by addition of iron(III) ion, yields a soluble tetraaquasalicylatoiron(III) complex. The intensely purple solution exhibits a strong absorption at 520 nm [9]. In the presence of excess iron, the following complex is formed [9]:

☆ Presented at the 12th International Conference on Flow Injection Analysis, Merida, Venezuela, 7–13 December 2003.

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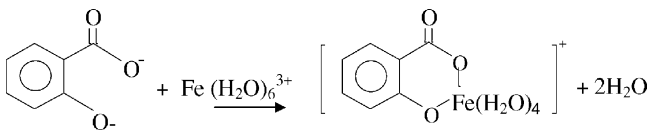
0039-9140/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.talanta.2004.03.067



(2)

But in the presence of excess salicylic acid, as here, the iron can be present as the $Fe(III)(sal)_3$ chelate in solution. The absorption maximum was at 520 nm.

We have investigated the use of such reactions for a simple FI system to assay the iron content in pharmaceutical preparations using cheap salicylate reagent obtained from aspirin. This is a less expensive source of salicylic acid than ACS +99% grade salicylic acid.

2. Experimental

2.1. Chemicals and reagents

All reagents were of analytical grade, unless otherwise stated. Deionised water (Milli Q, Millipore) was used throughout. A stock solution of 1000 mg l^{-1} Fe(III) was prepared by dissolving 0.8774 g of ferric sulphate in a portion of water. Concentrated sulfuric acid (0.5 ml) was added before making up to a volume of 100.00 ml. Working Fe(III) standard solutions were obtained freshly by appropriate dilutions of the intermediate 100 mg l^{-1} Fe(III), obtaining from the stock solution. A salicylate reagent solution (0.01 M) was prepared by dissolving 1.20 g aspirin drug (Tumjai, Osodsapa, commercially available in a drug store in Thailand) in 20 ml of 2 M sodium hydroxide. The solution was placed in a boiling water bath for 15 min before making to a volume of 500 ml with water.

2.2. FI manifold

A laboratory-made flow injection analysis system was employed. The system consisted of a peristaltic pump (Ismatec, Switzerland), a six port injection valve (Upchurch, USA), a home-made flow through cell, a controller and a recorder as shown in Fig. 1. The green LED and the photodiode were used as light source and sensor, respectively. The controller was a laboratory designed and assembled device with microprocessor and was used to control the operation of the pump and injection valve, and to collect and to transfer the data to a recorder.

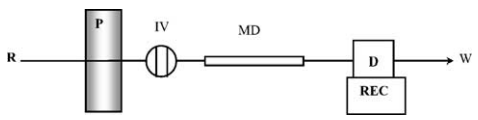


Fig. 1. FI manifold for determination of iron using salicylate: R: 0.01 M salicylate solution, P: peristaltic pump, IV: six-port injection valve, MD: mixing device, D: detector, REC: recorder, W: waste.

A series of Fe(III) standard solutions was injected into the salicylate reagent stream via an injection valve. The complex formed was continuously monitored. Calibration was made by plotting peak height versus Fe(III) concentration.

2.3. Assay of iron in pharmaceutical preparation samples

Pharmaceutical samples were taken from local drug stores. Sample preparation was adapted from AOAC 977.30 [10] as follows. Twenty tablets of a drug sample were weighed and powdered. A portion of the powder, equal in weight to the average of one tablet, was accurately weighed and transferred to a 250 ml flask. A 50 ml portion of water and 2 ml conc. hydrochloric acid were added. The mixture was boiled on a steam bath for 30 min before cooling to room temperature and was diluted with water to a volume of 100.0 ml. The mixture was filtered through Whatman No. 1 paper. A 1 ml aliquot of the filtrate was transferred to a 50 ml volumetric flask, and 0.5 ml of hydrogen peroxide was added before making to a volume of 50 ml with water. The samples were then analyzed by the proposed method.

3. Results and discussion

3.1. FI set optimization

A simple one-line manifold FIA system for iron determination using salicylate is depicted in Fig. 1. The parameters, namely, concentration, flow rate and pH of the salicylate reagent solution, mixing coil type and length were investigated. The injection volume (40 μl) and Fe(III) standard (0.5 mg Fe l^{-1}) were fixed for all studies.

Various concentrations (0.01, 0.05, 0.1 and 0.5 M) of salicylate were tried by using a flow rate of 4.0 ml min^{-1} and a mixing coil length of 100 cm. The higher the concentration of salicylate, the higher was the peak obtained. The Schlieren effect was pronounced when salicylate concentration was 0.05 M or more. Using a 200 cm mixing coil resulted in a similar observation. The mixing coil was replaced by a glass bead column (0.3 mm glass beads packed in 0.3 mm i.d. × 5 cm Tygon tube, in which both ends were plugged with 0.3 mm o.d. × 2.0 cm Tygon tubing for the connection to the system tubings) as a mixing device to promote better mixing [11]. A flow rate of 8.8 ml min^{-1} instead of 4.0 ml min^{-1} was tried, but a lower peak was obtained (0.68 cm instead of 0.75 cm).

A set of conditions was then selected: 0.01 M salicylate reagent, 40 μl injection volume, a glass bead column with the dimension described above and a flow rate of 4 ml min^{-1}.

3.2. Calibration

Using the selected conditions, a linear calibration was obtained in the range of 1.0–20.0 mg l^{-1}: peak height (cm) $= 0.4618[Fe(III)(mg\,l^{-1})] + 0.1627$, $r^2 = 0.9997$. Relative

Table 1
Assay of iron contents in pharmaceutical preparation samples by the proposed FI and USP method [13] (average of triplicate results)

Sample no.	Form of iron	Label (mg per tablet)	USP method		The proposed FI method			
					Modified AOAC 977.30 (HCl alone)[a]		Strong acid digestion (HNO_3 + $HClO_4$) with HCl[a]	
			mg g^{-1}	mg per tablet	mg g^{-1}	mg per tablet	mg g^{-1}	mg per tablet
1	Fumarate	200	459	221	455	219	443	213
2	Fumarate	90	67.7	90	69.8	93	72.0	96
3	Fumarate	200	446	214	436	209	481	231
4	Fumarate	200	390	197	376	190	395	200
5	Fumarate	400	424	415	405	396	400	391
6	Fumarate	200	352	194	355	196	375	208
7	Gluconate	200	509	330	296	192	330	213
8	Gluconate	150	231	161	205	157	179	138
9	Sulphate	200	322	149	438	202	444	205
10	Citrate	470.9	1094	604	688	380	909	502

[a] Sample preparation procedure

standard deviations ($n = 3$) over this range (1–20 mg l^{-1}) varied from 1.4–5.4%. A detection limit (3σ of the blank signal [12]) of 0.5 mg l^{-1} was estimated.

3.3. Application to pharmaceutical preparations

Pharmaceutical samples available in local drug stores were taken. They were in forms of fumarate, gluconate, sulphate and citrate. The sample preparation (Modified AOAC 977.30 procedure) described in the experimental section was employed. The obtained solution was analyzed following the proposed FI procedure. The results are summarized in Table 1.

Another sample preparation procedure with very strong acid digestion was employed for comparison and to confirm that iron was converted into iron(III) and dissolved in the solution. The procedure is as follows. Twenty tablets of a drug sample were weighed and powdered. A portion equal to the weight of one tablet was transferred to a 250 ml flask. A 25 ml volume of water, 15 ml conc. nitric acid and 5 ml conc. perchloric acid were added into it. The mixture was boiled on a hot plate until nearly dry, then cooled to room temperature before adding 4 ml conc. hydrochloric acid and 10 ml water and making to a volume of 100 ml with water. The mixture was filtered through Whatman No. 1 paper. A 1 ml aliquot of the filtrate was transferred to a 50 ml volumetric flask and diluted to the mark with water. It was found that the results from the both sample preparation procedures for FI were in good correlation (Table 1), except for the citrate form (sample #10). This indicates that iron in the sample can be quantitatively converted into free Fe(III) by the sample preparation procedure using HCl and H_2O_2 in place of HNO_3 and $HClO_4$ with HCl.

The USP method was employed as a reference for validating the proposed FI method. The procedures followed the USP 24 [13] for the drug samples in the forms of fumarate, gluconate and sulphate. The sample in citrate form was also analyzed by following the USP procedure for sulphate since there is no USP method for the citrate form. It was found that the results from the proposed FI methods using both sample preparation procedures agree with that of USP method as seen from the t test ($t = 1.37$ for that of the sample preparation procedure with HCl and H_2O_2 and $t = 1.44$ for the procedure using HNO_3 and $HClO_4$ with HCl at the 95% confidence level). And there was no significant difference between the two procedures for sample preparation ($t = 0.08$ at 95% confidence level). However, although the results from the t test indicated that there were no significant differences between the methods, it can be seen from Table 1 that the milligrams per tablet obtained by the USP method for sample numbers 7–10, of which iron contents are in the forms of gluconate, sulfate and citrate, differed from that of the labels, whereas that of the proposed FI method were practically the same, except for sample number 10 (citrate form). The errors from the USP method for gluconate forms may be from zinc dust (getting through the filter paper in a step to convert Fe(III) to Fe(II) [13]), which may be contaminated in the solution and which made the values higher than labeled. For the sulphate form, the results are lower than the labeled, and this may come from the long time of the filtration step, during which some of Fe(II) may be oxidized to Fe(III). For the citrate form, using FI, both the procedures for sample preparation are not suitable and the USP procedure for sulphate is not suitable for citrate form. However, the proposed method can be well applied to the samples in which the iron contents are in the forms of fumarate, gluconate and sulphate. Further study in more detail in such cases should be made.

This study demonstrates that the proposed FI procedure is simple. Other advantages gained, in comparing to the USP method, include economics (simple FI system, cheap

and readily available reagent, simpler sample preparation), less risk from toxic and hazardous chemical wastes. The procedure offers feasibility in automation.

4. Conclusions

A new cost-effective FI spectrophotometric assay of iron in pharmaceutical preparations (in a form of fumarate, gluconate or sulphate) is proposed. Salicylate is obtained from alkaline hydrolysis of the antipyretic drug, aspirin, which is cheap and easily available as reagent. Various advantages can be benefit from the proposed FI procedure, especially in some remote places.

Acknowledgements

Thanks are due to the Commission for Higher Education and Naresuan University for scholarships to YU; the Thailand Research Fund (TRF), the Postgraduate Education and Research Program in Chemistry (PERCH) for supports.

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Flow injection in-valve-mini-column pretreatment combined with ion chromatography for cadmium, lead and zinc determination

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Received 26 March 2004; received in revised form 28 May 2004; accepted 28 May 2004
Available online 25 August 2004

Abstract

A flow injection (FI) in-valve-mini-column packed with Chelex-100 resin is proposed for on-line sample pretreatment for some metal ions, namely, Cd(II), Pb(II) and Zn(II), prior to simultaneous determination using ion chromatography (IC). A solution containing a mixture of the cations was first passed through the in-valve-mini-column, followed by on-line elution. The eluate was then flowed further to an injection valve and was injected into an ion chromatograph. Conditions of the system were optimized. A single standard calibration was possible. The recoveries of cations were found to be in the range of 95–105%. The developed method was applied to the accurate analysis of zinc ore samples.
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Keywords: Sample pretreatment; In-valve-mini-column; Ion chromatography; Flow injection; Ores; Cadmium; Lead; Zinc

1. Introduction

Sample pretreatment is necessary when the analytical method cannot provide good separation and quantification due to interferences from sample matrix components [1]. Sample preparation has been a growing area in chromatography over the past several years, since samples containing interfering substances can affect chromatographic performance. These substances may mask the peak of interest or irreversibly retain on the analytical column, permanently damaging the column. To eliminate these problems, such samples need to be treated before injection [2]. A common problem in ion chromatography is poor resolution of an analyte ion from matrix ions that are present in relatively high concentrations [3–9]. Post column derivatization with a color reagent such as 4-(2-pyridylazo) resorcinol (PAR) or 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP) for visible spectrophotometric detection [10–13] has been used for good sensitivity and stable baselines. One of the most important aspects of developing an IC method is the ability to recognize when undesirable chromatographic effects are derived from sample matrix interferences. Sample matrix effects can include shortened retention times, poor peak efficiency, poor resolution, poor reproducibility and irregular baseline [1].

A flow injection (FI) system can be used to introduce samples into a detector such as atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectroscopy (ICP–AES), inductively coupled plasma-mass spectroscopy (ICP–MS) and ion-selective electrode (ISE) and

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doi:10.1016/j.talanta.2004.05.060

to enhance the pretreatment step. FI systems with on-line sample pretreatment are very beneficial. Automation of sample pretreatment and manipulation within the manifold have increased sample throughput and decreased the potential for sample contamination [14,15]. These include, for examples, column retention, gas diffusion, and solvent extraction [16,17]. The on-line column in a FI system could be used for sample pretreatment for complicated matrix samples prior to the determination step.

In the present work, a simple flow injection system with an in-valve-mini-column has been coupled with a simple ion chromatograph (IC) with a conductivity detector without suppressor, for on-line sample cleaning or pretreatment for the determination of Cd, Pb and Zn in a complicated matrix sample such as in a zinc ore sample, prior to their determination. An additional advantage of prolonging the life-time of the IC column is also gained.

2. Experimental

2.1. Standard solutions

Standard solutions of each cation were prepared by appropriate dilution of a stock standard solution (1000 mg l^{-1}, Merck, Germany). Ultrapure water (resistivity 18 mΩ cm^{-1}) was produced by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Buffer solutions and eluents

For the FI system, ammonium acetate buffer solution (1.0 M, pH 5.4) was prepared by mixing 57 ml of glacial acetic acid (99.8% (w/v), Merck, Germany) and 75 ml of 25% ammonia liquor (25% (w/v), Merck, Germany) and diluting to 1000 ml. Sodium citrate buffer solution pH 3 was prepared from reagent-grade chemical obtained from Merck (Germany). The eluent was 2.0 M HNO_3 (65% (w/v) HNO_3, Merck, Germany).

For the IC system, a mixture of tartaric acid and oxalic acid was employed as the eluent. The eluent concentration optimized for the best resolution was: 3 mM tartaric acid/1 mM oxalic acid.

All solutions were degassed using an ultrasonic bath before use.

2.3. FI in-valve-mini-column

Chelex-100 (sodium form, 50–100 mesh; Bio-Rad Laboratories) was packed in a mini-column, made of acrylic tubing (3 mm i.d. × 2 cm), similarly described in [16]. Teflon frits were placed at each end of the column to prevent the loss of the resin when a solution passed through the column, as shown in Fig. 1. The two ends of the column were connected to a six-port injection valve by replacing a sample loop of the valve.

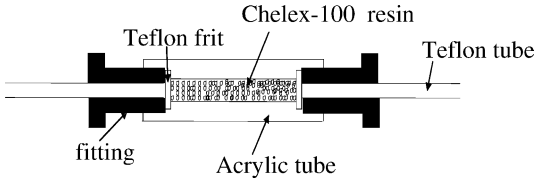

Fig. 1. Ion exchange mini-column.

2.4. Instruments

2.4.1. FI system

The FI system consisted of two peristaltic pumps (MP-3, EYELA, Tokyo Rikakikai, Tokyo, Japan and Ismatec, Glattbrugg-Zurich, Switzerland), and a Rheodyne (model 7725i) injection valve with in-valve-mini-column.

2.4.2. Ion chromatograph

The ion chromatographic system without chemical suppression (Metrohm Ltd., Switzerland) used this study consisted of a Metrohm isocratic pump, a six-port valve with 20 μl sample loop, and a Metrohm 732 conductivity detector (without suppressor). The analysis column used for the cation separation was a universal cation column (4.6 mm × 100 mm; Altech, USA). The column was packed with polybutadiene-maleic acid (PBDMA) coated on silica material. The chromatograms were recorded and handled with Metrohm software (Metrohm Ion analysis, Metrohm Ltd., Switzerland), which was also used for controlling the pump and injection valve.

2.5. Procedure

The FI–IC manifold (Fig. 2) operation was controlled by two injection valves under manual control for timing as indicated in Fig. 3. Standard/sample solution was mixed with buffer to adjust the pH for adsorption of cations on the resin, and flowed through the column with the FI-valve in the load position, with various loading times, while the unretained cations were passed to waste. After that the valve was switched to the injection position and 2.0 M HNO_3 was flowed through the mini-column to elute cations from the column. The eluate was neutralized with 2.0 M NaOH and then controlled for pH with citrate buffer. When the elapsed time was 24 s after switching the FI valve to the injection position, the zone of cations was moved into the sample loop and was injected into the IC via the IC valve.

2.5.1. Preparation of zinc ore samples

A portion (0.1 g) of a zinc ore sample was accurately weighed and digested with concenterated nitric acid (25 ml) by heating on a hot plate until it became clear (about 4 h). It was filtered and transferred into a 250 ml volumetric flask and made to the volume with 1% nitric acid.

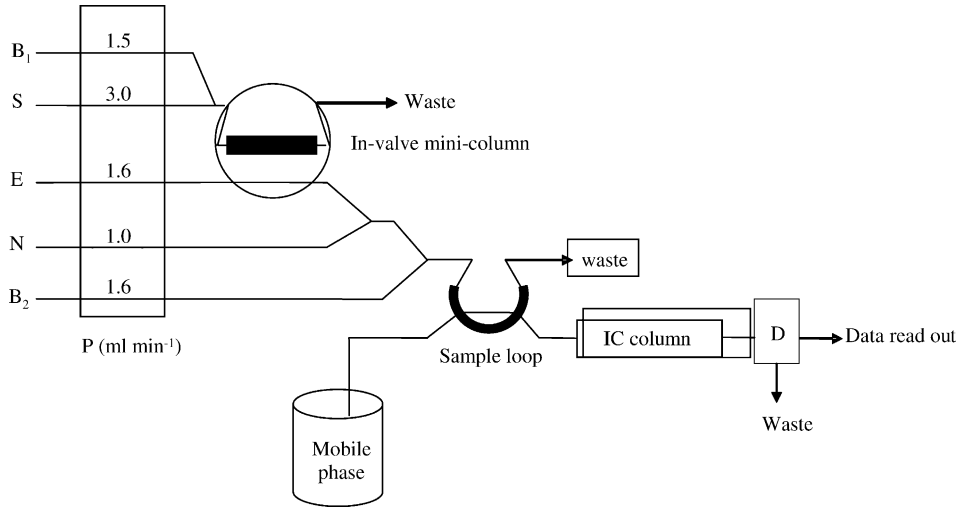

Fig. 2. Manifold of FI–IC combination; P: peristaltic pumps; B_1: ammonium acetate buffer; B_2: sodium citrate buffer; S: sample or standard solution; E: 2.0 M nitric acid; N: 2.0 M sodium hydroxide; mobile phase: mixture of tartaric acid and oxalic acid; sample loop: 20 μl loop of IC; IC column: universal cation column 100 mm × 4.6 mm; D: conductivity detector (without suppressor).

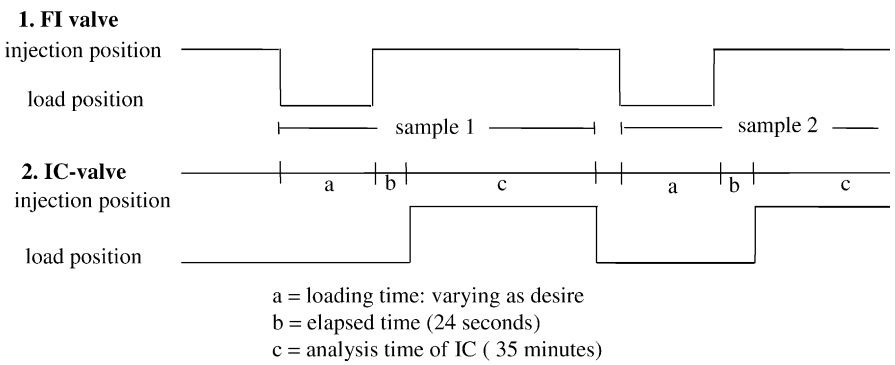

Fig. 3. Schematic diagram of timing control for operation of valves in the FI–IC system.

3. Results and discussion

3.1. Ion chromatography

The conditions, mobile phase concentration, and flow rate of mobile phase for ion chromatographic separation were optimized. Results are summarized in Table 1 and Fig. 4(a)–(d). For the flow rate studies (1.0, 1.5 and 2.0 ml min^{-1}), the lower the flow rate employed, the better sensitivity obtained, but a lower flow rate resulted in longer analysis time, as expected. Chromatograms of a blank solution and of cations obtained from conventional ion chromatography are shown in Fig. 5(a) and (b), respectively. Milli-Q water was used as a blank solution and a mixture of cations containing 3 mg l^{-1} Cu, 3 mg l^{-1} Zn, 5 mg l^{-1} Cd and 10 mg l^{-1} Pb in 20 μl was injected directly into the ion chromatograph with a mobile phase flow rate of 1.5 ml min^{-1} and an injection volume of 20 μl. In the chromatographic separation similar to a previous report [8], a mixture of 3 mM tartaric acid/1 mM oxalic acid was used as the mobile phase since this organic acid eluent can separate Cd, Cu, Pb and Zn on the PBDMA stationary phase, which is polybutadienemaleic acid coated on silica material of the

Table 1
Concentrations of tartaric acid and oxalic acid

Concentration (mM)		Retention time (min)				Total analysis time (min)	Comments
Tartaric acid	Oxalic acid	Cu	Zn	Cd	Pb		
3.0	2.0	1.1	4.4	11.8	12.9	17	Bad resolution of Cd and Pb (Fig. 4a)
1.5	2.0	1.1	5.2	15.4	15.4	20	Co-elution of Cd and Pb (Fig. 4b)
3.0	1.5	1.4	6.5	17.0	20.5	25	Good resolution of all cations (Fig. 4c)
3.0	1.0	1.9	9.4	23.0	31.3	35	Good resolution of all cations (Fig. 4d)

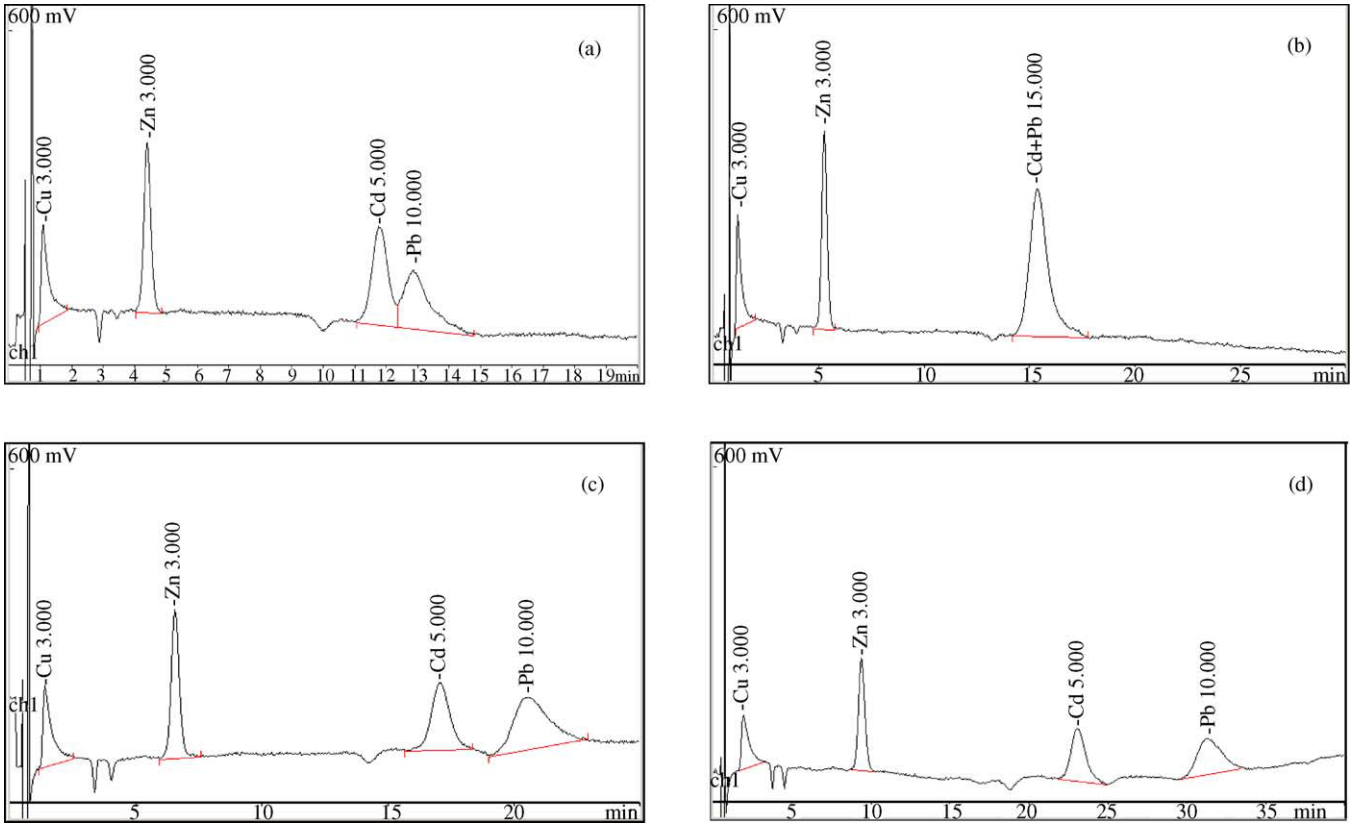

Fig. 4. Chromatograms of cations in Milli-Q water at different concentrations and ratios of tartaric acid and oxalic acid as a mobile phase: (a) 3 mM tartaric acid/2 mM oxalic acid, (b) 1.5 mM tartaric acid/2 mM oxalic acid, (c) 3 mM tartaric acid/1.5 mM oxalic acid and (d) 3 mM tartaric acid/1 mM oxalic acid. The values on the peak tops are concentrations (mg 1–7).

universal cation column. The retention time of the cations of interest in the presence of the mobile phase followed the order Cu < Zn < Cd < Pb, and the separation was achieved within 35 min at a flow rate of 1.5 ml min^{-1}.

3.2. Flow injection-ion chromatography

Using the FI–IC system and optimal conditions of the FI system in Table 2, the results in Fig. 6a show the chromatogram of a blank solution obtained on-line from the FI system to the ion chromatograph. A blank solution (without cations) was loaded on the in-valve-mini-column and followed by the on-line elution process with 2.0 M HNO_3 for FI operation. The eluate acid solution from the FI system was neutralized with 2.0 M NaOH and the pH controlled with sodium citrate buffer solution before flowing into the IC injection valve, because highly acid solution will affect the analytical column. The baseline of the blank solution in the chromatogram exhibits a large, early elution peak, which is that of a high concentration of unretained Na^+ ion. Because the conductivity of Na^+ ion is lower than that of H^+ ion, a baseline drift occurs from the change of the Na^+/H^+ ratio leading to stepwise fashion at the about 20–25th and 25–27th min.

Table 2
Proposed conditions for FI system with in-valve-mini-column

Parameters	Conditions
Buffer for adsorption process	1.0 M ammonium acetate buffer pH 5.4
Loading flow rate	3 ml min^{-1}
Eluent	2.0 M Nitric acid
Eluent flow rate	1.6 ml min^{-1}
Base for neutralized eluate	2.0 M sodium hydroxide
Buffer for eluate	Sodium citrate buffer pH 3

In Fig. 6b, the chromatogram of a mixture of cations (Cd, Cu, Pb and Zn) was obtained by using the same conditions as for the blank. From the chromatogram, the high conductivity of the unretained Na^+ ion causes a large peak and interferes with the Cu peak (t_R = 1.4 min).

Reproducibility tests were carried out for estimating the relative standard deviation (R.S.D.) of the chromatographic peak areas. This data was obtained from five repeated injections at a loading time of 40 s for a concentration of 1 μg ml^{-1} of all interested cations. Average R.S.D. values of 3.1, 3.5 and 5.1% for Zn, Cd and Pb, respectively, were obtained.

Calibration plots, with various loading times, were constructed with single standard calibration by using a plot of peak area (y-axis) and micrograms of cation loaded (x-axis). The microgram of cation was calculated by concentration (μg ml^{-1}) of a standard solution used × loading flow rate

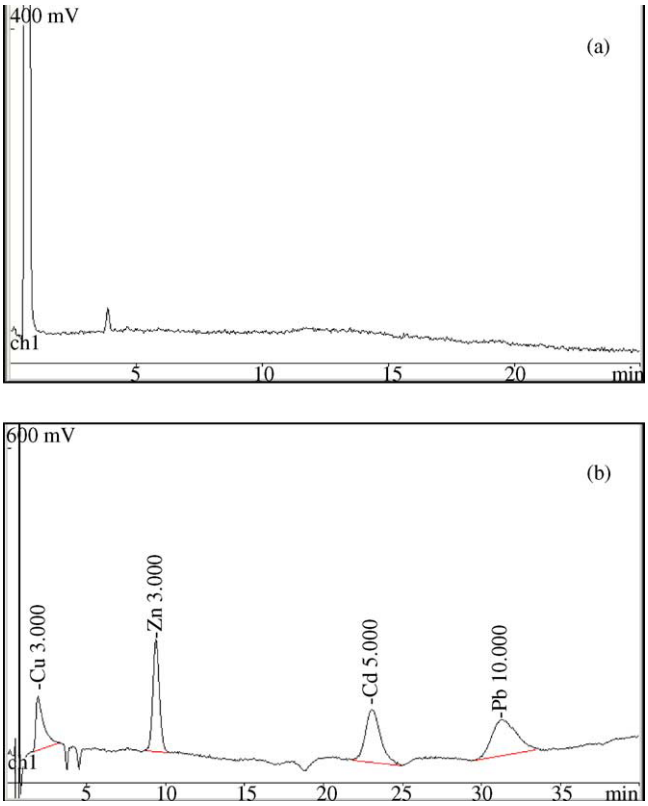

Fig. 5. Chromatograms: (a) blank solution without cations using Milli-Q water (b) cations (Cd = 5 mg l^{-1}, Pb = 10 mg l^{-1}, Zn = 3 mg l^{-1}) in Milli-Q water by direct injection into ion chromatograph without FI system. Peaks: Cd (t_R = 24.1 min), Pb (t_R = 32.5 min) and Zn (t_R = 9.8 min); column: universal cation (100 mm × 4.6 mm); eluent: 3 mM tartaric acid/1 mM oxalic acid; flow rate 1.5 ml min^{-1}; injection volume: 20 μl; detector: conductivity without suppressor.

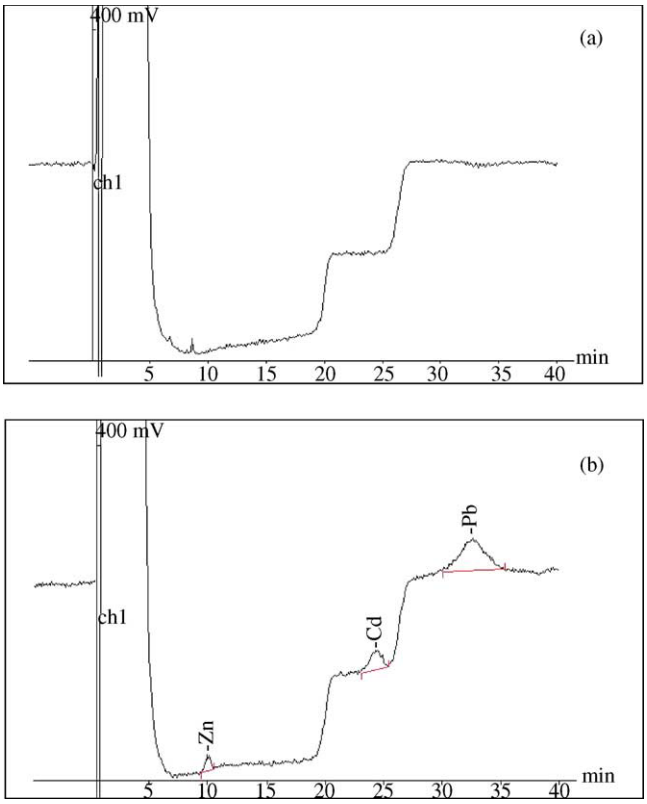

Fig. 6. Chromatograms: (a) blank solution without cations, using the FI–IC system (b) cations (Cd = 5 mg l^{-1}, Pb = 10 mg l^{-1}, Zn = 3 mg l^{-1}) by using the FI on-line sample pretreatment with loading time 1 min and loading flow rate 3 ml min^{-1} before injecting into ion chromatograph with elapsed time 24 s. Peaks: Cd (t_R = 24.3 min), Pb (t_R = 32.6 min) and Zn (t_R = 9.9 min); column: universal cation (100 mm × 4.6 mm); eluent: 3 mM tartaric acid/1 mM oxalic acid; flow rate 1.5 ml min^{-1}; injection volume: 20 μl; detector: conductivity without suppressor.

(ml min^{-1}) × loading time (min). The plots were found to be linear. For each cation, two breaks of linear calibration ranges were obtained; for Cd: $y = 105x - 99$ ($r^2 = 0.989$) (for 6−25 μg) and $y = 44x + 1035$ ($r^2 = 0.981$) (for 25–60 μg); for Pb: $y = 93x + 762$ ($r^2 = 0.983$) (for 18–54 μg) and $y = 43x + 3559$ ($r^2 = 0.992$) (for 54–180 μg) and for Zn: $y = 89x - 553$ ($r^2 = 0.986$) (for 60–200 μg) and $y = 59x + 5561$ ($r^2 = 0.992$) (for 200–600 μg). Under such conditions, detection limits were: 5, 3 and 4 μg for Cd, Pb and Zn, respectively.

The proposed method was applied to the determination of cation mixtures in zinc ore samples and ore samples with spiked cations. To determine Cd, Zn and Pb in zinc ore samples which have high matrix content, the digested sample solution was on-line pretreated using the in-valve column FI system. The pretreated sample zone was injected into the ion chromatograph. The results obtained agree with reference methods (AAS and ICP–AES), all from triplicate

Table 3
Analysis of zinc ore samples

Sample	Concentration found (%)								
	Zn			Cd			Pb		
	FI–IC[a]	ICP–AES[b]	AAS[b]	FI–IC[a]	ICP–AES[b]	AAS[b]	FI–IC[a]	ICP–AES[b]	AAS[b]
A	25.3 ± 0.6	30.4 ± 0.2	30.4 ± 0.2	0.27 ± 0.01	0.33 ± 0.02	0.32 ± 0.02	6.0 ± 0.2	5.8 ± 0.3	5.7 ± 0.3
B	49.5 ± 1.2	45.1 ± 0.5	45.1 ± 0.5	0.19 ± 0.01	0.20 ± 0.02	0.20 ± 0.02	5.4 ± 0.2	6.3 ± 0.3	6.3 ± 0.3
C	42.4 ± 0.8	45.1 ± 0.5	45.1 ± 0.5	0.20 ± 0.01	0.20 ± 0.02	0.19 ± 0.02	4.4 ± 0.2	6.3 ± 0.3	6.3 ± 0.3
D	26.9 ± 0.4	27.8 ± 0.2	27.8 ± 0.3	0.18 ± 0.01	0.19 ± 0.02	0.19 ± 0.02	4.6 ± 0.1	4.8 ± 0.3	4.8 ± 0.3
E	28.5 ± 0.4	27.9 ± 0.3	27.8 ± 0.3	0.18 ± 0.01	0.19 ± 0.02	0.18 ± 0.02	4.6 ± 0.2	4.8 ± 0.3	4.8 ± 0.3

Both (a) and (b) were from triplicate results: mean ± S.D.
[a] The proposed method.
[b] Analyzed by the Mineral Resources Region 3 (Chiang Mai).

determination with standard deviation as represented in Table 3. The recoveries of cations were found to be in the range of 95–105%. The proposed procedure offers simultaneous determination of Cd, Pb, and Zn. This is advantageous over the AAS, in which simultaneous determination is not possible. The proposed pretreatment using FI column reduces IC column damage. The FI–IC procedure is cheaper than the ICP–AES method, both in instrumentation and operation aspects.

4. Conclusion

In-valve column in FI system for sample pretreatment coupled to a simple IC with conductivity detector without suppressor for simultaneous determination of some cations in zinc ore samples which have high amounts of matrix interference, has been successfully applied. The on-line sample matrix interference removals are easily automated, and eliminate costly and time consuming off-line operations, and reduce IC column damage. The developed FI–IC method offers advantages over traditional analytical procedures. It allows a range of some cations to be simultaneously analyzed, and is accurate, reliable and uses readily available chemicals. The on-line sample preparation FI-column can also potentially be combined with IC using PAR/absorbance detection.

Acknowledgements

Thanks are due to the National Research Council of Thailand (NRCT) and the Thailand Research Fund (TRF) for research grant support, the Post Graduate Education and Research Program in Chemistry, the Commission for Higher Education and the Graduate School, Chiang Mai University, Thailand for partial support.

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Flow injection analysis of doxycycline or chlortetracycline in pharmaceutical formulations with pulsed amperometric detection

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Received 27 February 2004; received in revised form 16 April 2004; accepted 16 April 2004
Available online 27 July 2004

Abstract

A flow injection with pulsed amperometric detection for determination of doxycycline or chlortetracycline in pharmaceutical formulations is described. Doxycycline or chlortetracycline were studied at a gold rotating disk electrode with cyclic voltammetry as a function of pH of supporting electrolyte solution. The optimized PAD waveform parameters were obtained with a flow injection system. The optimized pulsed conditions of doxycycline were 1150 mV (versus Ag/AgCl reference electrode) detection potential (E_{det}) for 220 ms (150 ms delay time and 70 ms integration time), 1500 mV (versus Ag/AgCl reference electrode) oxidation potential (E_{oxd}) for 70 ms oxidation time (t_{oxd}) and 250 mV (versus Ag/AgCl reference electrode) reduction potentail (E_{red}) for 400 ms reactivation time (t_{red}). The optimized pulsed conditions of chlortetracycline were 1050 mV (versus Ag/AgCl reference electrode) detection potential (E_{det}) for 300 ms (200 ms delay time and 100 ms integration time), 1300 mV (versus Ag/AgCl reference electrode) oxidation potential (E_{oxd}) for 70 ms oxidation time (t_{oxd}) and 250 mV (versus Ag/AgCl reference electrode) reduction potentail (E_{red}) for 400 ms reactivation time (t_{red}). The optimized PAD waveform was applied to the determination of doxycycline hydrochloride and chlortetracycline hydrochloride standard solution and in pharmaceutical formulations. The linear dynamic ranges of doxycycline hydrochloride and chlortetracycline hydrochloride were 1 μM–0.1 mM. The sensitivity of this method was found to be 23 μA/mM for doxycycline hydrochloride and 33.76 μA/mM for chlortetracycline hydrochloride. The detection limit for both compounds is 1 μM. The doxycycline hydrochloride and chlortetracycline hydrochloride content in commercially available tablet dosage forms by the proposed method was comparable to those specified by the manufacturer.
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Keywords: Doxycycline; Chlortetracycline; Pulsed amperometric detection; Flow injection analysis

1. Introduction

Tetracyclines are well-known antibiotics used routinely in human and veterinary medicine for prevention and control of disease [1,2]. Chlortetracycline and doxycycline are antibiotics commonly used in food-producing animals because of their wide antibacterial spectrum, high potency and low cost. These raise the possibility that a residue of tetracyclines may remain in animal tissues intended for human consumption [3]. Moreover, they are important antibiotics widely used to control bacterial infections in human. Therefore, it is necessary to develop an assay method with high sensitivity and accuracy for monitoring the chlortetracycline and doxycycline. Many methods for determination of these compounds have been reported. These include microbiological assay [4,5]. These procedures are subject to problems such as high pH dependence, low sensitivity, low stability, as well as being time consuming. Recently, these antibiotics have been analyzed by HPLC with UV [6,7], fluorescence [8], MS [9,10] or electrochemical detection [11]. The peaks of these compounds tend to tail and exhibit low efficiency due to interaction with the residual silanol groups on silica-based packing materials. Also techniques such as spectrophotomety [12,13], chemiluminescence [14–18], spectrofluorimetry [19,20] or electrochemical methods [21,22] have been employed. However, one of the important limitations of

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doi:10.1016/j.talanta.2004.04.036

method based on spectrophotometer, chemiluminescence or spectrofluorimetry is the fact that these compounds are inactive species for direct detection. Therefore, the derivatization procedure is normally required that make the methods are tedious, expensive, and long time analysis. The high sensitivity and selectivity of electrochemical detection are desired for antibiotics determination. Electrochemical techniques are alternatives, which can be cheap, fast and simple. The working electrode, mercury, is extensively used for determination tetracyclines [23,24]. This electrode has some problems such as the toxicity and limited stability of responses. In recent years, the flow injection system has received much attention and some analytical applications have been reported. A flow injection system was introduced to conventional analytical instrument to improve sample throughput and sensitivity that are the requirement to develop the assay method in pharmaceutical industry. Thus, use of the flow injection system coupled to the mercury electrode is complicated. Moreover, problems associated with easily oxidized mercury electrode have to be considered. Voltammetry and amperometry are the techniques that offer the high sensitivity. Their disadvantage is deposition of the detection product or solution impurities on the electrode surface. Therefore, pulsed amperometric detection with alternated anodic and cathodic polarization to clean and reactivate the electrode surface, has been introduced to overcome this problem. PAD offers the possibility to clean and reactivate the electrode surface effectively after measuring cycle without mechanical polishing [25–28]. In the simplest implementation of PAD, the potential of the working electrode is stepped between the potentials for detection, E_{det}, cleaning, E_{oxd} and reactivation, E_{red}. All three steps of PAD require the following: (a) the oxidation of analyte during the detection step; (b) the oxidative desorption of adsorbed detection products or solution impurities at the cleaning step; (c) the cathodically dissolved of inert oxide product during reactivation step [29]. Pulsed amperometric detection has been used for the sensitive detection of numerous compounds [30–34]. It also has been successful for the determination of tetracycline in pharmaceutical formulation [35]. The goal of this work is extended the use of the PAD waveform for the determination of doxycycline or chlortetracycline in pharmaceutical formulations. In this research also employed the flow injection system with PAD to reduce time analysis and to obtain low detection limit. The present method has been proved to be simple, rapid, sensitive and suitable for automatic analysis.

2. Experimental

2.1. Chemicals

All the chemicals were analytical grade, and the water used was deionized water. Phosphate solutions (for pH 2–4.5) were prepared from 0.1 M potassium dihydrogen phosphate (Merck) and adjusted to the desired pH using 85% phosphoric acid (J.T. Baker). For the phosphate solutions (pH 5–10) were adjusted by 0.1 M sodium hydroxide. Standard doxycycline hydrochloride and chlortetracycline hydrochloride (Sigma-Aldrich) solutions were freshly prepared in 0.1 M potassium dihydrogen phosphate solution prior to use.

Stock standards of doxycycline (0.5 mM) and chlortetracycline (0.5 mM) were prepared by accurately weighing the hydrochloride of doxycycline or chlortetracycline into volumetric flasks and dissolved with 0.1 M potassium dihydrogen phosphate solution. To prepare solutions for the standard addition method, 2.5 ml of sample (prepared as described in Section 2.2) solution was pipetted in a set of five 10 ml volumetric flasks. Then, the 0, 1.0, 2.0, 3.0 and 4.0 ml of a stock solution of standards were added in sample solutions and the volume was adjusted by 0.1 M potassium dihydrogen phosphate solution.

2.2. Sample preparation

Doxycycline hydrochloride capsules (100 mg Medochemie, USA) and chlortetracycline hydrochloride capsules (250 mg, F. E. Sillic, Thailand) were used in this study.

A mass powder of ten capsules of doxycycline hydrochloride (Medomycin, 100 mg) or chlortetracycline hydrochloride (Aureomycin, 250 mg) were transferred to each 1000 ml volumetric flask then dissolved in 0.1 M potassium dihydrogen phosphate solution (pH 2 for doxycycline and pH 2.5 for chlortetracycline). Both of sample solutions were filtrated through a 0.45 μm Nylon membrane syringe filter. The filtered solutions were further diluted with 0.1 M potassium dihydrogen phosphate solution to obtain a final concentration of 240.45 and 257.65 $\mu g\,ml^{-1}$ (0.5 mM), respectively.

2.3. Electrode

The gold rotationg disk electrode (Au RDE, Metrohm, Switzerland) and gold disk electrode (Bioanalytical System, West Lafayete IN, USA) were pretreated by polishing with 0.05 μm of alumina/water slurries on a felt pad, followed by rinsing with ultrapure water prior to use.

2.4. Rotating disk voltammetry

Electrochemical measurements were carried out in a single compartment three-electrode glass cell. The rotation speed was held at 250 rpm. A Ag/AgCl electrode and a platinum electrode were used as the reference and auxiliary electrode, respectively. Cyclic voltammetry was performed with an Autolab Potentiostat 100 (Metrohm, Switzerland).

2.5. Flow injection analysis with pulsed amperometric detection

The flow injection analysis system consisted of a thin-layer flow-through electrochemical cell (Bioanalytical

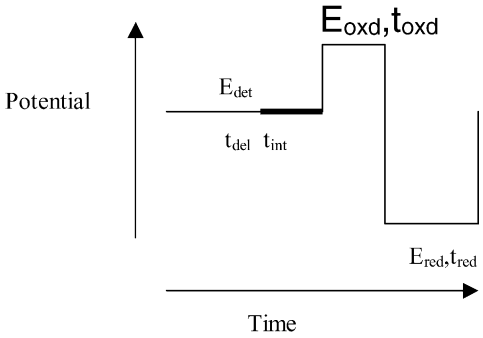

Fig. 1. Typical PAD waveform.

System, Inc.), an injector port (Rheodyne 7125) with a 20 μl sample loop, a peristaltic pump (BIO-RAD) and an electrochemical detector (PG 100). The carrier solution, 0.1 M potassium dihydrogen phosphate, was regulated at a flow rate of 1.0 ml min^{-1}. The thin-layer flow, through electrochemical cell consisted of a silicone rubber gasket as a spacer, a gold disk electrode as the working electrode, a Ag/AgCl electrode as the reference electrode and a stainless steel tube as the auxiliary electrode and the outlet from the flow cell. The experiments were performed in a faraday cage to reduce the electrical noise. The used PAD waveform to obtaine the FI-PAD response was depicted in Fig. 1.

The FI-PAD response was monitored for independent variation of all potential and time parameters. The electrode was condition in a solution of 0.1 M potassium dihydrogen phosphate solution and pumped through the flow system at a constant flow rate of 1.0 ml min^{-1} with the selected PAD waveform until a stable baseline was established. The sample was then injected into the flow injection system via an injection valve equipped with a fixed sample loop of 20 μl and the resulting peaks were recorded.

3. Results and discussion

3.1. pH dependence study

In our initial experiments, the electrochemical behaviors of both doxycycline or chlortetracycline were studied at Au RDE in 0.1 M potassium dihydrogen phosphate solution of pH 2–10. It was found that the best-resolved anodic signals for oxidation of doxycycline or chlortetracycline were obtained at pH 2 and pH 2.5, respectively. Therefore, these pHs were used for the rest of the experiments.

3.2. Cyclic voltammetry

The cyclic voltammetric *(I–E)* responses are shown in Fig. 2 for the Au RDE in 0.1 M potassium dihydrogen phosphate solution with and without 1 mM doxycycline (Fig. 2a) and 1 mM chlortetracycline (Fig. 2b). The background response for the supporting electrolyte exhibits an anodic wave

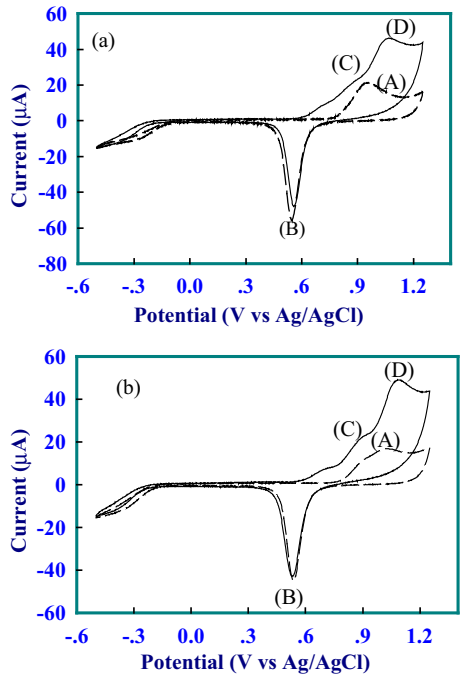

Fig. 2. Cyclic voltammetric response for (a) 1 mM doxycycline (pH 2) and (b) 1 mM chlortetracycline (pH 2.5) at the Au RDE (0.07 cm^2). Condition: 250 rpm rotation speed: 50 mV s^{-1} scan rate. The background cyclic voltammograms are also shown (dash line).

on the positive scan in the region of ca. +0.8 to +1.2 V versus Ag/AgCl (wave A). This background response corresponds to charging of the interfacial double layer and formation of a small amount of surface oxide. The cathodic peak obtained on the negative scan in the region of ca. +0.7 to +0.4 V versus Ag/AgCl (wave B) corresponds to dissolution of the surface oxide formed on the positive scan. In the presence of doxycycline or chlortetracycline, the two-step anodic signal for oxidation of them were observed on the positive scan beginning at ca. 0.6 V versus Ag/AgCl. The first and secon steps were occurred in the region ca. +0.6 to +0.9 V versus Ag/AgCl (wave C) and +0.95 to 1.15 V versus Ag/AgCl (wave D), respectively. The anodic responses for doxycycline or chlortetracycline on the positive scan were sharply inhibited by the onset of the surface oxide formation at potential greater than ca. +1.2 V versus Ag/AgCl. The decrease of signal on the subsequent negative scan in the region of ca. +1.25 to +0.8 V versus Ag/AgCl indicates the reduction of activity for the oxide covered gold surface.

3.3. PAD waveform optimization

The PAD waveform used in this experiment is described in Fig. 1. E_{det} is the detection potential applied for the time period t_{det} ($t_{det} = t_{del} + t_{int}$), and the electrode current is sampled by electronic integration over the time period t_{int} following a delay of t_{del} to allow the charging current to decrease to a negligible value. A positive cleaning potential (E_{oxd}) that removes the oxidizable contaminant on the elec-

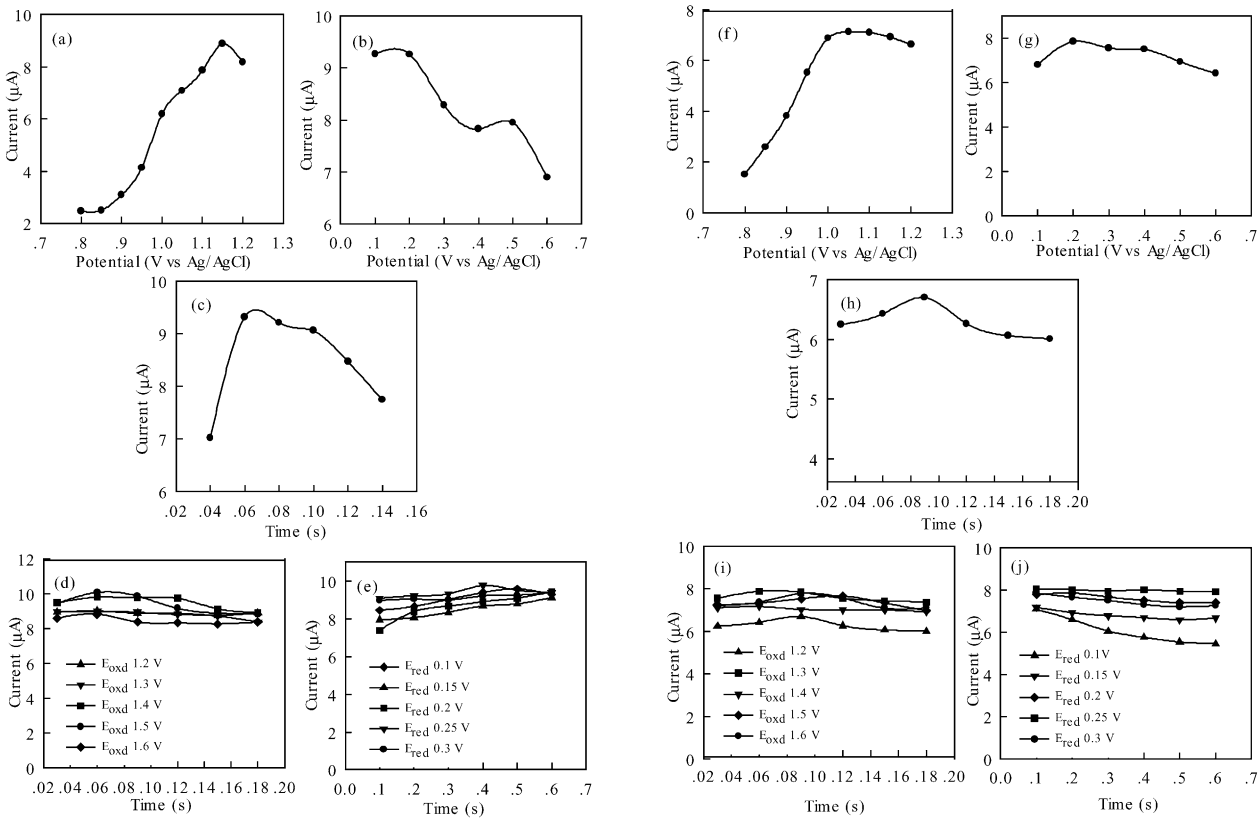

Fig. 3. FI-PAD response as a function of: (a) E_{det}; (b) t_{del}; (c) t_{int}; (d) E_{oxd} and t_{oxd}; and (e) E_{red} and t_{red}, for chlortetracycline and (f) E_{det}; (g) t_{del}; (h) t_{int}; (i) E_{oxd} and t_{oxd}; and (j) E_{red} and t_{red} for doxycycline in 0.1 M potassium dihydrogen orthophosphate solution (pH 2) at the Au RDE (0.07 cm^2).

trode surface is applied for the time period t_{oxd} following E_{det}. A negative reactivating potential (E_{red}) that dissolves the inert oxide product on the electrode surface is applied for the time period t_{red} following E_{oxd}. The optimization of each waveform parameter carried out in the FI system was studied while the other parameters were held constant. The average peak currents for each parameter were plotted versus the varied parameter. Observations of each parameter are discussed later.

3.4. Optimization of detection step (E_{det}, t_{int} and t_{del})

Fig. 3a and f show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline respectively according to E_{det} variation in the range +0.8 to +1.2 V versus Ag/AgCl in intervals of 0.5 V. The potential range used for E_{det} optimization was chosen from the potential region in the cyclic voltammogram (Fig. 2) that the oxidation of each doxycycline or chlortetracycline occurred. The optimum detection potential for doxycycline was obtained at E_{det} = 1.15 V versus Ag/AgCl and for chlortetracycline was obtained at E_{det} = 1.05 V versus Ag/AgCl.

Fig. 3b and g show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline with t_{del} variation from 100 to 500 ms. The t_{del} optimal values of doxycycline and chlotetracycline were 150 ms and 200 ms, respectively.

Fig. 3c and h show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline with variation of t_{int} from 40 to 140 ms. The optimal values of t_{int} for doxycycline was obtained 100 ms and chlortetracycline was obtained 70 ms.

3.5. Optimization of oxidation step (E_{oxd} and t_{oxd})

A clean electrode surface is progressively fouled by the detection products during application of E_{oxd} and to avoid this problem, t_{oxd} was applied to clean the electrode surface. Fig. 3d and i show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline as a result of the variation of t_{oxd} from 30 to 180 ms at intervals of 30 ms for difference E_{oxd} values in the range +1.2 to +1.6 V versus Ag/AgCl in interval 0.1 V. The optimal values of doxycycline was obtained E_{oxd} = 1.3 V versus Ag/AgCl and t_{oxd} = 70 ms. For chlortetracycline, the E_{oxd} = 1.5 V versus Ag/AgCl and t_{oxd} = 70 ms was recommended as optimal.

3.6. Optimization of reduction step (E_{red} and t_{red})

The formation of surface oxide at the electrode surface, which reduced the electrode surface activity, occurred during the oxidation step. Therefore, it is necessary that the values of E_{red} and t_{red} are chosen to achieve complete re-

Table 1
The optimal PAD waveform parameters of doxycycline and chlortetracycline

Parameter	Doxycycline	Chlortetracycline
E_{det} (V vs. Ag/AgCl)	1.05	1.15
t_{del} (ms)	200	150
t_{int} (ms)	100	70
E_{oxd} (V vs. Ag/AgCl)	1.3	1.5
t_{oxd} (ms)	7.0	70
E_{red} (V vs. Ag/AgCl)	0.25	0.25
t_{red} (ms)	400	400

ductive dissolution of the surface oxide. Fig. 3e and j show the FI-PAD response variations for 0.5 mM doxycycline and 0.5 mM chlortetracycline with variation of t_{red} from 100 to 600 ms at intervals of 100 ms for difference E_{red} values in the range +0.1to +0.5 V versus Ag/AgCl in interval 0.1 V. For doxycycline or chlortetracycline, the optimal values of reduction step were obtained the value of $E_{red} = 0.25$ V versus Ag/AgCl and $t_{red} = 400$ ms. To conclude, the potentials and times for the optimization are shown in Table 1.

3.7. Linear range, detection limit and repeatability

From a series of repetitive 20 μl injections of doxycycline or chlortetracycline in 0.1 M potassium dihydrogen phosphate solution under the optimum pH conditions and the optimized PAD waveform parameters described above provided well-defined signals as shown in Fig. 4. The current signal increased with increase in concentration. The calibration curves for doxycycline or chlortetracycline were obtained from using the optimized PAD waveform parameters. The analytical performance results are shown in Table 2. The dynamic linear working range of both compounds is the same and over two orders of magnitude.

3.8. Drug analysis of pharmaceutical formulations

The proposed PAD methods for doxycycline or chlortetracycline were applied to the determination of doxycycline or chlortetracycline in pharmaceutical formulations by standard addition method. In order to evaluate, these proposed methods for the determination of doxycycline or chlortetracycline in drug capsules, the recovery, and within-day and between-day studies were carried out. The results are summarized in Table 3.

Table 2
Linear range, detection limit and repeatability of doxycycline hydrochloride and chlortetracyline hydrochloride

Parameter	Doxycycline	Chlortetracycline
Linear range	1 μM–0.1 mM	1 μM–0.1 mM
Sensitivity (μAmM^{-1})	23.4	33.6
Regression equation	$Y = 23.354X + 0.0272$	$Y = 33.785X + 0.037$
Correlation coefficient (R^2)	0.9992	0.9994
Detection limit	1 μM	1 μM
Repeatability (%RSD)	3.17	2.18

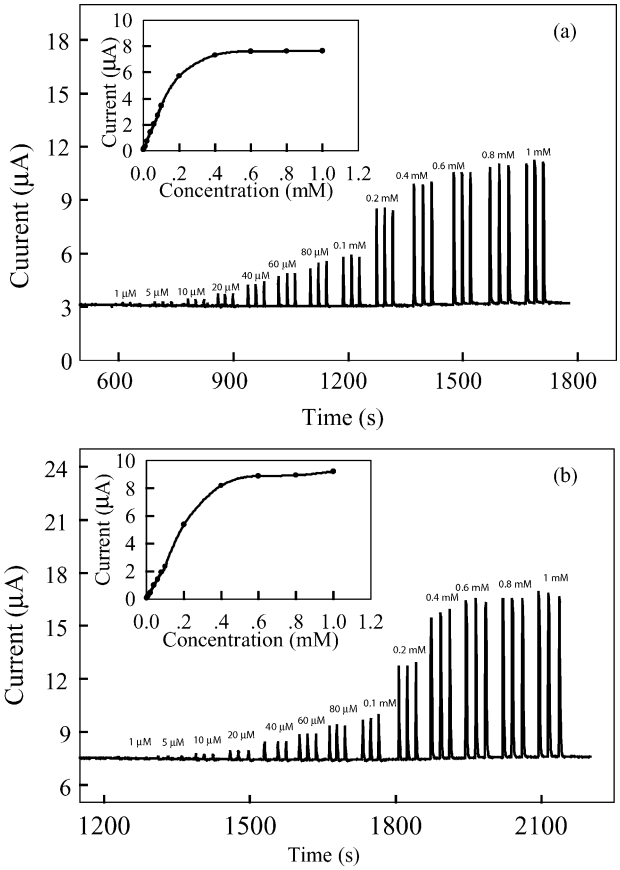

Fig. 4. FI-PAD results of (a) chlortetracycline and (b) doxycycline in 0.1 M potassium dihydrogen phosphate at gold disk electrode. The flow rate was 1 ml min^{-1}.

Table 3
Percent difference and percent recovery of doxycycline hydrochloride and chlortetracycline hydrochloride capsule samples

Parameters	Doxycycline	Chlortetracycline
% Difference	2.57 ± 1.77	7.28 ± 0.27
% Recovery of spiked standard solution	87–103	93–109

4. Conclusion

This is the first investigation of doxycycline or chlortetracycline using pulsed amperometric detection applied to a flow injection system to avoid a problem about fouling of products or interferents on the surface of a gold working electrodes. The optimized conditions, such as pH and the various potentials were investigated. The results showed that FI-PAD with optimized conditions can be used to determine doxycycline or chlortetracycline in pharmaceutical formulations. FI-PAD provided wide working concentration (0.001–0.1 mM), low detection limit (1 μM) and high re-

peatability (% RSD 3.17–2.18). The advantage of the proposed method is simple and time saving because the cleaning step occurs simultaneously during the measurement. The commercial drugs of doxycycline hydrochloride or chlortetracycline hydrochloride were also analyzed by proposed methods.

Acknowledgements

Acknowledgements are made to the Thailand Research Fund (Research Team Promotion Grant) and the Ratchadaphisek Somphot Endowment Grant, Chulalongkorn University.

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Flow-injection determination of iodide ion in nuclear emergency tablets, using boron-doped diamond thin film electrode☆

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Received 29 February 2004; received in revised form 5 April 2004; accepted 16 April 2004
Available online 28 July 2004

Abstract

The electrochemical determination of iodide was studied at boron-doped diamond thin film electrodes (BDD) using cyclic voltammetry (CV) and flow-injection (FI) analysis, with amperometric detection. Cyclic voltammetry of iodide was conducted in a phosphate buffer pH 5. Experiments were performed using glassy carbon (GC) electrode as a comparison. Well-defined oxidation waves of the quasi-reversible cyclic voltammograms were observed at both electrodes. Voltammetric signal-to-background ratios (S/B) were comparable. However, the GC electrode gives much greater in the background current as usual. The potential sweep rate dependence exhibited that the peak current of iodide oxidation at 1 mM varied linearly ($r^2 = 0.998$) with the square root of the scan rate, from 0.01 to 0.30 V s^{-1}. This result indicates that the reaction is a diffusion-controlled process with negligible adsorption on BDD surface, at this iodide concentration. Results of the flow-injection analysis show a highly reproducible amperometric response. The linear working range was observed up to 200 μM ($r^2 = 0.999$). The detection limit, as low as 0.01 μM (3σ of blank), was obtained. This method was successfully applied for quantification of iodide contents in nuclear emergency tablets.
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Keywords: Boron-doped diamond thin film electrode (BDD); Iodide; Flow-injection analysis

1. Introduction

Diamond itself does not have electrical properties in terms of being conductive or semiconductive. However, with the technology of a so-called chemically vapor deposition (CVD), film of diamond with specific dopants, such as boron, can be conveniently grown on a substrate [1–3]. This diamond film offers remarkable properties of being such an electrochemical sensor.

For electrochemical measurements, thin film of boron-doped diamond on Si wafer (BDD), has benefits over other electrodes especially those sp^2-bonded carbon electrodes, e.g., carbon paste and glassy carbon [1]. Examples [1–3] of the superior properties of the BDD include (i) low and stable background current which results in improvement in signal-to-background; (ii) considerably wide working potential window in both aqueous and non-aqueous media; (iii) very low capacitance; (iv) low adsorption of polar molecules, due to the hydrogen termination during the film growth (unlike the GC surface which is usually quite polar with covered film of oxide) [4]; (v) remarkable reproducibility [5] and (vi) morphological and micro structural stability although at over potential condition.

With respect to the outstanding properties of BDD film, quite a number of applications, based on use of this new material for electrochemical quantitation, have been reported [6–19]. This synthetic type of diamond film has already been applied as amperometric detectors in flow-injection (FI) analysis [6–14,19] and in liquid chromatography [15–19]. However, there has been no report of use of BDD electrode for determination of iodide. There is only a recent report for quantitative analysis of iodide, but on diamond paste based electrodes [20].

☆ Presented at the 12th International Conference on Flow Injection Analysis, 17–23 December 2003, Merida, Venezuela.

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doi:10.1016/j.talanta.2004.04.023

Presently, iodine is one of the supplements being widely publicized in our country. Campaigns have been raised against iodine deficiency disorder (IDD) in populations. Drinking water and/or table salt as well as foods are supplemented with iodide or iodate. Supplementation of iodine also comes in as multivitamins.

In the States, customers can purchase a pharmaceutical product used in the event of nuclear emergency. This product is basically potassium iodide, known as "potassium iodide tablet". The product is orally consumed to protect human thyroid from body absorption of radioactive iodine.

Since there are varieties of samples being the source of iodine, and somehow, intake of iodine should be concerned with extreme caution. Some people can be markedly sensitive to the element [21].

In the previous reports, determination of iodide can be made using several methods such as spectrometric analysis [22,23], catalytic spectrometric analysis [24,25] and by potentiometric detection [26]. Analysis of iodide using amperometric detection by other working electrodes has been well established especially for liquid chromatography [27–29]. Nevertheless, there is no report for detection on the BDD electrode.

This paper presents, for the first time, the electrochemical oxidation of iodide on thin film of boron-doped diamond. Cyclic voltammetric investigation was made for an iodide solution with comparison to the commercially known glassy carbon electrode. Amperometric determination of iodide on BDD was explored by applying the thin film for quantitative analysis in some pharmaceutical products.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade and deionized-distilled water was used throughout. A stock solution of iodide standard (0.1 M) was prepared by dissolving approximately 1.66 g (accurate weight) of potassium iodide crystals (Merck, Germany) in 100.0 ml of water. Working solutions of iodide were obtained by appropriate dilution with water. A phosphate buffer 60 mM, pH 5, was prepared by dissolving 8.9056 g of sodium dihydrogen phosphate (Fluka, Switzerland) and 0.4914 g of disodium hydrogen phosphate (Fluka) in water and making up to the mark in a 1-l volumetric flask. Phosphate buffers 60 mM, pH 4–9, were prepared similarly with pH adjustment using 1 M sodium hydroxide and 1 M hydrochloric acid solutions.

2.2. Sample preparation

Four commercial products of potassium iodide tablets, for radiation emergency, were employed in validation of the method. IOSAT™ (distributed by ANBEX, USA) and Thyro-Block™ (Wallace Pharmaceuticals, USA) are the samples, which contain 130 mg KI/tablet. NO-RAD™ (Body Gold, USA) and RAD BLOCK KI™ (distributed by USDPI, USA) both contain 65 mg KI/tablet. In the analysis, tablets were accurately weighed and dissolved in 500.0 ml of water. Suspension was removed by filtration through 0.25 μM pore of cellulose acetate membrane before analyses.

2.3. Cyclic voltammetry

Similarly to the former reports [6,11–15], an Autolab potentiostat 100 (Eco-Chemie, the Netherlands) with a single compartment three electrode glass cell, was used for all the cyclic voltammetric studies. A BDD electrode was pressed against the smooth ground joint at the bottom of the cell and was isolated by an o-ring, which resulted in 0.07 cm^2 of electrode area. Electric contact was carried out by placing the backside of electrode (Si substrate) on a brass plate. The electrochemical cell was housed in a faradaic cage to reduce electronic noise. A platinum wire and a Ag/AgCl with a salt bridge were used as the auxiliary and reference electrodes, respectively.

Cyclic voltammetric study was also carried out, in the same way, using glassy carbon as the working electrode.

2.4. Amperometric determination on BDD electrode by flow-injection

For routine analysis, we propose use of the BDD detection of iodide by flow-injection technique [30]. The system is displayed schematically in Fig. 1. An Ismatec peristaltic pump (model IS7610, Switzerland) was used for propelling the carrier stream (phosphate buffer). Arrangement of the amperometric detection with a BDD working electrode was the same as that described in previous works [6,11–15]. Again in this present work, the electrode area was utilized at 0.36 cm^2.

The final sample solutions were diluted (25 or 50 times) before injections. External calibrations with aqueous solutions of potassium iodide were adopted.

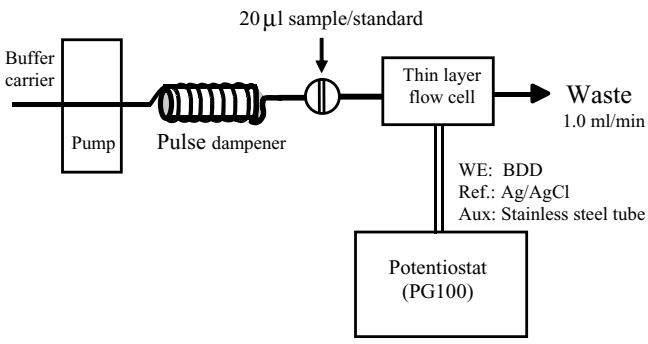

Fig. 1. The FI manifold with amperometric detection using BDD as the working (WE) electrode. Optimal condition: potential, 1.0 V; carrier, 60 mM phosphate buffer at pH 5.

2.5. Potentiometric analysis by ion selective electrode (ISE)

Accurate 10.00 ml of a sample solution was transferred into a 50 ml volumetric flask. To the flask, a 1.0 ml of 5 M $NaNO_3$ solution was added as an ionic strength adjuster. The mixture was made up to the level with water. The solution was measured for the potential developed across the employed iodide-ISE (Orion, USA) and the saturated calomel electrode (Orion). A digital Orion Ionanalyzer (model 601A) was used for the measurement.

2.6. The colorimetric method employed for comparison

Our colorimetric flow-injection method [23] which utilizes gas diffusion for selective determination of iodide was adopted for the validation. The detection is based on complex formation of I_3^-–starch. This method was previously validated against another technique.

3. Results and discussion

3.1. Cyclic voltammetry: BDD and GC electrodes

Fig. 2 shows the cyclic voltammograms that are obtained form an iodide solution together with the corresponding background voltammograms for BDD and GC electrodes. Peak-shaped oxidation responses are observed for both electrodes, but the background currents are dramatically different. The background current of the GC electrode is approximately 10 times greater than the background of BDD electrode. The BDD exhibits well-defined quasi-reversible oxidation peaks at 0.57 and 0.79 V versus Ag/AgCl, whereas the GC electrode provides the oxidation peaks at 0.48 and 0.74 V. To avoid using the high potential position, therefore, the first redox couple was considered. The potential differences between the first reduction peaks and the corresponding anodic peaks are at the order of 90 mV at GC electrode and 220 mV at BDD electrode. The peak separation suggested that the voltammetry is distorted by quasi-reversible behavior. The first anodic peak current, subtracted from background current, is 0.86 μA at BDD electrode and is 0.67 μA at GC electrode. We then expect that BDD electrode would provide a better sensitivity.

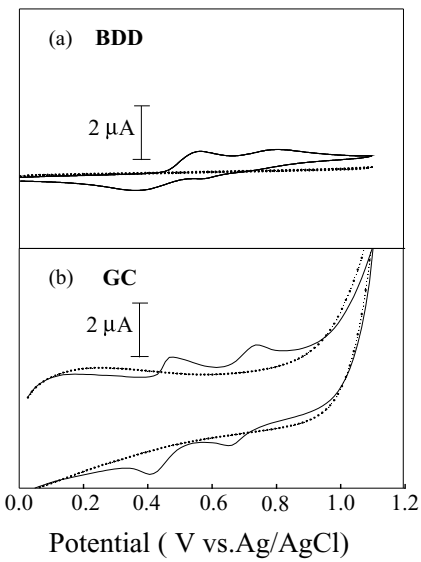

Fig. 2. Solid lines are the cyclic voltammograms that were obtained from batch experiment for 0.50 mM iodide in 60 mM phosphate buffer (pH 5). Background voltammograms (60 mM phosphate buffer, pH 5) are also shown as dotted lines for both electrodes. The scan rate was fixed at 0.02 V s^{-1}.

There have been some reports of cyclic voltammograms of iodide on platinum [31], gold and glassy carbon [32] electrodes. In these works, the authors also observed sequential oxidation of iodide, similarly to what we have seen in Fig. 2. Thus it is likely that the oxidation behaviors of iodide for both BDD and GC are the same as that reported in the systems studied by Hanson and Tobias [31] and by Qi and Hiskey [32]. These authors investigated and suggested that the oxidation sequence of iodide at the working electrodes can be represented by the following reactions:

$$3I^- \rightarrow I_3^- + 2e^- \quad (1)$$

$$2I_3^- \rightarrow 3I_2 + 2e^- \quad (2)$$

Our results also exhibit that the oxidation sequence is reversible for BDD and GC electrodes. For our work, we employed only the first oxidation peak current.

Buffer pH was also investigated from pH 4–9. The BDD and GC electrodes exhibit well-defined cyclic voltammograms of iodide in all pHs. It was observed that the oxidation peak currents slightly shift to positive potentials with increasing pH. The current background was enlarged when approaching the higher pHs. We chose pH 5 as the optimal pH for further studies on the BDD electrode.

3.2. Scan rate and concentration dependence study

Cyclic voltammograms of an iodide solution on BDD were investigated at different scan rates, the results are displayed in Fig. 3. As seen in the inset of Fig. 3, peak current varies linearly with the square root of scan rate. This indicates that the current is limited by semifinite linear diffusion of iodide to the BDD electrode.

The relationship between oxidation peak current (μA) and iodide concentration was examined from 0.50 to 10 mM. Linear calibration ($r^2 = 0.999$) was obtained with the slope of 6.07 μA mM^{-1}. These results have demonstrated that the BDD electrode is appropriate for the quantitation of iodide.

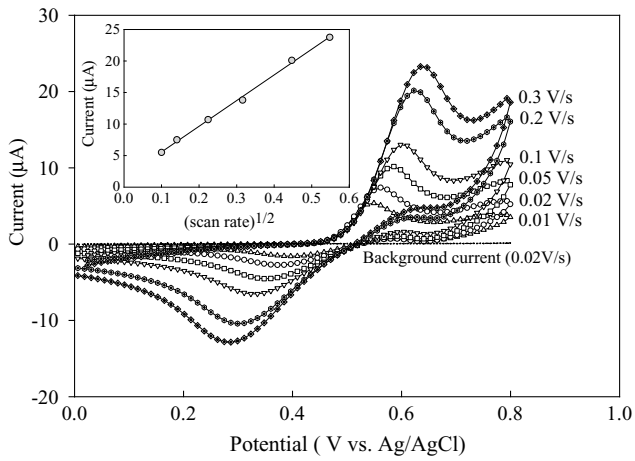

Fig. 3. Cyclic voltammograms, obtained at various potential scan rates for 1 mM iodide in 60 mM phosphate buffer (pH 5) at BDD electrode. The inserting figure shows the relationship between the oxidation current and the square root of the scan rate with regression figures of 40.74 μA $(s/V)^{1/2}$ (slope) and 0.998 (r^2).

3.3. Flow-injection analysis with amperometric detection

3.3.1. Optimal potential

For routine analysis, it is often more convenient than the potentiometric mode to use hydrodynamic injection of samples with amperometric detection. Optimization was first carried out using the FI manifold in Fig. 1 to find the most sensitive oxidation potential. The results are presented in Fig. 4. According to the results, the maxima S/B ratio was observed at 1.0 V. Therefore, this potential was chosen for amperometric detection in all FI experiments.

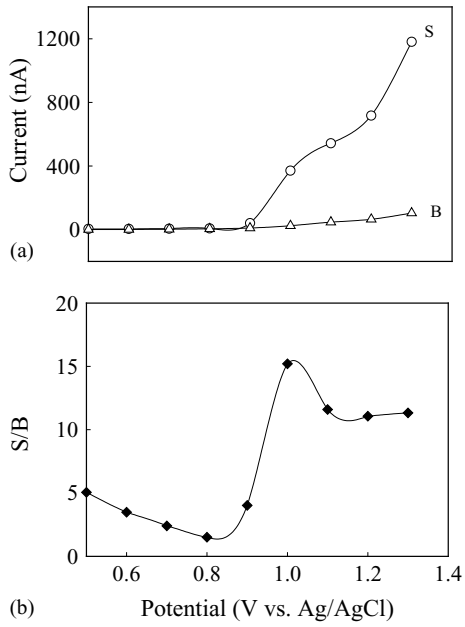

Fig. 4. Results obtained from the system of amperometric-FI (Fig. 1): (a) hydrodynamic signal of the carrier, 60 mM phosphate buffer or the background current (B) and peak current from injections ($n = 5$) of 10 μM of iodide into the carrier stream (S) and (b) plot of the signal-to-background ratio (S/B) and the applied potential.

3.3.2. Analytical features

We examined analytical performance of the manifold shown in Fig. 1. The system was operated at 1.0 V. Linear calibration was observed between 0.8 and 200 μM. The regression equation is $y = 37.41x - 2.28$ ($r^2 = 0.999$), where y and x are the area of peak current (nA s) and iodide concentration (μM), respectively. The detection limit (3σ) is as low as 0.01 μM. The system provides an impressively good precision (%R.S.D. = 2.2) for 20 μl injections ($n = 30$) of 0.1 mM iodide. Throughput of sample is 85 samples h^{-1}.

3.3.3. Performance on pharmaceutical applications

3.3.3.1. Nuclear emergency tablets. Samples of potassium iodide tablets were determined using our electrochemical method (Fig. 1). The results were compared with the values obtained from an ISE method and a colorimetric flow-injection method [23] as shown in Fig. 5. According to the ANOVA test [33], the results for IOSAT™, Thyro-Block™ and NO-RAD™ are not significantly different at 95% confidence [$F_{observed}$ are 0.1037 (IOSAT™), 0.7862 (Thyro-Block™) and 3.493 (NO-RAD™) where $F_{critical} = 4.256$]. The results of RAD BLOCK™ determined by all the method are not significantly different at 99% confidence ($F_{observed} = 6.667$, $F_{critical} = 8.022$). It can also be observed in Fig. 5 that all of our results, with three different analytical methods, agree well with the manufacture's contents.

For these samples, there is no evidence of interferents from sample matrices. Recovery studies made for all the samples have shown that the extracts' matrices do not have influence on electrochemical oxidation of iodide (86–103% recovery). For our method, the samples can be directly analysed after the following steps: water extraction, filtration, dilution and finally injection.

3.3.3.2. Multivitamin tablets. Multivitamins have always been our sample of interest. Usually these samples contain iodine at much lower levels (e.g., 150 μg I/tablet) than the raidoactive protection tablets (e.g., 49 mg I/tablet). With appropriate extraction (for example, one vitamin tablet is extracted with 100.0 ml of water, (approximately 1 mg I L^{-1})) we should be able to get reasonable signal currents from this type of sample.

Our results demonstrated that, although with C-18 clean up, the extracts still contain other electroactive species that cause positively erratic results (can be up to 500 times greater in signal current). An experiment was then carried out to study effects of foreign ions, including compounds that are likely to exist in aqueous vitamin extracts. The results are summarized in Table 1. According to these results, at least two ingredients (Vitamin B_6 and ascorbic acid) interfere the quantitative analysis of iodide.

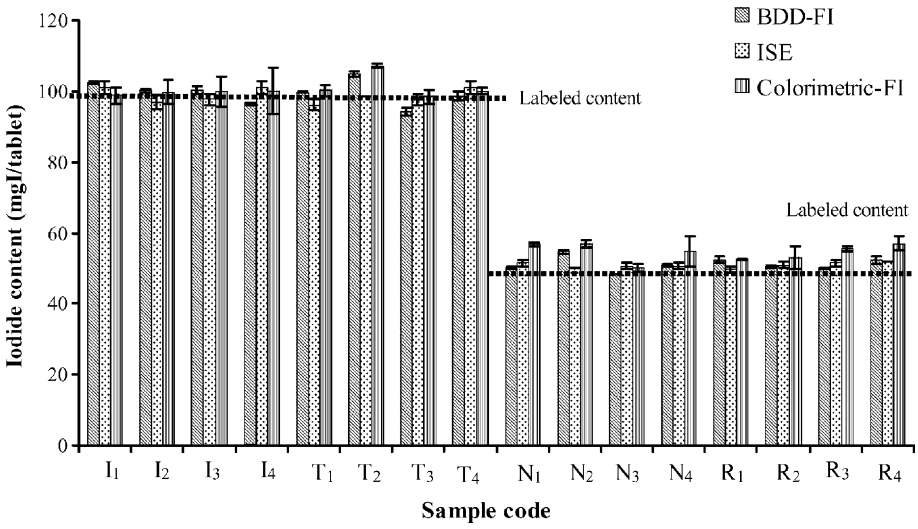

Fig. 5. Comparison of the iodide contents found in potassium iodide tablets, which were, obtained by the proposed method (BDD-FI), ISE method and colorimetric-FI method [20]. Determination by each method was carried out in triplicate for a sample. I: IOSAT, T: Thyro-Block, N: NO-RAD and R: RAD BLOCK KI.

Table 1
Effect of foreign ions on the alteration of FI signals obtained from replicate injections ($n = 5$) of standard potassium iodide 0.01 mM

Foreign species/added as	Investigated concentration ($mg\,L^{-1}$)[a]	Results[b]
1. Vitamin B_1 or aneurine hydrochloride/ $C_{12}H_{17}ClN_4OS{\cdot}HCl$	21–42	Does not interfere
2. Vitamin $B_2/C_{17}H_{20}N_4O_6$	24–48	Does not interfere
3. Vitamin B_6 or adermine hydrochloride/ $C_8H_{11}NO_3{\cdot}HCl$	30–60	Strongly interfere (e.g. 22% alteration even at $2\,mg\,L^{-1}$)
4. Vitamin C or L(+)ascorbic acid/$C_8H_8O_6$	900–1200	Strongly interfere (e.g. 19% alteration even at $1.6\,mg\,L^{-1}$)
5. Cl^-/NaCl	180–363	Does not interfere (studied up to $200\,mg\,L^{-1}$)
6. F^-/NaF	–	Does not interfere (studied up to $800\,mg\,L^{-1}$)
7. Br^-/NaBr	–	Does not interfere (studied up $200\,mg\,L^{-1}$)

[a] Likely concentrations of the foreign species, no. 1–5, in vitamin extracts.
[b] Greater than ±5% signal alteration is classified as interfering condition.

4. Conclusions

This work presents, for the first time, application of the BDD for quantitative analysis of iodide. For routine, we recommend amperometric detection in flow-injection mode. The flow-injection system is simply a single channel manifold with flow-through electrochemical cell of the working diamond electrode. With this construction, analysis of samples is very rapid (85 samples h^{-1}). Wide dynamic range (0.8–200 μM) was obtained, with good precision (R.S.D. = 2.2%).

The method is suitable for determination of iodide in nuclear emergency tablets. The BDD-FI method was proved to be valid for this type of samples. However, for multivitamins, separation prior the amperometric detection is required, since some of the ingredients have shown to interfere quite strongly. Utilization of the amperometric BDD as the detector for liquid chromatography is at present being investigated for multivitamins.

Acknowledgements

This work was supported by grants from the Thailand Research Fund, Ratchadapisek Somphot Endowment Grant and the Post Graduate Education and Research Program in Chemistry. The authors would also like to thank the TJTTP-OECF. Special thanks are extended to Miss Nattakarn Wangfuengkanagul for her kind training of the potentiometric measurement.

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Sequential injection with lab-at-valve (LAV) approach for potentiometric determination of chloride☆

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Received 13 June 2004; received in revised form 10 August 2004; accepted 11 August 2004
Available online 16 September 2004

Abstract

Sequential injection with "Lab-at-Valve (LAV)" approach is demonstrated for potentiometric determination of chloride. The LAV flow-through electrode system consists of two Ag/AgCl electrodes: one as a reference electrode, silver chloride activated surface-silver wire soaked in a constant-concentration chloride ion solution in a small tube covered with a polymer-membrane, another as a working electrode (a similar silver chloride activated surface-silver wire) placed in a flow channel. The electrode system is attached at one port of a 10 port multiposition valve. A modified autoburette was used as a propelling device. Using SI operation via a program written in-house, based on LabVIEW®, a standard/sample is inserted, via the selection valve, in potassium nitrate as an electrolyte and water is used as a carrier. The zones are transported from the holding coil to the flow cell to monitor the difference in potential due to concentration cell behavior. The potential difference is then recorded as a peak. Peak height is proportional to logarithm of chloride concentration. The SI-LAV for chloride determination is very simple, fast, precise, accurate, automatic and economical. Applications to mineral drinking water and surface water have been made. The results agree with those of IC and titrimetric methods.
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Keywords: Sequential injection; Lab-at-valve; Potentiometry; Chloride.

1. Introduction

Environmental-friendly analytical procedures are in demand for today's analytical chemistry. Green analytical chemistry concerns the use of non-toxic chemicals, with smaller amounts of reagent and hence less waste generated, and with high throughput analysis with a high degree of automation and portability. To meet these requirements, various approaches based on flow analysis have been developed to miniaturize and automate analytical systems, such as flow injection analysis (FIA) [1–3], multi-syringe FIA [4], sequential injection analysis (SIA) [5,6], SIA with lab-on-valve (LOV) [7] and micro total analysis systems (μTAS) or lab on a chip (LOC) [8].

The μTAS concept initiated by the group of the late Widmer [8] in 1990 has become an active research area today. Although, this approach provides fast analysis using minute amounts of sample and reagents, the production of the chip platform needs advanced, expensive facilities, which are not affordable by most laboratories, and the application of the technique to real samples is still limited.

Ruzicka introduced a SI-LOV concept [9] in 2000, to perform wet chemistry at microlitre levels with a relatively large bore conduits, to avoid surface contamination and clogging. The SI-LOV was designed to integrate sample processing channels with a multipurpose flow cell, and machined to perfectly fit on top of a selection valve by replacing a stator

☆ Presented at the 12th International Conference on Flow Injection Analysis, December 7–13, 2003, Venezuela.

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0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2004.08.007

plate and connection port of the valve. In this way, various analytical processes, namely sampling, sample pretreatment, reaction and separation, and detection, can be incorporated onto an SIA valve. This approach is more tolerant to dirty real samples than chip based approaches.

A simpler approach, SI with lab-at-valve (LAV) concept, has been proposed [10,11]. This is employed by attaching a device integrating sample processing and detection units on a port of a multiposition selection valve, without taking apart components of a purchased valve. This makes the LAV simpler than the LOV. The LAV unit can be built using an ordinary and less precise machine tool, to have suitable functions for chemistries of interest and with a nut that can plug in a port of the valve in the usual way. Such an integrated LAV device compiling analytical process taking place in it should be compact and economical comparing to a normal SIA system. Various advantages similar to those of the LOV should be gained by using the LAV. In this paper we demonstrate SI-LAV approach for potentiometric determination of chloride with a simple made chloride ion selective electrode (ISE), which has been introduced earlier for use in FI [1–3], but with modification to use a Ag rod instead of tubular Ag to make the electrode more easy to clean and replenish the AgCl film, and to store after use. The proposed potentiometric system is simpler than the previously reported spectrophotometric ones and does not require expensive or toxic reagents, e.g., silver nitrate [5] or mercuric thiocyanate [6,7].

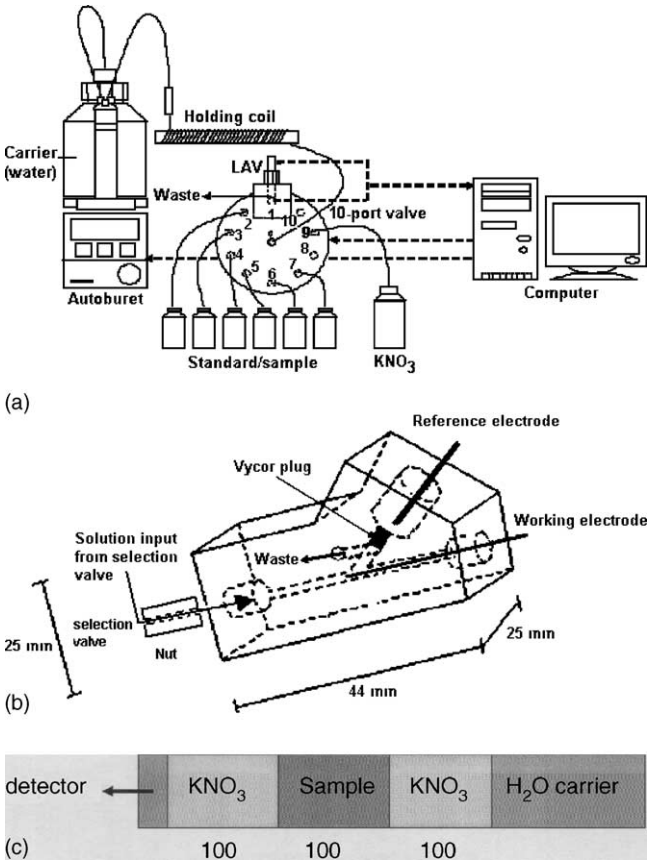

Fig. 1. (a) Schematic diagram of the SI-LAV system; (b) Potentiometric flow cell (LAV unit), the LAV plugged onto a port of the selection valve (45 mm distance from the port to the working electrode, 1.0 mm i.d. channel); and (c) Sequence of solutions aspirated into the system; the values indicating volumes of the solutions (μl).

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Deionized water (obtained by a system of Milli-Q, Millipore, Sweden) was used throughout. A chloride standard stock solution (0.2 M) was prepared by dissolving 5.8440 g of sodium chloride (Merck, Darmstadt, Germany) in water and making upto a volume of 500 ml in a volumetric flask. Working standard solutions of appropriate concentrations were obtained by diluting the stock solution with water. A potassium nitrate stock solution (0.10 M) was prepared by dissolving 5.05 g of potassium nitrate (Merck, Darmstadt, Germany) in 500 ml of water. Working electrolyte solutions of appropriate concentrations were prepared by diluting the stock solution with water. A ferric chloride solution (0.5 M) in 1 M hydrochloric acid was prepared by dissolving 8.11 g of $FeCl_3$ anhydrous (Merck, Darmstadt, Germany) in 100 ml of 1 M HCl.

2.2. Potentiometric flow through cell

A potentiometric flow through cell was designed to be put on a side of a selection valve by plugging into one port of the selection valve (the cell acts as the LAV unit) as shown in Fig. 1(a). The cell was made from a Perspex plastic block (see Fig. 1 (b)) by cutting and drilling to form channels for inserting a Ag/AgCl wire working electrode (WE) and another Ag/AgCl wire reference electrode (RE), and for carrier solution inlet and outlet. The channel for RE was separated from the carrier solution channel by a small VYCOR plug (BAS, Indiana, USA), acting as a salt bridge.

2.3. SI-LAV manifold

The SI-LAV system used is schematically depicted in Fig. 1(a). It consisted of an autoburette (765 Dosimat, Metrohm Ltd., Herisau, Switzerland) equipped with a 10 ml exchange unit for the pumping system, connected to a personal computer via an RS232C interface, a ten-port multiposition valve with a microelectric actuator (C25-3180EMH, VICI, Texas, USA), and a LAV unit with two Ag/AgCl electrodes, which are connected directly or via a potentiometer (744 pH meter, Metrohm Ltd., Herisau, Switzerland) to the input terminals of a data acquisition board (AT-MIO-16X-50 equipped with SCB-68, National Instruments, Texas, USA). The autoburette was connected to the center of the selection valve via a holding coil (Tygon® tubing, 0.8 mm i.d., 4.0 mm o.d., 4.5 m long, Cole-Parmer Instrument Company, Illinois, USA) and the LAV unit was placed at

port-1 of the selection valve. Both instrumental control and data acquisition were manipulated via programs written in-house based on LabVIEW®software (Version 6; National Instruments, Texas, USA). This software provided control of the volume to be dispensed or aspirated by the autoburette, flow rate, selection of different valve positions and performance of data acquisition. The SIA-gram data were evaluated for peak height by using Microsoft® Excel 97 software (version 8.0, Microsoft, Arizona, USA).

2.4. Preparation of a Ag/AgCl electrode

A silver wire (0.5 mm in diameter) obtained from a local jewelry shop was used to prepare an Ag/AgCl electrode. The wire was polished and cleaned just before immerging it into a solution of 0.5 M ferric chloride in 1 M hydrochloric acid, to form a AgCl film on the electrode. The electrode was then washed with water and used as a working or a reference electrode by assembling each into the flow through cell described above. A VYCOR plug served as a salt bridge for the reference electrode, and the reference electrode was immersed in 1.40 mM KCl/0.01 M KNO_3 solution, unless otherwise stated.

2.5. Procedure

The solution aspiration sequence of the SI-LAV system is summarized in Table 1 and Fig. 1(c). First, the carrier (H_2O) was drawn to fill all tubes. Then, a 100 μl portion of potassium nitrate, 100 μl of standard/sample and 100 μl of potassium nitrate solution, respectively, were sequentially aspirated into a holding coil. Finally, the stacked zone was sent to the LAV unit to monitor for potential difference between WE and RE as the zone passed the WE, recording as a SIA-peak. The potential difference is based on a concentration cell, a difference in concentrations of chloride in the WE and RE half-cells. A calibration graph was plotted of the peak height obtained as a function of the logarithm value of concentration of the chloride standard. Chloride concentration in a sample was evaluated from the calibration graph. In spite of the slow operation of the autotitrator, the whole operating cycle took only about 70 s for a sample. Replacing with a more efficient pumping device would result in even a higher sample throughput.

3. Results and discussion

3.1. SI-LAV components

The SI-LAV concept was demonstrated for the determination of chloride. A LAV unit, a flow through cell for potentiometric detection with a simple home-made chloride ion selective electrode, could be fabricated small enough to put at one port of the selection valve (see Fig. 1). The upper port position was the most appropriate one to attach the flow cell to the valve because it is easier to remove any air bubble, which may be in the line out of the cell. A polymer VYCOR plug was inserted between the reference electrode and the flow channel in order to prevent leaking of chloride from the reference solution. The LAV is compact in size and has various advantages that are similarly offered by a LOV system [9]. However, the LAV can be assembled without taking apart components of a purchased valve and is easy to build to be suitable for chemistries of interest. Moreover, it can be fabricated with relatively low-cost, less precise machine tools. In this work, an autoburette was employed as an alternative inexpensive solution propelling device in place of a syringe pump. However, a solution volume of less than 100 μl cannot be aspirated due to the default setting of the autotitrator to aspirate at least 100 μl volumes each time. The WE and RE were connected directly to the signal and ground poles, respectively, of an analog input of the interfacing board. Identical signal profiles were obtained by either connecting the electrode directly to the input of the interface card or by inserting a potentiometer between them. This indicates that an impedance of the interfacing board input terminal was high enough to detect the cell potential correctly.

3.2. Optimization

Due to limitation of the autoburette used, some parameters were fixed: volume of electrolyte solution and sample, 100 μl each, flow rate for aspiration and dispensation of the solution, 15 ml min^{-1}. The effects of concentration of the electrolyte (KNO_3) solution and sodium chloride in the RE half cell on the slope of the calibration graph for chloride were investigated. It was found that the electrolyte concentration did not significantly affect to the slope, intercept and R^2 of the calibration graph, for the injection of a series of standard solutions as shown in Table 2. However, the 0.01 M KNO_3 was selected to minimize the effect of ionic species due to

Table 1
SI-LAV operating sequence

Step	Operation	SV port[a]	Volume (μl)	Flow rate (ml min^{-1})	Flow direction
1	Aspirate electrolyte solution	9	100	15	To HC
2	Aspirate standard/sample solution	2–7	100	15	To HC
3	Aspirate electrolyte solution	9	100	15	To HC
4	Dispense to LAV unit and record signal	1	2000	15	To LAV

[a] SV = Selection valve.

Table 2
Effect of potassium nitrate concentration

$[KNO_3]$ (M)	Calibration equation (0.60–2.80 mM Cl^-)	R^2
0.005	$y = 0.053x - 0.047$	0.999
0.010	$y = 0.051x - 0.029$	0.999
0.025	$y = 0.049x - 0.038$	0.998
0.050	$y = 0.053x - 0.045$	0.999
0.100	$y = 0.051x - 0.045$	0.999

sample matrices, which may affect the total ionic strength of the solution passing through the WE. For mineral drinking water and surface water samples, this concentration of the electrolyte is sufficient for control of ionic strength of the sample.

Chloride concentration in the reference half-cell did not affect to the slope, intercept and R^2 of the calibration graph (a plot of peak height (V) (y) versus log (mM concentration of chloride standard solution) (x)). Using a reference solution containing 0.01 M KNO_3 with 0.30, 0.80, 1.40 or 2.80 mM potassium chloride, the calibration equations: $y = 0.051x - 0.030$, $R^2 = 0.998$, $y = 0.051x - 0.032$, $R^2 = 0.998$, $y = 0.051x - 0.032$, $R^2 = 0.999$ or $y = 0.051x - 0.030$, $R^2 = 0.998$, respectively were obtained. A 1.40 mM chloride solution with 0.01 M KNO_3 in the RE half cell was selected.

3.3. Analytical characteristics

Three linear calibration graph ranges, 0.10–0.80 mM ($y = 0.059x + 0.120$), $R^2 = 0.998$), 0.80–8.40 mM ($y = 0.056x - 0.105$, $R^2 = 0.998$) and 10–120 mM ($y = 0.060x - 0.128$, $R^2 = 1.000$) were obtained. Each injection consumed 200 μl of 0.01 M KNO_3 and 100 μl of standard/sample, with the whole analysis time of 70 s, which corresponds to sample throughput of about 50 h^{-1}. Relative standard deviations for 11 replicate injections of 10–40 mM chloride standards were in range of 0.7–1.3%. Higher %RSDs may be obtained for solutions containing chloride concentrations out-side of the range.

The stability of the electrode was studied by continuous injection (about 300 injections) of a series of concentrations of chloride standard solutions (0.10–0.80 mM) and some samples, for a period of time about 6 h. The slope of the calibration graphs was quite constant (0.06 ± 0.01), indicating a good stability of the electrode for at least 6 h. Although, the effects of some interferences have not been investigated here, the literature [12–14] indicates that some halides (I^- and Br^-), sulfide, cyanide and some metal ions (Fe^{3+} and Al^{3+}) have some interferences in the analysis by this kind of chloride ion selective electrode. However, these ions are usually present in natural waters at relatively low concentrations compared to chloride and do not significantly interfere, otherwise sample pretreatment is needed [12–14].

Table 3
Chloride contents in mineral drinking water (triplicate determination)

Sample	Chloride concentration (mM)	
	IC [15][a]	SI-LAV[b]
1	0.30	0.37 ± 0.01
2	0.14	0.15 ± 0.01
3	0.65	0.73 ± 0.02
4	0.37	0.35 ± 0.01
5	0.08	<0.10
6	0.06	<0.10
7	0.02	<0.10
8	0.02	<0.10
9	0.17	0.20 ± 0.01
10	2.36	2.26 ± 0.01
11	0.02	<0.10

[a] Analysis by routine laboratory of the Science and Technology Service Center, Faculty of Science, Chiang Mai University.
[b] Using the calibration equation of the range 0.10–0.80 mM Cl^-. The limit of quantitation is 0.10 mM.

Table 4
Chloride contents in surface water samples taken around a pickling industry (triplicate determination)

Sample[a]	Chloride concentration (mM)	
	Titration [16]	SI-LAV[b]
1	3.0 ± 0	2.0 ± 0.3
2	86 ± 1	86 ± 1
3	85 ± 2	86 ± 2
4	86 ± 3	88 ± 1
5	66 ± 0	69 ± 1
6	66 ± 1	68 ± 1
7	71 ± 2	71 ± 2
8	50 ± 1	45 ± 2

[a] Sample 1 = supplied pond water for the factory usage, 2–8 = surface water around the factory.
[b] Using the calibration equations of ranges of 0.80–8.40 and 10–120 mM Cl^-.

3.4. Application to water samples

The developed SI-LAV system was applied for the determination of chloride in different commercially available mineral drinking water samples from a market place in Chiang Mai, and surface water samples collected from around the vicinity of a pickling factory in Hang Dong district of Chiang Mai. Analyses by ion chromatography [15] and Mohr titration [16] were also carried out for comparison. The results (means of triplicate determinations) obtained are summarized in Tables 3 and 4. The results from the pairs of the two methods correlate well with each other, except at low concentrations of chloride. However, the t-test at 95% confidence level indicated that the results obtained from the techniques are not significantly different [17]. The chloride contents in surface water around the pickling factory were found to be significantly higher than the raw water used by the factory, indicating that salt may be introduced to the surface water via discharge water. More close investigation on this will be carried out in our laboratory.

4. Conclusion

A novel SI-LAV concept is demonstrated. Here we describe potentiometric determination of chloride in some water samples. Various advantages of the proposed LAV are similar to those of the LOV, such as economical and integrated instrumentation, small amounts of reagent consumption, rapidity, and automation in analysis. However, the LAV unit can be more easily fabricated with relatively low price materials and available instrument/machine tools, so the LAV could be a cost effective alternative. Other LAV developments such as solvent extraction and bead injection are in progress [18,19].

Acknowledgements

The authors thank the Thailand Research Fund (TRF) and the Post-graduate Education and Research Program in Chemistry (PERCH) for research grant support. The authors also thank Miss Ton Thu Giang for conducting some experiments and Professor Gary D. Christian for useful discussion.

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A dynamic liquid–liquid interfacial pressure detector for the rapid analysis of surfactants in a flowing organic liquid

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Received 22 April 2004; received in revised form 27 July 2004; accepted 28 July 2004
Available online 27 August 2004

Abstract

Design and development of a dynamic interfacial pressure detector (DIPD) is reported. The DIPD measures the differential pressure as a function of time across the liquid–liquid interface of organic liquid drops (i.e., *n*-hexane) that repeatedly grow in water at the end of a capillary tip. Using a calibration technique based on the Young–Laplace equation, the differential pressure signal is converted, in real-time, to a relative interfacial pressure. This allows the DIPD to monitor the interfacial tension of surface active species at liquid–liquid interfaces in flow-based analytical techniques, such as flow injection analysis (FIA), sequential injection analysis (SIA) and high performance liquid chromatography (HPLC). The DIPD is similar in principle to the dynamic surface tension detector (DSTD), which monitors the surface tension at the air–liquid interface. In this report, the interfacial pressure at the hexane–water interface was monitored as analytes in the hexane phase diffused to and arranged at the hexane–water interface. The DIPD was combined with FIA to analytically measure the interfacial properties of cholesterol and Brij®30 at the hexane–water interface. Results show that both cholesterol and Brij®30 exhibit a dynamic interfacial pressure signal during hexane drop growth. A calibration curve demonstrates that the relative interfacial pressure of cholesterol in hexane increases as the cholesterol concentration increases from 100 to 10,000 $\mu g\,ml^{-1}$. An example of the utility of the DIPD as a selective detector for a chromatographic separation of interface-active species is also presented in the analysis of cholesterol in egg yolk by normal-phase HPLC-DIPD.
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Keywords: DIPD; DSTD; Flow-based technique; Chromatography; FIA; Liquid–Liquid interface; Cholesterol; Brij®30

1. Introduction

Surfactants are chemical species that concentrate and align preferentially at an interface due to the presence of both hydrophilic and hydrophobic moieties within their structure. Alignment at the air–liquid surface (surface tension), solid–liquid and liquid–liquid interfaces (interface tension) often results in the lowering of surface tension at the given interface. Due to their unique properties (i.e., the ability to lower surface tension, foam, and create emulsions), surfactants and other interface-active species are widely used in many industries including food and beverage, personal care products, cosmetics, metal working, mining, agriculture, paper, and leather. Furthermore, surface-active chemicals are used in large quantities for many household applications.

Several methods have been developed for the measurement of surface tension. Most of these methods can be divided into three main groups: force methods (Du Noüy ring and Wilhelmy plate), shape methods (sessile drop, pendent drop, and spinning drop), and pressure methods (small bubble

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doi:10.1016/j.talanta.2004.07.040

surfactometer) [1]. When a surfactant-containing solution forms a fresh interface, a finite time interval must elapse before equilibrium surfactant concentrations can be partitioned between the interface and the bulk solution. The aforementioned methods actually measure the static surface tension since the measurement is made after the interface-active analytes attain equilibrium at the air–liquid interface. The non-equilibrium interface tension is known as the dynamic interface tension. Most surfactant-driven processes, such as foaming, emulsification, coating flows and wetting, take place under non-equilibrium conditions, and therefore, dynamic interface tension information is an imperative parameter for understanding these processes. In principle, some of the surface tension measurement methods described above can be used to measure dynamic surface tension, however, most are not appropriate for high-throughput measurements. A few of the most common dynamic surface tension techniques are maximum bubble pressure [2], oscillating jet [3] and fast forming drop [4].

In the study of surfactants, another area of interest is the interfacial tension at the liquid–liquid interface. Specifically, adsorption at liquid–liquid interfaces plays a key role in the dynamics of multiphase systems. Though the principles are similar to adsorption at the air–liquid interface, measurements at the liquid–liquid interface require unique instrumentation. Ravera et al. reviewed the use of several techniques for liquid–liquid interfacial pressure measurements and found that while in theory many of the techniques commonly employed for air–liquid measurements can also be used for liquid–liquid interface studies, in practice, however, many obstacles, such as solvent miscibility, density, and viscosity came to the forefront [5]. Thus, relative to measurements at the air–liquid interface, few instruments are available for liquid–liquid interface studies and even fewer of these measure dynamic interfacial tension. The lack of methods to measure liquid–liquid interfaces is likely due to the difficulty of designing the instrumentation.

Most of the research in the area of liquid–liquid interfacial measurements focuses on the physics of adsorption at the interface, and the development and testing of theories to understand the kinetics and thermodynamics of adsorption at the interface [4–11]. Instead, we focus on the dynamic analytical signal resulting from adsorption at the liquid–liquid interface and have developed a simple and robust instrument to detect and quantify interface-active analytes in organic samples. The technique presented here offers a mechanically simple and inexpensive means to measure the liquid–liquid interface. This instrument can also be used to qualitatively describe the adsorption kinetics of interface-active analytes and the interactions of mixtures that affect the interfacial pressure [12,13].

Another important attribute of surface and interfacial tension measurement methodologies is the ability to carry out real-time analysis on dynamic systems containing complex and interacting surfactants. Previously, the dynamic surface tension detector (DSTD) was developed as a detection system for flow-based techniques, such as high performance liquid chromatography (HPLC), flow injection analysis (FIA), and sequential injection analysis (SIA). The DSTD responds to the surface activity of analytes at the air–liquid interface and offers a mechanically simple and inexpensive means to measure the air–liquid interface of a wide variety of samples, including samples that would foam using a bubble pressure technique [12–17]. The design and construction of the DSTD has previously been discussed in detail [12–19]. The DSTD has been used for the detection and study of species, such as common surfactants, polymers and proteins at the air–liquid interface. The most recent DSTD design is based on measuring the interior pressure throughout drop growth of repeatedly forming drops, which are pneumatically detached from a capillary tip.

Herein, we present an adaptation of the DSTD to form a dynamic interfacial pressure detector (DIPD) for the detection and analysis of species that are interface-active at the liquid–liquid interface. The DIPD complements the DSTD for evaluating chemical species that are either insufficiently soluble in aqueous media, or samples with unique liquid–liquid interface properties, or samples in which the dynamic interactions at the liquid–liquid interface are vital to their performance. In the presented experiments, the DIPD was coupled with FIA and HPLC for the study of the dynamic interface properties of typical surface-active species at the hexane–water interface.

2. Theory

The DIPD measurement follows principles similar to the DSTD [12]. In the DIPD, the internal pressure of repeatedly growing drops is measured relative to the pressure of the bulk solution phase in which the drops are grown, using a pressure transducer (see Figs. 1 and 2). Pressure is measured throughout the growth of the drop and the resulting drop pressure profiles are used to calculate the relative interfacial pressure.

The time-dependent Young–Laplace equation relates to the measured drop pressure, and was applied to calculate the

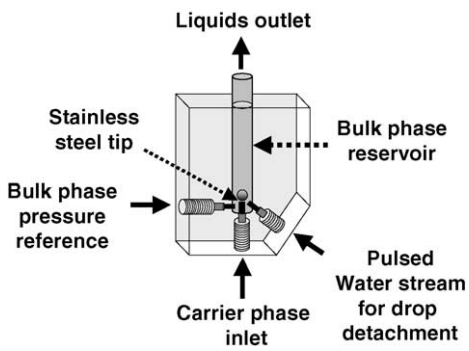

Fig. 1. A schematic diagram of the DIPD flow cell.

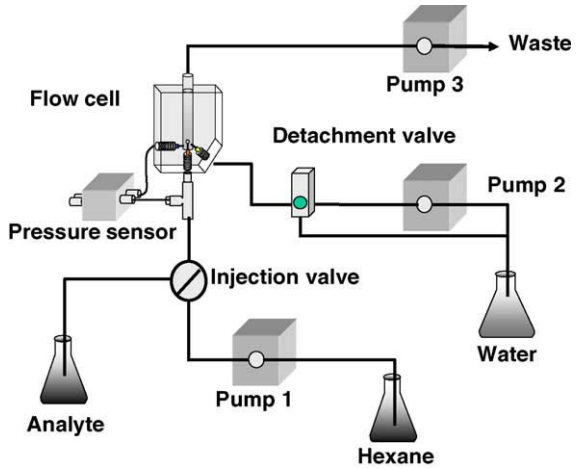

Fig. 2. A schematic diagram of FIA combined with DIPD for the liquid–liquid interfacial pressure measurement. Pump 1: piston pump at flow rate of 60 μl min^{-1} *n*-hexane, Pump 2: piston pump at flow rate of 4 ml min^{-1} water.

interfacial pressure from the pressure measurements:

$$P(t) = \frac{2\gamma(t)}{r(t)} + P_C \quad (1)$$

In Eq. (1), P is the drop pressure referenced to the water bulk phase pressure, γ is the liquid–liquid interfacial tension, r is the drop radius, and P_C accounts for viscous losses in the capillary tubing. The parameters, P, γ, and r, are functions of time, t, throughout the drop growth.

Drop pressure "profiles" are the raw signals obtained for the mobile phase ($P(t)_M$) (i.e., carrier stream), standard ($P(t)_S$), and analyte ($P(t)_A$). Combined with the dynamic interfacial pressure of the standard ($\pi(t)_S$), these three signals allow the dynamic interfacial tension of the analyte ($\pi(t)_A$) to be calculated using Eq. (2):

$$\pi(t)_A = \frac{\pi(t)_S\left[P(t)_M - P(t)_A\right]}{\left[P(t)_M - P(t)_S\right]} \quad (2)$$

The dynamic interfacial tension of the analyte ($\pi(t)_A$), calculated relative to the dynamic interfacial tension of the standard ($\pi(t)_S$), contains information regarding the diffusion and arrangement of analyte at the liquid–liquid interface.

It would be ideal to calibrate the DIPD sensor using a standard solution with known interfacial pressure $\pi(t)_S$. Likewise, it is advantageous to have a standard such that $\pi(t)_S$ is constant at a given standard concentration and independent of drop time, i.e, $\pi(t)_S$ is simply π_S. Unfortunately, due to the difficulty of liquid–liquid interfacial tension measurements, the liquid–liquid interfacial pressure of a suitable standard acting at the interface of *n*-hexane and water which has been confirmed by multiple techniques or multiple research groups could not be found in the literature [4,7,8,20–23]. For this reason, we report the relative interfacial pressure values, defined by Eq. (3):

$$\pi_R(t)_A = \frac{\pi(t)_A}{\pi_S} = \frac{\left[P(t)_M - P(t)_A\right]}{\left[P(t)_M - P(t)_S\right]} \quad (3)$$

Relative interfacial pressure is simply the interfacial pressure of the analyte divided by the interfacial pressure of the standard. Since the standard is consistent, the relative interfacial pressures of analytes can be compared to understand their interfacial properties and to analyze different analytes. In this work, isopropanol in *n*-hexane and *n*-octanol in *n*-hexane were evaluated, and applied, as standard solutions to achieve a relative interfacial pressure for analytes of interest from the DIPD, using Eq. (3). We found that both isopropanol and *n*-octanol align rapidly at the water–hexane interface thus providing a π_S independent of drop time.

3. Experimental

3.1. Materials

HPLC grade *n*-hexane, *n*-octanol and 2-propanol (isopropanol) were obtained from Fisher Scientific (Fisher Scientific international Co., Fair Lawn, NJ). Cholesterol (98% purity) and Brij®30 were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI). All chemicals were used as received. Deionized (DI) water, demineralized to greater than 18 MΩ with a Millipore system (Millipore, Bedford, MA), was used as the bulk phase and in the preparation of all solutions. The water was degassed using an ultrasonic bath (Cole-Parmer Instrument Company, Vernon Hills, IL) with vacuum system for 15 min prior to use.

3.2. Dynamic interfacial pressure detector and its flow cell

A flow cell, fabricated from acrylic sheet, was designed in-house for this detector. A block of acrylic sheet was selected as the flow cell material because it is transparent, easily machined, and reasonably chemically hearty for demonstrating the detection principle. A schematic diagram of the DIPD flow cell is shown in Fig. 1. The flow cell is comprised of the following parts:

(1) Bulk phase reservoir
A 0.795 cm inside diameter (i.d.) × 2.22 cm long cylindrical hole was drilled into the block of acrylic sheet to serve as the bulk phase reservoir. In this work, water was used as the bulk phase and *n*-hexane was used as the carrier phase. To prevent *n*-hexane from adhering to the acrylic sheet flow cell wall, the bulk phase reservoir was lined with a 0.795 cm outside diameter (o.d.) × 4.40 cm long Teflon® tube (Brinkmann Instruments, Inc., Westbury, NY). This allowed the detached hexane drops to rise to the surface and be drawn to waste.

(2) Carrier inlet
The buoyant force operating on the carrier phase droplets must be sufficient to overcome the adhesive forces acting between the carrier phase and the tubing tip. The tubing tip material is chosen to minimize the adhesive forces, which consequently, minimizes droplet shape distortion. Since the DSTD uses an aqueous carrier phase, poly(etheretherketone), or PEEK™, is chosen as the tubing tip material. With the DIPD and a *n*-hexane carrier phase, however, it is more appropriate to use stainless steel as the tubing tip material. The stainless steel tip is situated inside a threaded inlet provided at the base of the flow cell. The stainless steel capillary sensing tip was made by cutting a syringe needle (Becton Drive 25 G × 11/2 in BD™, Franklin Lakes, NJ) to a length of 10 mm and polishing both ends of the tip until smooth. As can be seen in Fig. 2, a side-arm just outside the carrier inlet is connected to the pressure sensor (Validyne DP15-40-2430, Northridge, CA). With this design, most organic solvents with densities less than water and immiscible with water may be used as a carrier stream (mobile phase), such as *n*-hexane used herein.

(3) Hydraulic drop detachment
Hexane drops were hydraulically detached by degassed water flowing at a high flow rate and directed at the base of the forming hexane drop. The drop detachment stream, which flows water at 4 ml min^{-1} when briefly actuated, was regulated by an injection valve, and actuated at 2 s intervals. The hexane drop size can therefore be controlled by the detachment interval.

(4) Water pressure reference
The reference side of the pressure sensor was connected to the water reference inlet in the flow cell reservoir to measure the pressure in the bulk phase. The *n*-hexane carrier, under constant flow rate conditions, was delivered through the capillary sensing tip to form drops at the end of the tip. The drops were either detached with the hydraulic water stream or would ascend to the surface as buoyant forces overcome the forces of molecular attraction between the drop and the tip. When hydraulic detachment is employed, drops are shot with a pulsed stream of water and detached from the tip well before drops would have risen to the surface due to the buoyancy force. Hydraulic detachment is advantageous because it increases data density, improves drop time repeatability, and allows relative interfacial pressure to be calculated in real-time.

A pressure transducer continuously measured the pressure signal. The signal was conditioned with a high gain carrier demodulator (Validyne CD12, Northridge, CA). The raw pressure signal was collected at the rate of 100 Hz and instrument control was performed on a PC (1.4 GHz AMD Duron™ processor, Sunnyvale, CA) with a data acquisition board (AT-MIO-16XE-50, National Instruments, Austin, TX), using software written in-house (LabVIEW Version 6, National Instruments, Austin, TX). Data analysis was performed with MATLAB 6.0 (The MathWorks, Natick, MA).

3.3. Flow injection analysis (FIA)

A diagram of the FIA system coupled with the DIPD is shown in Fig. 2. A piston pump (Beckman, Model 114 M, Berkeley, CA) was used to introduce the carrier and sample to the flow cell at a flow rate of 60 μl min^{-1}. A second pump (Beckman, Model 114 M, Berkeley, CA) was required to control the flow of water into the flow cell chamber for hydraulic drop detachment. As the hexane drops are formed at the end of the stainless steel tip, they were detached with the pulse of water created by switching a valve in-line with the flow at a frequency of 0.5 Hz (2 s per drop). The detached hexane drops then rose to the surface and were aspirated out of the flow cell by a vacuum pump along with excess water from the hydraulic detachment process. This allowed the vacuum pump to maintain a stable bulk phase fluid level in the water reservoir.

The principles of FIA were utilized to introduce sample to the DIPD. A sample volume containing the interface-active analyte in *n*-hexane was injected into the *n*-hexane carrier using an injection valve (EC10W, Valco Instruments Co. Inc., Houston, TX) equipped with the appropriate length of PEEK™ tubing (Upchurch, Oak Harbor, WA) loop size. The sample plug then passed through PEEK™ tubing into the detector flow cell for analysis.

3.4. Normal phase high performance liquid chromatography

To demonstrate the operation of the DIPD as a selective HPLC detector, the FIA-DIPD instrument was modified to include a normal phase NP-HPLC cyano column (2.1 mm × 30 mm, Spheri-5, 5 μm, Brownlee Columns, Applied Biosystems, Inc., Foster City, CA) placed in between the DIPD flow cell and the injection valve. The *n*-hexane mobile phase was run at a flow rate of 60 μl min^{-1}. Cholesterol in egg yolk was separated by NP-HPLC and selectively detected by DIPD. To extract cholesterol from egg yolk, one egg yolk was mixed with 100 ml of *n*-hexane and the resulting biphasic mixture was shaken for two hours. Upon shaking, the cholesterol along with other organophilic compounds from the egg yolk partitioned into the *n*-hexane supernatant. Aliquots of the supernatant were injected into the NP-HPLC-DIPD system for chromatographic separation and detection.

4. Results and discussion

To test the response of the DIPD, and to ascertain the suitability of standards, a 100 μl plug of 0.5% isopropanol in hexane and a 100 μl plug of 0.1% octanol in hexane were injected separately into the water bulk phase of the DIPD

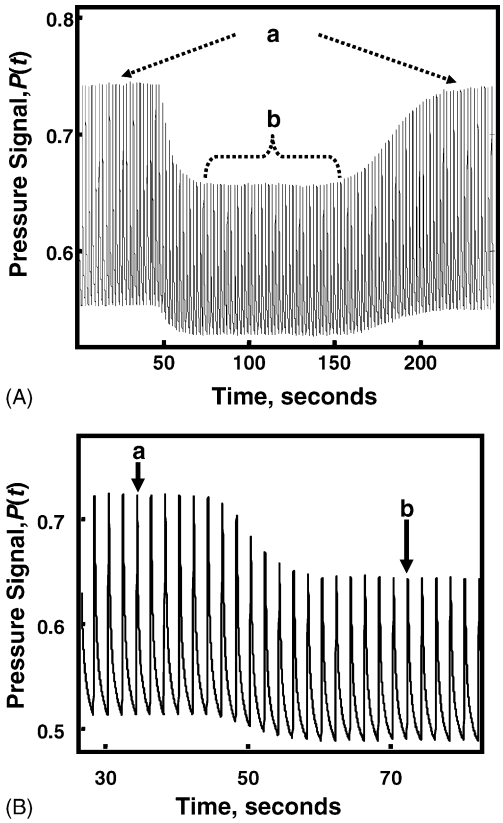

Fig. 3. (A) Raw pressure data, $P(t)$, obtained from the DIPD for a 100 μl injection of 0.1% *n*-octanol in *n*-hexane: (a) baseline signal of the *n*-hexane carrier phase; (b) steady-state concentration of 0.1% *n*-octanol in *n*-hexane. Each drop was detached from the end of the stainless steel tip at 0.5 Hz or every 2 s. (B) An enlargement of raw pressure data, $P(t)$, from Fig. 3(A). Each peak referred to the measured pressure of each drop that formed and detached at the end of the sensing tip every 2 s: (a) baseline signal of *n*-hexane; (b) steady-state concentration of 0.1% *n*-octanol in *n*-hexane.

system. Fig. 3(A) shows the raw drop pressure data, $P(t)$, of 0.1% *n*-octanol in *n*-hexane. During the first 50 s labeled "a", only *n*-hexane was present at the liquid–liquid interface. When the solution of 0.1% *n*-octanol in *n*-hexane reached the DIPD, labeled "b", the pressure signal, $P(t)$, decreased due to the lower interfacial tension (i.e., higher interfacial pressure) and achieved a steady-state signal that lasted for approximately 100 s.

In Fig. 3 (B), an enlargement of raw drop pressure data in Fig. 3 (A) is shown for clarity. The maximum of each of the sharp $P(t)$ profiles represents the maximum pressure of an individual drop. This maximum pressure, P_{max}, occurs when the liquid at the end of the sensing tip begins to form a new drop. Drops were detached from the capillary sensing tip every 2 s. Drop pressures decreased as the drop volume increased and were at their minimum values, P_{min}, just before detachment, according to Eq. (1).

Single drop pressure profiles of hexane, 0.5% isopropanol in hexane, and 0.1% octanol in hexane forming in the water bulk phase are shown in Fig. 4. These profiles were averaged from 10 raw $P(t)$ drop profiles. Comparing the pressure drop

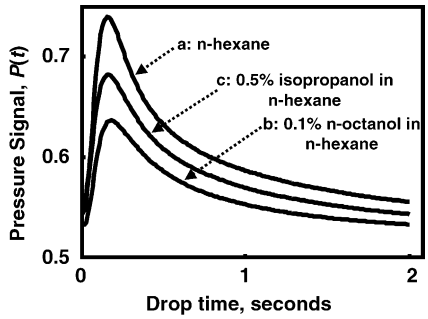

Fig. 4. Average drop pressure profiles of *n*-hexane (mobile phase carrier stream), 0.5% isopropanol in *n*-hexane, and 0.1% *n*-octanol in *n*-hexane from 10 drops under steady-state concentration conditions: (a) pure *n*-hexane drop; (b) 0.1% *n*-octanol in *n*-hexane; (c) 0.5% isopropanol in *n*-hexane.

profiles of *n*-hexane, 0.1% *n*-octanol in *n*-hexane, and 0.5% isopropanol in *n*-hexane illustrates that injecting small quantities of *n*-octanol or isopropanol into the *n*-hexane carrier dramatically lowers the interfacial tension (increases the interfacial pressure), and therefore, leads to a decrease in both P_{max} and P_{min}. The exquisite sensitivity of the DIPD is realized by noting that a volume of only 100 nl (equal to 0.1% of 100 μl) of *n*-octanol was injected into the *n*-hexane carrier, and since *n*-octanol achieved a steady-state concentration for a period of 100 s, *n*-octanol was measured with a high signal-to-noise ratio as it passed the DIPD at a rate of only 1 nl of *n*-octanol per second.

The drop pressure profiles of analytes that can be used as standards run parallel to the pure solvent drop pressure profiles from the pressure maximum to the drop detachment as shown in Fig. 4. Thus, *n*-octanol and isopropanol are good candidates as standards. Fig. 5 shows the relative interfacial pressure of 0.1% *n*-octanol and 0.5% isopropanol calculated using the drop pressure profiles shown in Fig. 4 and Eq. (3). The relative interfacial pressure of 0.1% *n*-octanol was calculated using 0.5% isopropanol as the standard and, likewise, the relative interfacial pressure of 0.5% isopropanol was calculated using 0.1% *n*-octanol as the standard. Fig. 5 shows that the relative interfacial pressure of both analytes is not

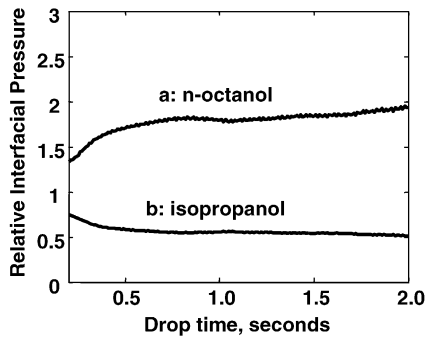

Fig. 5. Relative interfacial pressure of the analytes, after the drop pressure profile data in Fig. 4 were applied to Eq. (3): (a) the relative interfacial pressure of 0.1% *n*-octanol in *n*-hexane when 0.5% isopropanol in *n*-hexane was used as the standard; (b) the relative interfacial pressure of 0.5% isopropanol in *n*-hexane when 0.1% *n*-octanol in *n*-hexane was used as the standard.

dynamic over the measured drop growth (for drop time range of ∼0.5–2 s) [16], therefore, both 0.1% *n*-octanol in *n*-hexane and 0.5% isopropanol in *n*-hexane were used as the standard solutions for sensor calibration, with 0.5% isopropanol used for calibration of the results reported herein. The calibration method defined in the Theory section (Eq. (3)) enables real-time calculation of relative interfacial pressure in a flowing system without the need of cumbersome optical measurements [16].

To demonstrate that the DIPD could be used to study the adsorption kinetics of interface-active molecules in *n*-hexane, the behavior of Brij®30 at the *n*-hexane–water interface was evaluated. The non-ionic surfactant, Brij®30 (Polyoxyethylene(4)lauryl ether), is a long chain polymer molecule that is soluble in both water and *n*-hexane. The Brij®30 hydrophobic chain is a lauryl group and the hydrophilic head group is polyoxyethylene ether. The characteristics of Brij®58 at the water/oil interface at constant drop size had been studied; although, in that work, the surfactant was dissolved in the aqueous phase [24]. The relative interfacial pressure of a 100 μl injection of 60 μg ml^{-1} Brij®30 in *n*-hexane is shown in Fig. 6(A) as a three-dimensional signal. Brij®30 shows a dynamic signal at this concentration and flow rate, with a relative interfacial pressure that changes by a factor of approximately four relative to 0.5% isopropanol in *n*-hexane as the standard, over the 2 s drop lifetime.

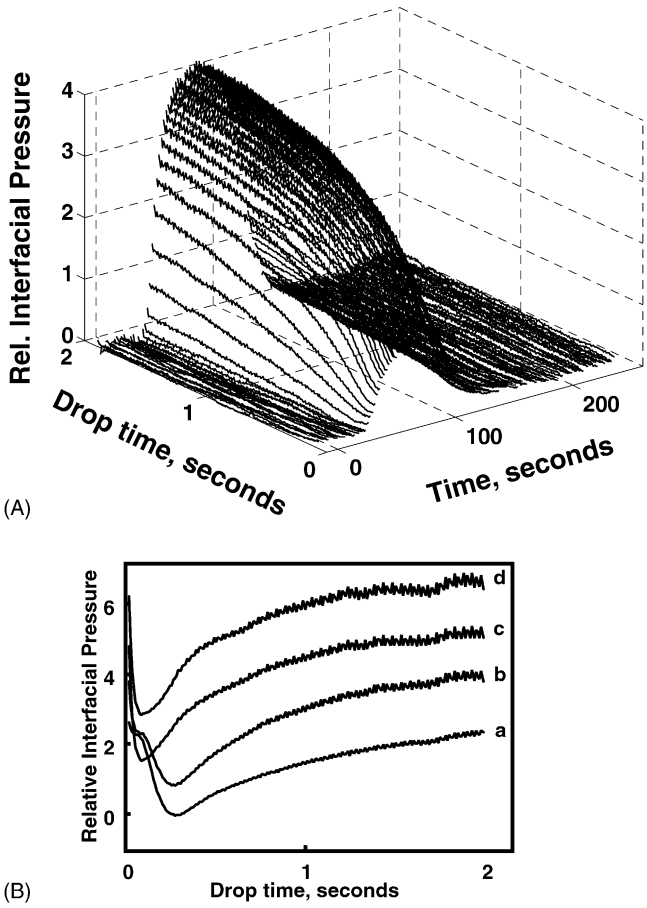

Fig. 6. (A) A three-dimensional relative interfacial pressure plot of 60 μg ml^{-1} Brij®30 in *n*-hexane using 0.5% isopropanol as standard. (B) A relative interfacial pressure plot of various concentrations of Brij®30 in *n*-hexane: (a) 20 μg ml^{-1}; (b) 40 μg ml^{-1}; (c) 60 μg ml^{-1}; and (d) 80 μg ml^{-1}.

The DIPD was designed to detect interface-active analytes in a sample and give selective signals for analytes that do not quickly reach equilibrium at the interface. Due to the size of the Brij®30 molecule, as well as the concentrations studied, Brij®30 was in the process of diffusing and arranging at the interface during the DIPD measurement. Fig. 6 (B) shows the individual drop pressure profiles of Brij®30 at four concentrations. An increase in the Brij®30 concentration is accompanied by an increase in the relative interfacial pressure measured by the DIPD. The consistency in the shape of the last three-quarters of the interfacial pressure profile indicates that DIPD may be useful for analyte identification and to understand the dynamic processes at the liquid–liquid interface.

The kinetic effect in the relative interfacial pressure during the drop lifetime is similar to the phenomena observed at the air–liquid interface by the DSTD. When the relative interfacial pressure is calculated as shown in Eq. (3), subtracting the carrier phase pressure signal and dividing by the pressure signal of the standard accounts for the increase of the surface area during drop growth. Thus, the relative interfacial pressure as the drop grows is related to the concentration of the analyte at the interface. Factors that affect the shape of the drop time profile include the concentration of the analyte in solution, analyte diffusion constant, molecule arrangement at the interface, equilibrium concentration at the interface and transfer of analyte to the interface [4,7,8,20].

The DIPD and DSTD are complementary detectors in that the DSTD detects interface-active analytes that are soluble and present in aqueous samples, whereas the DIPD detects analytes that are soluble and present in organic solvents. Examples of organic-soluble analytes include petroleum products, cooking oils, hydrocarbons and high molecular weight alcohols. In this report, cholesterol was examined. Samples of cholesterol in *n*-hexane ranging in concentration from 100 to 10,000 μg ml^{-1} were evaluated with the FIA-DIPD system. A three-dimensional FIA-gram of a 100 μl injection of 1000 μg ml^{-1} cholesterol is shown in Fig. 7 as an example. The time axis shows the FIA injection profile. The drop time axis shows the relative interfacial pressure of cholesterol at the water–hexane interface using 0.5% isopropanol in *n*-hexane as the standard. The increase in relative interfacial pressure with drop time is the result of the dynamic transfer of analyte to the interface. Due to the size of the cholesterol molecule, it is suspected that much of the kinetic signal is due to the diffusion of molecules from the bulk phase (inside drop) to the interface. Fig. 7 indicates that the cholesterol response is indeed kinetically hindered possibly due to diffusion and rearrangement processes at the drop interface, and appears to then nearly reach interfacial equilibration. Future studies might involve investigation to determine whether the time required to reach a state near equilibrium can be used to

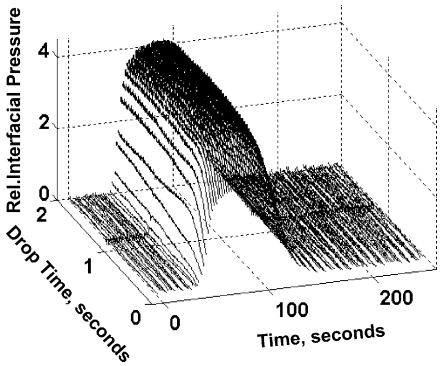

Fig. 7. A three-dimensional relative interfacial pressure plot of 1000 μg ml^{-1} cholesterol in *n*-hexane using 0.5% isopropanol as standard.

estimate the diffusion constant. Again, it is evident that the shape of an analyte interfacial pressure profile during drop growth might be useful for analyte identification.

The FIA profiles for multiple concentrations of cholesterol are given in Fig. 8(A). The interfacial pressure signals are an average of the relative interfacial pressure signals from 1.8 to 2.0 s in the drop profile (right before drop detachment, so the maximum relative interfacial pressure). The relative interfacial pressure increased with concentration of cholesterol. In Fig. 8 (B), the calibration curve of the concentration series of cholesterol from Fig. 8 (A) is shown. Fig. 8 demonstrates that the DIPD can be used to quantify the cholesterol concentration, and quantitative analysis for any surface-active analyte is possible in principle.

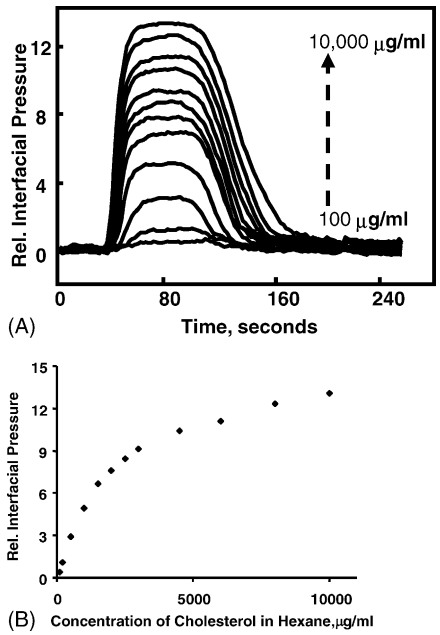

Fig. 8. (A) Overlay FIA-gram plots of relative interfacial pressure of various concentration of cholesterol over the range 100–10,000 μg ml^{-1} in *n*-hexane. The concentrations were 100, 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 6000, 8000 and 10,000 μg ml^{-1}. (B) A calibration curve of the cholesterol data is plotted against relative interfacial pressure and concentration of cholesterol in *n*-hexane.

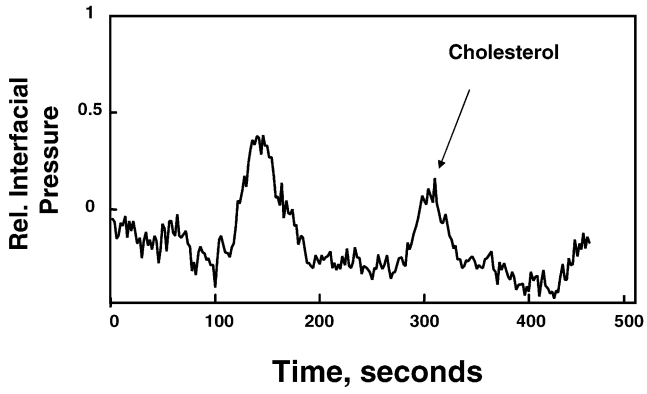

Fig. 9. Separation of cholesterol from egg yolk by NP-HPLC-DIPD. Experimental conditions are given in the text, and using 0.5% isopropanol as standard.

An example of the use of the DIPD as a selective detector for NP-HPLC was demonstrated with the extraction and normal phase liquid chromatographic separation of cholesterol from egg yolk. Because the DIPD selectively detects only interface-active compounds, only a few compounds in the sample must be separated to measure cholesterol in egg yolk. After the egg yolk was shaken with *n*-hexane, a 15 μl injection of the supernatant was injected into the *n*-hexane carrier phase of the NP-HPLC-DIPD system. The resulting chromatogram, given in Fig. 9, shows that cholesterol, with a retention time of approximately 300 s was separated from other organophilic egg yolks constituents, which eluted at approximately 150 s. The selectivity of the DIPD results in a relatively simple chromatogram, whereas use of another detector, such as absorbance or refractive index would be either not selective enough or not sensitive enough or both. The method of standard additions was used to confirm the retention time and concentration (250 μg ml^{-1}) of the cholesterol peak in the egg yolk extract, and the other organophilic egg yolk constituent remained unidentified.

5. Conclusions

An instrument known as the DIPD was designed and developed for making analytical measurements of surface-active species at the water–hexane interface. In principle, any combination of two immiscible liquids with different densities could be used with the DIPD. As a drop grows from a stainless steel capillary tip, the DIPD can be used to monitor the diffusion and arrangement of interface-active analytes at the liquid–liquid interface. The relative interfacial tension response can be used to identify and quantify surfactants and understand their behavior at liquid–liquid interfaces. The DIPD is a novel detector because it selectively measures the interfacial activity of analytes within drops eluting from a flowing stream into another immiscible liquid, and therefore, is a suitable detector for flow-based analytical techniques, such as FIA and NP-HPLC. Furthermore, the DIPD serves

as a detector for analyte quantification, as demonstrated by a calibration of cholesterol.

Acknowledgements

We thank the Center for Process Analytical Chemistry (CPAC), the Thailand Research Fund (TRF) and Postgraduate Education and Research program in Chemistry (PERCH) for financial support.

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Sequential injection-ELISA based system for online determination of hyaluronan

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Received 21 September 2004; received in revised form 29 November 2004; accepted 29 November 2004
Available online 4 January 2005

Abstract

Sequential injection-bead-based immunoassay system has been developed. The main purpose is to make immunoassay process more automated by manipulating the precise delivery of micro-volumes of reagents and the precise timing of incubation and washing steps with a computer program that controls the bi-directional syringe pump. The manifold was designed with the aims of reducing back pressure from beads that act as solid surfaces for immobilization of the target substance, reducing dispersion and dilution of the reagent during incubation, and maximizing signal while minimizing incubation time. This was done by introducing air segment to separate the reagent zone from the carrier stream and by using a suitable sensitive detector which, in this case, was an amperometer. In this study, hyaluronan (HA) was used as a target analyte because of its clinical significance as a potential biomarker for liver, bone and cancer diseases. Amount of hyaluronan was determined using competitive enzyme linked immuno sorbent assay (ELISA) based technique where immobilized HA and HA in solution compete to bind with a fixed amount of biotinylated HA-binding proteins (b-HABPs). Upon separation of the two phases, anti-biotin conjugated with enzyme and a suitable substrate were introduced to follow the binding reaction of the immobilized HA and b-HABPs whose degree of binding is indirectly proportional to the amount of HA in solution. A calibration curve was constructed from a series of concentrations of HA standards. Lowest detectable concentration was found to be 1 ng/mL with the dynamic working range of 1–5000 ng/mL and R.S.D. of intra-assay ($n = 7$) and inter-assay ($n = 3$) of various HA concentrations were 4–10% and 9–12%, respectively. Used beads could be reused by washing with 2 M guanidine. Total analysis time for this automatic assay was about 30 min as compared to the 5–8 h used in conventional batch well ELISA. The system could be applied to assay HA in human serum.
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Keywords: Sequential injection analysis; Bead ELISA; Online immunoassay; Hyaluronan

1. Introduction

Immunoassay is an analytical technique that utilizes the specific molecular recognition between antigen and antibody to selectively determine the presence of the antigen. The detection of the binding reaction between antibody and antigen can be followed using different available labels. Examples of recently used labels are radioactive isotope [1,2] chemiluminescent compounds [3,4], metals [5,6] and enzymes [7,8]. Among all the labels, the use of enzymes has gained high popularity due to their amplification properties in which they can convert substrate to product as long as the substrate is present. Assay with an enzyme label, known as enzyme linked immuno sorbent assay (ELISA), has been widely used.

Conventional immunoassay is normally carried out in a micro-well where antigen or antibody is immobilized onto the surface of the well. The rate of binding reaction between antigen and antibody depends upon the diffusion rate of antigen or antibody in the bulk solution to the immobilized ones. Conventional immunoassay, therefore, requires a long incubation

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doi:10.1016/j.talanta.2004.11.032

time, mostly overnight or at least 5–8 h to ensure maximum binding. There have been attempts to reduce the analysis time of immunoassay [9–13]. Using microbeads as solid surface is one way that has been gaining popularity [14–18]. Now that there are many chemically modified beads commercially available, different formats of bead-based immunoassay have been reported [9,19–23]. The main advantages of bead-based immunoassay include increasing the surface area for immobilization of antigen or antibody and reducing the incubation time due to less diffusion distance needed between species in bulk solution and the ones immobilized on beads that are suspended in the solution. Magnetic microbeads also facilitate the phase separation during the washing steps [24,25].

The combination of beads and ELISA is very useful and has been used in many applications [9,23,24,26–28]. However, immunoassay always involves many steps of incubation and washing which are tedious and subjected to human error, mostly due to imprecise timing on each step. The small amount of each of the reagents used in the analysis process is also another difficult matter to handle by an inexperienced person. There is rarely any development on a true online immunoassay where the whole process from the first step of incubation to the last step of detection is automatic. This is due to the lack of a system that can deliver a very small volume of reagents and precisely control the incubation time. The new generation of flow injection technique called sequential injection analysis (SIA) is now available [29–33], and a true online immunoassay is possible. One very interesting work is the combination of bead injection technique with the lab-on-valve (LOV)-sequential injection analysis system for carrying out ELISA [34]. However, this work involves the sophisticated LOV device that attaches as a part of the multi-selection valve of the commercial SIA system.

The attempt of this work is to make the immunoassay process more automated and more precise than batch process by utilizing the most simple, and cost effective system as much as possible. This was done by utilizing the sequential injection analysis (SIA) system controlled by computer software to deliver micro-volumes of reagents and to precisely time the incubation and washing steps. Beads were used as solid surfaces. The system was developed with the aims of reducing back pressure in the flow line which commonly occurs when using a bead packed column, as well as to minimize the incubation time and to maximize the signal as much as possible. An air segment was introduced to separate the reagent zone from the carrier stream [35] in order to reduce the dispersion of the reagent during the incubation time when the pump was stopped. Amperometry was chosen as a detection means because of its high sensitivity and low susceptibility to the differences in non-electrochemical properties of reagents. For example, the Schlieren effect which is usually encountered when using spectrophotometric detection can be avoided when electrochemistry techniques are used.

The SIA-bead ELISA based system was developed for quantification of hyaluronan (HA) in serum. Hyaluronan is a glycoprotein, composed of gluconate acetylglucosamine disaccharide repeating units, that has been reported to be a potential biomarker for liver, cancer and bone diseases [36–43]. It can bind selectively to the proteins called HA-binding proteins (HABPs). In this study, the competitive ELISA based technique was carried out. Immobilized HA on beads and HA in solution competed to bind with a fixed amount of biotinylated HA-binding proteins (b-HABPs). Upon separation of beads from solution, anti-biotin conjugated with enzyme horseradish peroxidase (anti-b-HRP) and a suitable substrate (tetramethylbenzidine, TMB-H_2O_2) were introduced to follow the binding reaction of the immobilized HA and b-HABPs. The degree of binding is indirectly proportional to the amount of HA in solution. The demonstration of the potential use of the system with real samples was shown by performing a recovery study of the amount of spiked HA in bovine and human serum samples. The system offers an automated immunoassay process with good sensitivity and precision as compared to using a batch process, even though the incubation time was reduced to decrease the analysis time and increase sample throughput.

2. Experimental

2.1. Apparatus and reagents

2.1.1. SIA system

The system is composed of a syringe pump (CAVRO Scientific Instrument Inc., XL 3000) that can draw solution up to 2.5 mL, two 10-ports selection valves (Valco Instrument Co. Ltd., C25-3180EMH), a computer (Microsoft Window XP professional, CPU-AMD Athlon^{TM} XP 2000 + 1.67 GHz 256 MB of RAM) and SIA control software (FIAlab 3000, FIA Instrument). Holding coil is made of tygon tubing. All other tubings are PTFE tubings. The detection system was an amperometer (μAutolab Type II Eco Chemie B.V. Utrecht, The Netherlands) with its control software (Autolab Software—General Purpose Electrochemical System version 4.9). A BAS flow cell with glassy carbon working electrode, Ag/AgCl reference electrode and stainless steel auxiliary electrode was used. The applied potential was set at 100 mV for detection of the reduction of TMB product, produced from the enzyme–substrate reaction, back to TMB.

2.1.2. HA coated beads

EAH-sepharose 4B beads (Amersham Biosciences) size 45–165 μm (mean bead size 90 μm) were used as solid surfaces for immobilization of HA. These beads were washed with saline solution at the ratio of 0.5 M NaCl 250 mL: deionized water 100 mL: bead 10 mL (drained gel) and were filtered prior to use.

One of the many sources of HA is umbilical cord, which has an abundant amount of HA [44], that is also commercially available. HA was extracted from umbilical cord by mixing 0.0300 g umbilical cord (Sigma, containing approximately

30 mg HA) with 0.0010 g bovine testicular hyaluronidase (Sigma) and 10 mL of 0.15 M NaCl (BDH) in 0.1 M sodium acetate buffer solution pH 5.0 (Sigma). The mixture was shaken to let the reaction take place for 3 h before bringing it to boil for 10 min to end the reaction.

The beads and HA from previous steps were mixed and about 30 mL deionized water and 0.5751 g *N*-ethyl-*N*′-3-dimethylaminopropyl carbodiimide hydrochloride (Sigma) were added to the mixture. After mixing thoroughly, 0.1 M HCl (Sigma) was used to adjust the pH to about 4.5. This mixture was continuously shaken for 24 h at room temperature. After that, the remaining un-substituted amino groups were blocked with 1 mL glacial acetic acid and allowed to stand for 6 h. The beads were washed with 400 mL each of different solutions in the order of 1 M NaCl, 0.1 M Tris–HCl (Sigma) pH 8.1, 0.05 M formate buffer (Sigma) pH 3.1, and deionized water, respectively. In each washing step, beads were separated from washing solution by centrifugation. Finally, the beads were kept in 0.5 M sodium acetate buffer pH 5.7 at 4 °C for future use.

2.1.3. Biotinylated HABPs

HABPs was extracted from cartilage using 4 M guanidine. The extract was dialyzed against water and then was liophilized. The powder was partially digested with tripsin before dialyzation and liophilization again. Up to this step, HA that was previously bound to HABPs in cartilage is still present with HABPs. To separate out the HABPs from HA in solution, bead HA was added. HABPs can bind to HA on bead better than HA in solution. This may be because of higher amount of HA on beads and also because some portions of HA in solution were partially digested and therefore cannot bind effectively to HABPs. After that, HABPs was eluted out from bead HA using 4 M Guanidine.

Biotinylation of HA-binding proteins was performed by a standard method [45]. Briefly, HABPs which had been prepared was dissolved in 0.1 M sodium hydrogen carbonate buffer pH 8.5 and with *N*-hydroxysuccinimidobiotin (Sigma–Aldrich Chemical, 34.1 g/L in DMSO) mixed at a ratio of 3:1 (w/w) at room temperature for 1–2 h. The mixture was then introduced to a Sephadex G-25 column and was eluted with PBS pH 7.4. The excluded protein peak was collected, aliquoted and stored at −20 °C as a stock solution of the biotinylated HABPs (b-HABPs).

2.1.4. Anti-biotin conjugated with HRP

Anti-biotin HRP (Zymed) was diluted to 1:1000 ratio with PBS buffer solution pH 7.4.

2.1.5. HA standard solutions

HA standard solution (IAL®-F) has the concentration of 20 mg/2 mL which is equivalent to 10×10^6 ng/mL. Stock solution of 1 mL at the concentration of 10,000 ng/mL was made by diluting the HA standard solution with 6% BSA to match the approximate amount of protein contained in real blood. This stock solution was further diluted into a series of different concentrations; 5000, 2500, 1000, 500, 250, 125, 50, 25, 5 and 1 ng/mL.

2.1.6. Serum samples

Bovine serum (Sigma) in the powder form was dissolved in PBS to obtain a final concentration of 6% (w/v). This is to mimic the real blood in liquid form that contains approximately 6% protein.

Human serum was obtained from Chiang Mai University Hospital. Volunteers had not taken food and drink for 8 h prior to the blood drawing. These bloods were centrifuged to separate out the blood cells. Serum samples were kept at −20 °C and were used without further dilution.

2.2. Manifold design

The manifold used in this study is as shown in Fig. 1. A pipette tip (100–1000 μL blue tip, Eppendorf) with cotton wool at the tip end was used as a reservoir for beads. It was directly connected to one of the ports on the selection valve. Since one end was opened to air, there was no problem of back pressure as normally found in a packed column. Other reagents were kept in microcentrifuge tubes and light sensitive ones such as TMB were kept in a dark color tube. All tubes were closed and only a small hole was made for the tubing from selection valve to go through for solution drawing. This was to avoid air contact that might oxidize and degrade

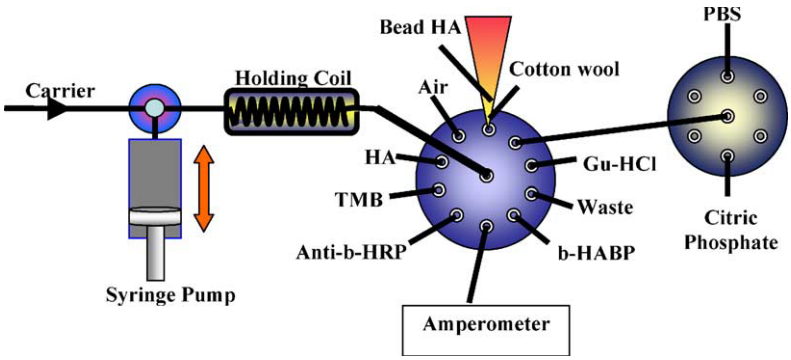

Fig. 1. SIA-bead ELISA based system for determination of HA.

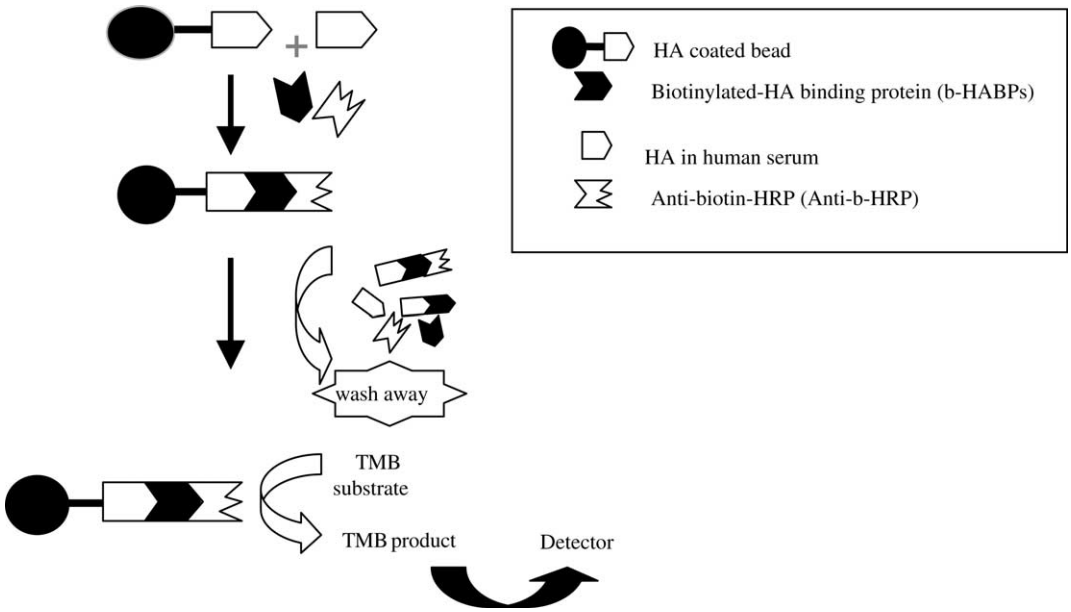

Fig. 2. Diagram illustrate bead ELISA based technique for determination of HA.

reagents. Holding coil was made of tygon tubing that can hold 2.5 mL of solution.

2.3. Operation steps

The bead-based ELISA process for determination of HA is illustrated in Fig. 2. Table 1 summarizes the operation steps. First, exactly 20 μL of HA coated bead suspension was pipetted into the pipette tip reservoir, see Fig. 1. The computer software program was started. The beads were washed once with 500 μL of PBS. Exactly 30 μL of standard HA solution or serum sample was drawn at the flow rate of 100 μL/s to mix with the bead HA in the reservoir. The mixed b-HABPs and anti-b-HRP zone was made by first drawing an air segment of 10 μL, followed by 10 μL of 1:1000 anti-b-HRP, 60 μL of 1:100 b-HABPs, and again 10 μL of anti-b-HRP. Total volume of the reagents drawn was 80 μL. The air segment helps to reduce dispersion of the zone during the reagent drawing and mixing process and the presence of anti-b-HRP on both sides helps to promote mixing. The zone was moved back and forth twice before sending 80 μL to the reservoir to mix with HA coated bead and sending the air segment to waste. These reagents were incubated with bead HA for 5 min before the unbound species were removed by washing the beads four times with PBS pH 7.4, 500 μL each time, followed by washing four times, with citric phosphate buffer pH 5.0, 500 μL each time. After that the buffer solution in the tubing leading to the detector was changed to citric phosphate buffer pH 5.0. Then TMB-H_2O_2 300 μL was drawn into the holding coil of which 250 μL was pushed into the reservoir at the flow rate of 50 μL/s to incubate with HRP that was bound to the bead HA. The rest of the TMB-H_2O_2 solution was moved to the detector to adjust the baseline. The mixing of TMB-H_2O_2 and (bead HA)-(HABPs-b)-(anti-b-HRP) was done by moving the solution in and out of the reservoir 20 times at the moving volume of 20 μL and the flow rate of 50 μL/s which resulted in a total mixing time of 200 s. The mixture was allowed to stand for another 100 s. Total incubation time was 5 min. The product of enzyme HRP and substrate TMB that

Table 1
Summarization of operation steps

Step no.	Operation	Volume (μL)
1	Pipette HA coated bead into the reservoir	20
2	Wash bead with PBS	500
3	Load HA standard solution or sample to the holding coil and then send it to mix with HA coated bead in the reservoir	30
4	Drawing air to the holding coil	10
5	Drawing anti-b-HRP to the holding coil	10
6	Drawing b-HABPs to the holding coil	60
7	Drawing anti-b-HRP to the holding coil	10
8	Mixing anti-b-HRP and b-HABPs by moving solution back and forth twice	
9	Send mixture to HA coated bead reservoir	80
10	Discard air	
11	Incubate HA solution, b-HABPs and anti-b-HRP with HA coated bead for 5 min	
12	Remove unbound species by washing beads with PBS four times followed by washing beads with citric phosphate buffer four times	500
13	Fill the detector line with citric phosphate buffer	
14	Draw TMB-H_2O_2 to the holding coil	300
15	Send some TMB-H_2O_2 to the detector to adjust baseline	50
16	Send the rest of TMB-H_2O_2 to bead reservoir and incubate for 5 min while moving solution back and forth at the end of the reservoir to promote mixing	250
17	Send the solution from the reservoir to the amperometer to detect product	
18	Change new bead reservoir to start new analysis	

occurred was detected when sending the solution to the amperometer at the flow rate of 20 μL/s. The resulting signal was the comparison of the TMB product with the TMB that had been sent previously to the detector. Used beads were collected to be washed for reuse.

2.4. Optimization

This study aims to improve the efficiency of the immunoassay technique by reducing reagent usage and time consumption as much as possible. Two main parameters studied here are the amount of b-HABPs for the given amount of HA coated beads and the incubation time.

2.5. Standard curve

The standard curve was constructed from various concentrations of standard HA solutions (1, 5, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL) in 6% BSA. Lowest detectable concentration and working range were estimated from this curve.

2.6. Recovery study

To demonstrate the performance of the system on analysis of real samples, bovine serum and human serum were used. The effects of matrices were tested by spiking 50 μL of standard HA solutions of different concentrations into 950 μL of 6% bovine serum to gain the final added concentrations of HA at 5, 50 and 250 ng/mL and do the same with human serum to obtain the final added concentrations of HA at 5, 125 and 250 ng/mL. Percent recoveries were calculated from the results obtained from the standard curve as compared to the expected value from calculation.

3. Results and discussion

3.1. Optimization

Amount of b-HABPs is the most critical parameter in this immunoassay process for the selected amount of HA coated beads used in the experiment. Insufficient amount of b-HABPs may be used up by the HA in solution and therefore no signal would be produced. An excess amount of b-HABPs would accommodate all the HA present both in the solution and immobilized HA on beads, therefore no significant signal with the different levels of HA in solution would be observed. Thus, in this study the optimum amount of b-HABPs was determined by varying the concentration of a 60 μL b-HABPs from 0, 1:200, 1:100, 1:50 to 1:10 dilution in phosphate buffer while amounts of beads, anti-b-HRP and TMB-H_2O_2 were fixed at 20, 20 and 300 μL, respectively. The signals were increased with the increased b-HABPs concentration but at the concentrations higher than 1:100, there was no significant change of signal as shown in Fig. 3. This was due to the limited amount of HA on the beads. The results indicated that 60 μL of 1:100 b-HABPs was suitable to be used with 20 μL of HA coated bead.

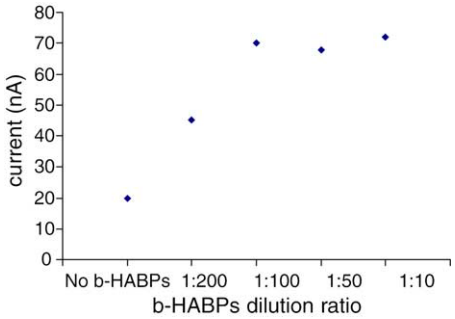

Fig. 3. Determination of optimum concentration of b-HABPs (60 μL) for the set of conditions: 20 μL HA coated bead, 20 μL anti-b-HRP and 300 μL TMB-H_2O_2.

Another important parameter that affects the sensitivity of the system is the incubation times between HA coated beads and b-HABPs and also between enzyme HRP and TMB-H_2O_2. Without stopping time for incubation, analytical signals could be observed. However, the longer the incubation time was, the better the sensitivity obtained. To compromise the analysis time and the sensitivity, the incubation time in both steps were limited to 5 min.

3.2. Standard curve and figures of merit

Standard curve is the sigmoidal (logistic) fit obtained from the computer software Origin version 7.0 as shown in Fig. 4. This standard curve is represented as $Y = [(A_1 - A_2)/(1 + (X/X_0)^P)] + A_2$ and $R^2 = 0.9963$ where Y is current in μA, X is concentration of HA in ng/mL, A_1 is initial Y value = 3.5, A_2 is final Y value = 2.5, X_0 is X value at Y equal to half of the limit A_1 and $A_2 = 77.78$ and P is power = 0.83. The lowest detectable concentration

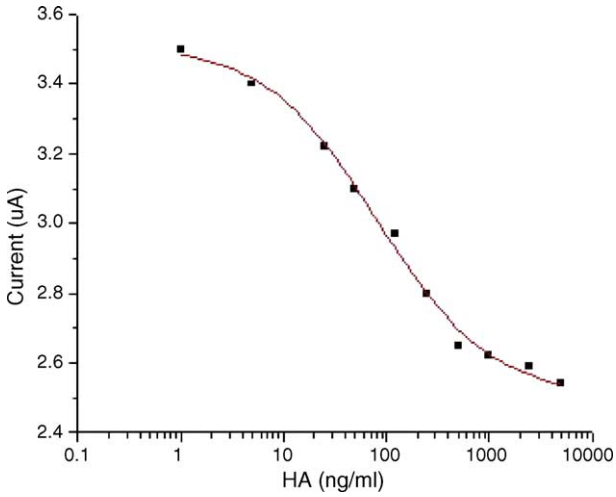

Fig. 4. Standard curve for determination of HA using the SIA system.

Table 2
Percent recovery of spiked HA in bovine serum and human serum samples

Sample	Obtained (HA, ng/mL)	Expected (HA, ng/mL)	% Recovery
Bovine serum	54	54	–
Bovine serum + 5 ng/mL standard HA	57 ± 6	59	97 ± 9
Bovine serum + 50 ng/mL standard HA	90 ± 5	104	87 ± 4
Bovine serum + 250 ng/mL standard HA	281 ± 15	304	92 ± 5
Human serum	47	47	–
Human serum + 5 ng/mL standard HA	60 ± 5	52	115 ± 9
Human serum + 125 ng/mL standard HA	188 ± 7	172	109 ± 4
Human serum + 250 ng/mL standard HA	303 ± 12	297	102 ± 4

of HA was found to be 1 ng/mL and the working range was 1–5000 ng/mL. The lowest detectable concentration was determined as the lowest distinguishable analytical signal ± S.D. with 95% confidence from the blank signal while the highest detectable concentration was determined similarly on another end of the calibration curve. This range can very well covers the HA level reported to be found in normal human at 28 ± 17 ng/mL [46] and at various elevated level in patients with liver and cancer diseases.

In this study, it was assumed that there were insignificant level of degraded HA too small to be unrecognized by HABPs. All serum samples have been kept at −20 °C and therefore HA should not be degraded during the sampling and assay process. It has also been reported that HABPs can interact with HA of the minimum 6–10 monomers and larger [47]. Small fragments of HA of 3–25 disaccharide units have been found in urine which is the last body fluid source before being discarded out of the body [48]. However, if HA macromolecules in serum were to be degraded with enzymes or other conditions, the degraded HA in serum should not be smaller than the ones found in urine and therefore the chance of having HA molecule of less than 6–10 monomers is possible but should be insignificant.

Precision of the system configured as % R.S.D. of intra-assay ($n = 7$) of various concentrations of HA in the working range was between 4 and 10% and inter-assay ($n = 3$) was between 9 and 12% comparing to % R.S.D. reported with the automated microparticle photometric agglunation assay where CV of intra-assay ($n = 10$) was 3.0–8.4% and CV of inter-assay ($n = 3$) was 4.8–8.9% [49]. The ones of batch well ELISA were found to be 6 and 21% for intra-assay ($n = 24$) and inter-assay ($n = 15$), respectively. Each analysis takes about 30 min, much less than the 5–8 h normally used with conventional batch well ELISA.

3.3. Recovery study

Table 2 shows the percent recoveries of spiked standard HA solutions in bovine and human serum samples. The recoveries were 87–115%. It is unclear at this time what matrices may cause positive error in human serum. Further study should be done to identify them.

4. Conclusion

The SIA-online bead-based immunoassay system has been developed. It can be employed to assay amount of HA in serum samples. Reuse of HA coated bead is possible by washing beads with guanidine solution to remove other reagents that have been added and leave HA attached on the bead surface. Analysis time per sample can be greatly reduced as compared to batch well ELISA without any decrease in sensitivity. Precision on drawing small amounts of reagents and timing the incubation and washing steps is improved with the use of computer control. Research has been in progress for a system performing multi-sample analysis by changing the configuration of the manifold in order to gain even better analysis time and to further develop the system into the lab-at-valve (LAV) device where integrating sample processing and detection unit are attached onto a port of a multiposition selection valve [50,51]. This would lead to an alternative cost effective μTAS for ELISA based multi-sample analysis in the future.

Acknowledgements

This research is supported by grant No. MRG4580015 from Thailand Research Fund (TRF) and the Commission for Higher Education (CHE). The authors also would like to acknowledge the TRF Senior Research Scholar grant No. RTA/08/2544 and No. RTA4780010 and Postgraduate Education and Research Program in Chemistry (PERCH) for additional support.

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Talanta 65 (2005) 1149–1161
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Review

Review on screening and analysis techniques for hemoglobin variants and thalassemia

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Received 2 March 2004; received in revised form 22 September 2004; accepted 23 September 2004
Available online 10 November 2004

Abstract

Thalassemia involves gene mutation that causes the production of an insufficient amount of normal structure globin chains while Hb variant involves gene mutation that causes the change in type or number of amino acid of the globin chain. It has been reported that some 200 million people worldwide had hemoglobinopathies of some sort. Attempts to develop effective and economical techniques for screening and analysis of thalassemia and Hb variants have become very important. In this review, we report the different techniques available, ranging from initial screening to extensive analysis, comparing advantages and disadvantages. Some indirect studies related to thalassemia indication and treatment follow-up are also included. We hope that information on these various techniques would be useful for some scientists who are working on development of a new technique or improving the existing ones.
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Keywords: Hemoglobinopathies; Hemoglobin variants; Thalassemia; Screening techniques

Contents

1. Introduction . . . 1150
2. Initial screening techniques for thalassemia . . . 1152
   2.1. Complete blood count (CBC) . . . 1152
   2.2. Osmotic fragility test (OFT) . . . 1153
3. Conventional confirmatory tests for thalassemia and Hb variants . . . 1153
   3.1. Deoxyhemoglobin solubility test . . . 1154
   3.2. Hemoglobin precipitation test . . . 1154
   3.3. Brilliant cresyl blue test or new methylene blue test . . . 1154
   3.4. Alkaline resistant hemoglobin test . . . 1154
   3.5. Acid elution stain (modified Kleihauer–Betke test) . . . 1155
   3.6. Ion exchange micro-column . . . 1155

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doi:10.1016/j.talanta.2004.09.013

4. Instrumental techniques for determination of thalassemia and Hb variants . . . 1155
4.1. High performance liquid chromatography (HPLC) . . . 1156
4.2. Electrophoresis . . . 1156
4.3. Isoelectric focusing (IEF) . . . 1156
4.4. Flow cytometry . . . 1157
5. Extensive analysis techniques for thalassemia and Hb variants . . . 1157
5.1. Polymerase chain reaction (PCR) with different formats of gel electrophoresis . . . 1157
5.2. DNA technology: DNA probe/DNA microchip . . . 1157
6. Indirect studies related to thalassemia indication and treatment follow-up . . . 1158
6.1. Ferritin . . . 1158
6.2. Skin tissue Fe concentration . . . 1158
6.3. Magnetic behavior of erythrocytes . . . 1158
6.4. Nuclear magnetic resonance spectroscopy . . . 1158
7. Conclusion . . . 1159
Acknowledgements . . . 1159
References . . . 1159

1. Introduction

Hemoglobin (Hb) is the molecule that carries and transports oxygen all through the body. It is composed of tetraglobin chains, two alpha and two non-alpha chains. The alpha (α) chains are encoded by the two closely related genes, alpha 1 and 2, on chromosome 16. The non-alpha chains – beta (β), gamma (γ) and delta (δ) – are encoded by a cluster of genes on chromosome 11. A fetus has a high amount of HbF ($\alpha_2\gamma_2$) as compared to other types of Hbs. A newborn has about 80% HbF and at about 1 year of age, HbF reaches a normal level. A few weeks after birth, the production of HbA ($\alpha_2\beta_2$) becomes dominant with the elevated level of HbA_2 ($\alpha_2\delta_2$) [1–3]. In normal adults, HbA is the main type of hemoglobin (96–98%) while HbA_2 and HbF are only present in 2–3% and less than 1%, respectively [4,5].

The failure in hemoglobin synthesis is a main cause of microcytosis and anemia in many population groups around the world. Hemoglobin variants (Hb variants) are characterized by the gene mutation of the globin chains that form hemoglobin (i.e., the replacement of different amino acids at a certain position). Thalassemia, which is slightly different from Hb variants, involves the gene mutation that causes production of an insufficient amount of normal structure globin chains. All types of thalassemias are considered quantitative hemoglobin disease.

Many types of hemoglobin variants have been found, depending on racial background [1–5]. Some types are not at all a problem, for example HbE heterozygous, while some types can cause severe anemia with serious clinical manifestation, for example HbS homozygous or sickel cell disease. Normally, Hb variants carriers, especially heterozygous, have no symptoms. However, combination of Hb variants and thalassemia gene on the same globin chain may result in severe symptoms. For example, combination of HbE with β-thalassemia gene becomes double heterozygote that shows symptoms similar to homozygous β-thalassemia, and combination of Hb constant spring (CS) with α-thalassemia gene will cause symptoms similar to HbH disease.

Thalassemia can be categorized into three classes – major, intermediate and minor – according to the severity of the symptoms [2,3]. The two main thalassemia syndromes (thalassemia major) are α and β thalassemias, which involve homozygous genetic defect in the α globin and β globin chain production, respectively. β-Thalassemia and sickel cell anemia have a wide distribution in tropical areas due to natural selection by malaria [6–8]. α-Thalassemia is most commonly found in Southeast Asia and Africa [1]. Related thalassemia minors or carriers are α-thal-1 (2 out of 4 globin gene deletion) and α-thal-2 (1 out of 4 globin gene deletion). Type 1 has insignificant but observable anemia while type 2 is a silent carrier without any symptoms shown [2]. Compound heterozygotes of α-thal-1 and α-thal-2 result in HbH disease. HbH is composed of β_4 chains instead of $\alpha_2\beta_2$ chains as in normal HbA. HbH is a relatively mild form of thalassemia and may go unnoticed. However, combination of HbH with HbCS, a type of hemoglobin variant of the alpha gene that has an elongated alpha chain with 31 extra amino acids, is severe and blood transfusion may be necessary. Homozygous α-thal-1 causes Hb Bart's hydrops fetalis which consists of γ_4 chains instead of $\alpha_2\gamma_2$ chains as in normal HbF. Unborn infants with Hb Bart's hydrops fetalis normally die before birth or within a short time after birth.

β-Thalassemia major is not as widely spread throughout Southeast Asia as α-thalassemia, but the β chain hemoglobinopathy HbE and HbTak are quite common. In HbE, the 26th amino acid of a normal β chain, glutamine, is replaced by lysine [1,2,4]. HbTak is another common Hb variant found in Asian population which is due to an insertion of the dinucleotide CA into codon 146 [CAC → CA(CA)C] resulting in elongation of the β chain by 11 amino acids. HbS and HbC are the main Hb variants in Africa but the

Table 1
Causes of different types of thalassemia and examples of hemoglobin variants and the areas commonly found

Thalassemia/Hb variants	Type	Cause/severity	Prevalent regions
Alpha-thalassemia	α-thal 2 trait or heterozygous α-thal 2 (−α/αα or α^+)	Only one out of four genes is deleted/No symptoms (silent carrier)	Southeast Asia, Africa
	α-thal 1 trait or heterozygous α-thal 1 (–/αα or α^0)	Two deleted genes are on the same chromosome/observable anemia	
	homozygous α-thal 2 (−α/−α)	Two deleted genes are on different chromosomes/observable anemia	
	α-thal 1 /α-thal 2 or HbH disease (–/−α or α^+)	Three out of four genes are deleted and excess β-chains form β_4/significantly big liver and spleen	
	α-thal 1 /α-thal 1 or homozygous α-thal 1 or Hb Bart's hydrops fetalis (–/–)	There is no alpha chain production and infants have γ_4 instead of normal HbF ($\alpha_2\gamma_2$)/die before or at birth	
Hb variants related to mutation in alpha globin chain	HbCS	Alpha chain has extra 31 amino acids	Southeast Asia
	HbMahidol (HbQ)	$\alpha^{74Asp\rightarrow His}$	Southeast Asia
	HbThailand	$\alpha^{56Lys\rightarrow Thr}$	Thailand
Beta-thalassemia	β^+ thal heterozygote (β^+/β)	Less production of beta chain/insignificant symptoms	Mediterranean, Middle East, India, Southeast Asia, and also found in West and North Africa, West Asia, Italy, Greece, East and Central Europe and USA
	β^+ thal homozygote (β^+/β^+)	Less production of beta chain/need some blood transfusion	
	β^0 thal heterozygote (β^0/β)	Complete failure on beta chain production/need some blood transfusion	
	β^0 thal homozygote (β^0/β^0)	Complete failure on beta chain production/severe symptoms and need blood transfusion often	
Hb variants related to mutation in beta globin chain	HbE	$\beta^{26Glu\rightarrow Lys}$	Southeast Asia
	HbC	$\beta^{6Glu\rightarrow Lys}$	Africa, USA, Mediterranean and Caribbean
	HbS	$\beta^{6Glu\rightarrow Val}$	

most common major hemoglobinopathy is HbS homozygosity. Both HbS and HbC affect the solubility of the hemoglobin by polymerization. HbC forms crystals and makes the red blood cell rigid which causes hemolytic anemia, while deoxygenated HbS polymerizes and forms fiber structure. Similar to HbE, the glutamine at the sixth position on the β chain in HbC and HbS is substituted by lysine and valine, respectively [4,9]. Table 1 summarizes the causes of different types of commonly found Hb variants and shows the areas of their prevalent existence [8].

In 1995, it was reported that nearly 200 million people worldwide had thalassemia or Hb variants of some type [1]. Intermarriage causes various combinations of globin chain defects. Even though carriers of some types of hemoglobin defection do not have any health effect, if in combination with thalassemia trait, then there would be a 25% chance that their children could have thalassemia symptoms [10].

Thalassemia patients in severe cases normally have iron overloading of tissues and malfunctioning liver and spleen due to increased iron collection from blood transfusion treatment [11,12]. The symptoms (i.e., abnormal facial features, big tummy and pale) and the treatment processes (i.e., regular blood transfusion) usually have an affect mentally, physically and economically on the patients and their society [13,14]. The lack of education and knowledge about the disease, along with the neglect of couples to have family planning, causes this genetic disease to spread widely. Therefore, in many countries, couples are now encouraged to have their health checked before or at the very beginning of pregnancy [15,16]. Attempts to develop effective and economic techniques for thalassemia screening and analysis have become very important, especially in the countries that have populations with high percentage of thalassemia trait, high birth rate and low funding [17,18].

There are many techniques that have been used to screen and diagnose for hemoglobin variants and thalassemia, mostly done in combinations. In this article, the authors attempt to report the different techniques available, ranging from initial screening to extensive analysis, including a few indirect studies. Screening techniques are the group of techniques that can initially indicate a defect in hemoglobin synthesis. Positive results from these tests need confirmation by a more extensive analysis technique. Negative results normally help in cutting down the number of subjects that need to be further diagnosed by a more advanced and complicated testing. Extensive analysis techniques can give more precise information about types of Hb variants or types of thalassemia. They normally involve higher technologies and instrumentation, and therefore are more expensive than screening techniques. In some cases, it is necessary to perform a more extensive test instead of a combination of many cheaper techniques, such as in prenatal diagnosis where sampling procedures are difficult and the amount of sample is limited. The flow chart shown in Fig. 1 summarizes the techniques for diagnosis of hemoglobinopathies that are commonly used in most laboratories. Different laboratories may have differ-

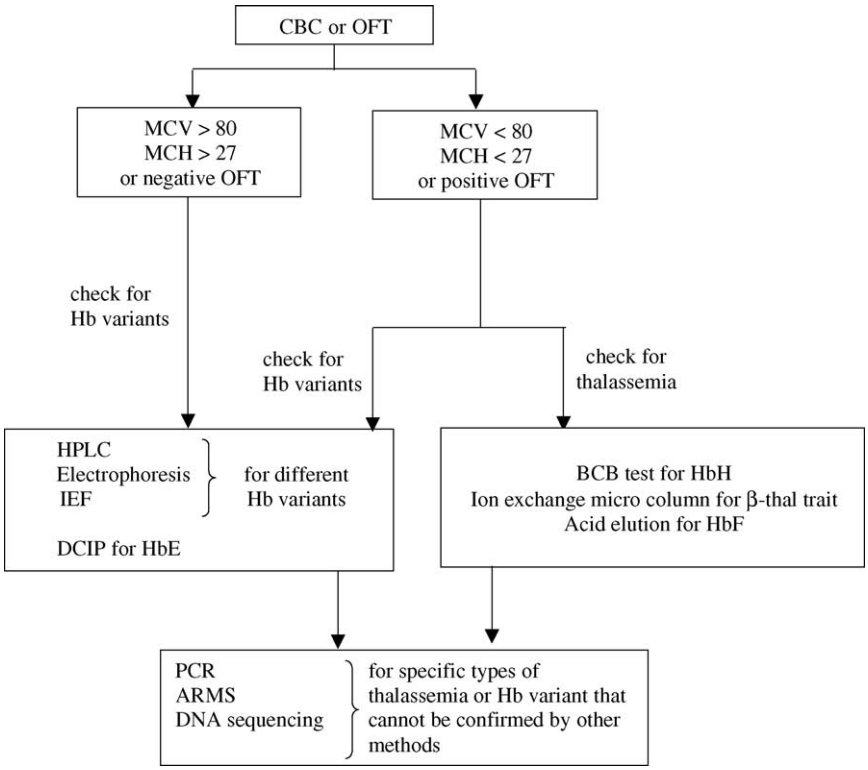

Fig. 1. Flow chart summarizes the normal process of thalassemia and Hb variants diagnosis (see text).

ent choices of analysis techniques, depending on availability of instrumentation and funding. In the developing countries where economic restrictions do not allow for the use of the relatively more expensive technologies, the development of low cost analysis techniques will always be needed. It is hoped that this will be useful information for those who are interested in learning about thalassemia and Hb variants diagnosis techniques for general knowledge, for the development of a new technique or for the improvement of the existing ones.

2. Initial screening techniques for thalassemia

Initial screening techniques are defined as techniques that are simple and relatively low cost which can indicate the possibility of having thalassemia. These techniques should involve the least sample pretreatment and be rapid, and may not need special instrumentation. This would lead to low cost and high sample throughput analysis. They provide a "yes/no" type answer. Positive samples need further confirmatory test while negative samples can be eliminated from further complicated and expensive testing. The complete blood count (CBC) or the alternative osmotic fragility test (OFT) can be used to screen for thalassemia. The negative result eliminates the possibility of having thalassemia, but does not completely exclude the possibility of having Hb variants. Therefore, if necessary, Hb variants testing is needed. Positive results reveal the possibility of having either thalassemia or Hb variants. These screening techniques cannot provide the information on the exact type of hemoglobinopathies, but can help in cutting down the number of samples from unnecessary complicated and expensive testing.

2.1. Complete blood count (CBC)

Complete blood count, a screening test involving the measurement of important characteristics of the blood, has been used as part of the diagnosis process for many diseases, including blood disorders, heart disease, kidney problems and nutritional status [1,19]. The main features of the blood normally tested in the CBC are the total white blood cell count (WBC), red blood cell count (RBC), hematocrit (Hct), hemoglobin (Hb), red cell distribution width (RDW), peripheral blood smear and other important erythrocyte indices (EI), namely mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) [19–22]. Among these parameters, MCV and MCH are the most important ones that can indicate the existence of thalassemia trait, i.e., when MCV < 80 and MCH < 27.

Table 2 summarizes the tests performed in the CBC, the calculation needed for each and the approximate normal cutoff level. However, due to the similar low red blood cell count between the patients with thalassemia (iron overloading) and the ones with iron deficiency, it has been suggested that in the geographic regions where iron deficiency rate is high, the cutoffs for thalassemia interpretation should be adjusted to more suitable values by using a receiver operator char-

Table 2
Summary of tests done for CBC and the interpretation of results for thalassemia and hemoglobinopathies indication

Test	Activity or calculation	Normal level
WBC[a]	Number of white blood cells in cubic millimeter (microliter) of blood	4500–10000 cells μl^{-1}
RBC	Number of red blood cells in cubic millimeter (microliter) of blood	Male 4.7–6.1, female 4.2–5.4 million cells μl^{-1}
Hct	MCV × RBC	Male 40.7–50.3%, female 36.1–44.3% (varies with altitude)
Hb	Spectrophotometric measurement at 540 nm of the cyamethemoglobin (Hb bound with cyanide)	Male 13.8–17.2 g dl^{-1}, female 12.1–15.1 g dl^{-1}; using 64,500 g/mole Hb
MCV	10 (Hct/RBC)	80–95 fl
Mentzer index	MCV/RBC	Less than 13 favors thalassemia over iron deficiency
MCH	(10 Hb)/RBC	27–31 (>30) pg per cell
MCHC	(100 Hb)/Hct	28–33%
Peripheral smear	Visual inspection for shape and number of red blood cells by staining with colored chemicals	
Erythrocyte sedimentation rate[a]	The rate that RBCs settled in the tube within 1 h (mm h^{-1})	Varies with age, gender and pregnancy; male age/2, (female age + 10)/2
Platelet count[a]	–	100000–300000 cells μl^{-1}
Fibrinogen[a]	–	200–400 mg dl^{-1}

[a] Parameters that are normally tested in CBC, but are not directly related to determination of thalassemia and Hb variants.

acteristic (ROC) curve [23]. The ROC curve is the plot of the true-positive results (Y) against the false-positive results (X) for the various sets of results used for constructing the decision threshold. It is used to statistically determine an optimal cutoff point for the medical tests [24–27], which in this case should better differentiate thalassemic microcytosis from non-thalassemic ones (i.e., iron deficiency patients). Other tests such as erythrocyte sedimentation rate, platelet count, and fibrinogen may also be done along with the CBC [28,29].

Many laboratories use an automated CBC machine which can provide many blood parameters in one run. However, the high cost limits its use in many hospitals around the world. Osmotic fragility test, an alternative screening test for thalassemia, is therefore performed instead.

2.2. Osmotic fragility test (OFT)

The main purpose of this technique is to diagnose the hereditary spherocytosis and it is also useful for screening of thalassemia. This simple test utilizes osmosis, the movement of water from lower to higher salt concentration region, to test for the osmotic resistance of the red blood cell [30]. A single hypotonic saline solution can be prepared from dilution of a Tyrode's solution, which is composed of NaCl, KCl, $CaCl_2{\cdot}6H_2O$, $MgCl_2{\cdot}6H_2O$, $NaHCO_3$, NaH_2PO_4, glucose and distilled water [31]. Whole blood is thoroughly mixed with this solution. In a hypotonic condition, the concentration of salt on the outside of a cell is lower than that on the inside, resulting in net water movement into cells. Normal red blood cells are broken within 1–2 min and the mixture then turns clear and reddish. Abnormal red blood cells have deviated osmotic resistances as compared to normal red cells. Spherocytes and erythrocytes with various membrane defects may show decreased osmotic resistance. However, red blood cells of thalassemia have higher osmotic resistance and thus have slower rupture rate, therefore the mixture remains turbid even after 1–2 h [32]. This technique can be carried out in one test tube and it is also called one tube method. Different laboratories may be using slightly different recipes for preparation of hypotonic salt solution, but all are normally based on the same concept of kinetic osmotic fragility. The OFT is a quick preliminary and very economic test before performing further studies of the blood cells.

3. Conventional confirmatory tests for thalassemia and Hb variants

These are useful tests to confirm the existence of certain Hb variants or abnormal level of some Hb types. Confirmatory tests for Hb variants include deoxyhemoglobin solubility test (DST) for detection of HbS and dichlorophenol indophenol precipitation test (DCIP) for detection of HbE. HbH disease which relates to α-thalassemia can be detected by DCIP and brilliant cresyl blue test (BCB). Alkaline resistant hemoglobin test (ART) and acid elution stain (AES) are used for detection of abnormal levels of HbF, which can help identify some types of thalassemia. The ion exchange microcolumn technique is used to quantify the amount of HbA_2 and HbF to identify β-thal trait, E-trait and EE homozygotes. These conventional techniques are relatively low cost and do not require complicated instrumentation. However, some of these techniques may need a highly experienced operator to translate the results. Therefore, availability of more modern instrumentation that can provide more precise information with less requirement of an experienced operator and less usage of toxic chemicals diminishes the use of some of these conventional techniques such as DST and ART.

3.1. Deoxyhemoglobin solubility test

This is a simple test for HbS based on its insolubility in a potassium phosphate saponin buffer solution (composed of K_2HPO_4, KH_2PO_4, saponin and distilled water). Turbidity would be observed within 5 min if the whole blood containing HbS were mixed with sodium hyposulfite and saponin buffer. This test can discriminate samples with HbS from samples with almost all other hemoglobins except Hb Bart's and some rare sickle Hb such as C-Georgetown and S-Travis. Therefore, if a positive test result is shown (i.e., high enough turbidity that newsprint cannot be seen through the test mixture when placed behind the tube), then a follow-up test by electrophoresis is recommended. A false-negative result may be from a high anemic condition [33–35].

3.2. Hemoglobin precipitation test

Some hemoglobin variants such as HbH (β_4 with α-thalassemia) and Hb Köln ($\beta^{98Val\rightarrow Met}$) are classified as unstable hemoglobins which can be precipitated by heating or adding a chemical such as isopropanol or dichlorophenol indophenol [2,36]. The heat stability test can be carried out at either medium temperature (50 °C) for 1–2 h or at high temperature (68 °C) with chemical reaction aids for 1 min. Although taking longer time, the medium temperature stability test is very simple. The clear supernatant of erythrocyte hemolysate in Tris buffer medium, obtained after removing plasma, hemolyzing with distilled water and removing stroma, is placed in the 50 °C water bath for 1 h. Normal hemolysates remain completely clear, while unstable hemoglobins cause flocculation of various turbidities. The test can be done much faster by using chemicals, i.e., KCN and $K_3Fe(CN)_6$, to form hemolysate cyanmethemoglobin. In a phosphate buffer medium, this hemolysate cyanmethemoglobin is agitated rapidly in the 68 °C hot water bath. After 1 min, normal hemolysate may show slight cloudiness and therefore this high temperature method, even though very fast, may need high experience in interpretation in order to avoid a false-positive reading [19,37].

Another way to demonstrate the instability of Hb is with isopropanol precipitation. Packed erythrocytes, cold deionized water and CCl_4 (1:1:1.5 ratio) are placed in a closed tube and vortexed for a few minutes, followed by centrifugation. The clear supernatant is then mixed with isopropanol–Tris buffer at a control temperature of about 37 °C. Unstable hemoglobins cause more turbidity over time, while normal hemoglobins remain clear for at least 30 min. The isopropanol test is reported to have some limitations on the subjects that contain ≥5% HbF, and those that are inappropriately preserved (i.e., unrefrigerated or too old samples) may give false-positive results. Adding anticoagulating reagent can help reduce the false reading but it is suggested that the samples with high HbF should be tested by heat stability, as it is not interfered by HbF [19,38,39].

Similarly, the dichlorophenol indophenol (DCIP) precipitation test is also used widely to screen for HbE and HbH. DCIP can oxidize HbE and HbH faster than any other type of hemoglobin, and therefore it can be used to screen for HbE and HbH. Interpretation of results can be difficult since it involves observing the cloudiness in a deep blue color of DPIC solution. However, a reducing agent may be added to overcome this problem. For example, in the AOAC standard titration method for ascorbic acid, the color of an oxidant DCIP is changed from dark blue to light blue on the way to the end point pink [40]. Therefore, if a small amount of ascorbic acid were added to the DCIP thalassemia test, then the observation could be made more accurately under the light blue condition.

Hemoglobin precipitation tests can be used to screen for some hemoglobin variants but they may not be able to speciate the types of hemoglobins (i.e., HbE and HbH show similar results). Further tests are needed to pinpoint the exact type.

3.3. Brilliant cresyl blue test or new methylene blue test

Both tests are based on the same procedures but with different reagents. They are specifically performed for HbH diagnosis, which cannot be indicated using other techniques such as affinity column and electrophoresis. Polymerase chain reaction can be used to diagnose for HbH, but the cost is higher than these simple color tests. HbH is unstable and it precipitates in the red cells, giving the appearance of many small golf balls inside the cells that can be observed when staining the blood film with brilliant cresyl blue ($C_{17}H_2OClN_3O$) or new methylene blue ($C_{18}H_{22}ClN_3S{:}SClZnCl_2$) [41,42]. The incubation time of blood and the reagents (brilliant cresyl blue in sodium citrate media) takes about 1 h in a controlled temperature setting of about 37 °C [43]. This test is very useful to confirm for α-thalassemia involving HbH inclusion body. However, the technique yields low sensitivity for α-thal trait and therefore it should only be used as a confirmatory test, but not for screening of α-thalassemia.

3.4. Alkaline resistant hemoglobin test

This is a test for abnormal level of fetal hemoglobin (HbF). Normally hemoglobins are denatured at alkaline pH such as in NaOH solution and they can be precipitated readily with saturated ammonium sulfate ($(NH_4)_2SO_4$) solution. However, HbF is not denatured as easily and remains soluble. Differences in alkaline resistance of the normal Hb and fetal Hb allow for rapid testing for the amount of HbF in blood. The procedure consists of a few experimental and calculation steps as reported before [19,44,45]. A suspended mixture of Hb-cyanide–ferricyanide (or cyanmethemoglobin) is prepared by adding packed red cells, obtained from centrifugation of whole blood in isotonic saline solution, into a cyanide–ferricyanide solution (KCN and $K_3Fe(CN)_6$ in distilled water). Then NaOH is added and the solution is mixed

for a few minutes before adding the saturated $(NH_4)_2SO_4$ solution. Coagulated protein can be removed by filtering the mixture until a clear filtrate is obtained. The percent of alkaline resistant hemoglobin is calculated based on the absorbance of the filtrate (Df) and the absorbance of the 1:10 dilution of the original cyanmethemoglobin without NaOH and $(NH_4)_2SO_4$ added (Db) at 540 nm, using the following equation: (100 Df)/(10 Db). In a normal person more than 1 year old, the percentage of HbF should be expressed as being less than 1–2% by using this method. Higher levels of HbF will be suspected of having a hemoglobin disorder of some kind. Although the method was found to mistakenly yield lower results for a subject with HbF higher than 30% of total hemoglobin, such as in umbilical cord blood of newborns, this method was sufficiently sensitive and reproducible for measuring 1–10% HbF, providing that final cyanmethemoglobin concentration is higher than 480 mg/100 ml [46]. In the cases where high amount of HbF is present, an alternative method such as immunological determination of HbF, e.g., by the gel precipitation or immuno-diffusion, involving the use of monoclonal antibody against HbF, may be used to avoid incorrect results obtained from the alkaline resistant hemoglobin test [8,47,48].

3.5. Acid elution stain (modified Kleihauer–Betke test)

This is a simple test for HbF and Hb Bart's. After smearing a blood sample on the slide and letting it dry, the slide is immersed in an 80% alcohol solution (ethyl, methyl or propyl alcohol) for 2–3 min. After that, the slide is immersed in a staining solution of Amido Black 10B ($C_{22}H_{14}N_6O_9S_2Na_2$) prepared in alcohol with pH adjusted to 2.0. After 3 min, the slide is washed under running water for 1 min. In the acidic condition, HbA, HbA_2, HbE, and HbH will be eluted out of the blood cells, leaving the cells empty (ghost cells) and showing no color. HbF and Hb Bart's can tolerate acid and are stained by the Amido Black 10B, showing dark blue color of the cells which can be observed under the microscope.

There are a few precautions that need to be taken when working with this technique. If the slide is left dry for too long, HbA will not be eluted out. The concentration of alcohol is also important because alcohol higher than 85% will cause HbA to stay in the cell, while alcohol lower than 65% will cause vacuolization of HbF. In addition, if the pH of the solution is higher than 2.5, HbA will not be eluted. All these cases will show false results [49,50].

The drawbacks of this technique are time consuming and subjected to human error. Another possible way of detection of HbF is flow cytometry which is more precise as described later.

3.6. Ion exchange micro-column

In the regions where economic restriction does not allow for the use of a relatively higher cost instrument such as HPLC, a cheaper method such as this ion exchange micro-column along with other inexpensive tests can be used in combination to diagnose the type of thalassemia. This technique is based on ion exchange chromatography as a simplified version of high performance liquid chromatography. The use of diethylaminoethyl DEAE anion exchanger, packed in a relatively cheap and small syringe, and Tris–HCl mobile phase can be adapted to separate HbA_2 and HbF effectively. The relative amounts of these Hbs can be estimated by calculating the peak areas of the absorbance, measured at 415 nm, of fractions eluted from the column. It has been shown that the results obtained from the batchwise micro-column are in agreement with those from HPLC, though the method lacks automation and yields lower precision [51–55]. However, the result from ion exchange micro-column technique is acceptably accurate and precise and can be used to confirm some types of thalassemias such as β-thalassemia trait. In addition, with its simplicity and low cost, some laboratories perform this technique together with the OF tests as regular screening techniques, especially where thalassemia cases related to abnormal ratio of HbA_2 and HbF is commonly found such as in Thailand.

It has been estimated that the cost for chemicals and materials per test of the micro-column technique is approximately five times less than that of HPLC. Even though the total analysis time per run is longer than automated HPLC (4 h versus 20 min), many ion exchange micro-columns can be set up and run at the same time. Therefore, the total analysis time of, e.g., 50 tests using multiple micro-columns at one time is less than performing 50 continuous HPLC runs (16 h using HPLC and 4 h using ion exchange micro-columns). In addition, an attempt to reduce the analysis time per run and to make the micro-column technique more automated has been carried out. A flow injection analysis system was joined together with a much smaller ion exchange column to improve the analysis time for hemoglobin typing as compared to the batch process [56]. More work needs to be done, but the preliminary results have suggested that the flow based and reduced volume ion exchange column system has the potential to improve the analysis time per run and to greatly reduce the amount of blood sample needed for the analysis.

4. Instrumental techniques for determination of thalassemia and Hb variants

These techniques involve modern technologies of complicated instrumentation. They can be automated and are usually faster and more reliable but more expensive than the conventional techniques. Even though these techniques can provide detailed information and can help in diagnosis of many types of Hb variants, there are a few exceptional Hb variant cases that cannot be identified with these techniques, and more extensive confirmatory tests are needed. Most instrumental techniques can perform qualitative and quantitative analysis, but with limited ways to accurately quantitate the signals, such as in gel electrophoresis, these techniques have been

used mainly for diagnosis of Hb variants rather than for detection of abnormal level of Hbs in thalassemia diagnosis, as shown in the flow chart in Fig. 1.

4.1. High performance liquid chromatography (HPLC)

In high performance liquid chromatography, particle size of the stationary phase packed in the column is quite small (about 2–5 μm). High pressure is required to force the mobile phase to continuously flow through the column. As the sample solution flows with the liquid mobile phase through the stationary phase, the components of the sample will migrate according to the non-covalent interactions of the compounds with the stationary phase. The degree of interactions determines the degree of migration and separation of the components (i.e., the component with a stronger interaction with the mobile phase than with the stationary phase will have a shorter retention time and thus will be eluted from the column first and vice versa) [57,58].

HPLC has become a very important tool for thalassemia and Hb variants diagnosis because of its ability to accurately and rapidly qualitate and quantitate different types of Hbs. However, in most laboratories, HPLC has been used for diagnosis of Hb variants rather than for quantification of normal Hb or thalassemia diagnosis, except for the case of prenatal analysis. The HPLC technique requires a very small amount of blood samples (μl), therefore, it is very suitable for prenatal diagnosis of thalassemia [59–63] where sample may be limited and difficult to obtain. There are many reports showing the agreement of results obtained from HPLC and those obtained from other techniques such as the globin synthesis technique, isoelectrofocusing, carboxymethylcellulose chromatography and DNA sequencing [59–64].

Anion exchange resin DEAE and gradient Tris–HCl buffer solution, pH 8.5–6.0, is a widely used stationary–mobile phase system for HbA_2 and HbF quantification to effectively diagnose β-thalassemia and Hb Bart's hydrop fatalis that occur frequently in Southeast Asia [62,65]. The system can also separate other Hb variants such as HbS, HbC and HbJ [66,67]. Cation exchangers, such as CM-cellulose (CMC) and silica supported with carboxylic acid residues with bis-Tris–KCN developer, can also be used for the same purpose [60,68,69]. The system based on carboxymethylated poly(vinyl alcohol) resin and sodium phosphate buffer solution as a stationary–mobile phase has been developed for separation and quantification of St-HbA1c, which is a marker of blood glucose regulation in diabetic patients [70].

The ratio of different globin chains (e.g., β:γ for β-thalassemia diagnosis) can also be determined with HPLC using a reverse phase C18 column and shows similar results to those obtained from CMC which is normally employed for this purpose [59]. Determination of Hb types using HPLC has gained high popularity over globin chains determination using CMC because of HPLC's relatively easier and faster analysis which is a result of its having fewer sample preparation steps and an automated data analysis system [61]. HPLC has an overall performance better than electrophoresis.

4.2. Electrophoresis

Electrophoresis is one of the widely used techniques for analyzing hemoglobin variants based on the movement of different Hb or different globin chains, containing different charges, in the electric field. At an alkaline pH, Hb is negatively charged and will move toward the anode (positively charged) terminal. Electrophoresis of total Hb is different from electrophoresis of separated globin chains. To perform electrophoresis of globin chains, a few steps need to be done in order to obtain free globin chains. First, heme is removed from hemoglobin by treating with mercaptoethanol. Then the four globin chains are split apart without denaturing them using 8 M urea. A cellulose acetate membrane is mainly used in alkaline pH electrophoresis. Normal operating voltage is about 250 mV and the approximate run time is about 90 min. After that, the membrane needs to be stained, de-stained and air dried before separation of globin chains can be observed. The main limitation of electrophoresis at alkaline pH is the inability to differentiate HbA_2, HbC, HbO and HbE from one another, nor can HbD, HbG and Hb Lepore be differentiated from HbS [33,71,72]. Therefore, it is normally used to screen for some types of Hb variants. The confirmatory test can be done using electrophoresis in acidic media.

At a lower pH of about 6.0, a better separation of different hemoglobins is obtained. Those Hbs that co-migrate in alkaline pH electrophoresis can be separated in acidic media. Nevertheless, the main technique for Hb quantification by densitometric scanning of the gel is still somewhat difficult and unreliable [33] and therefore electrophoresis technique has been used mainly for detection of Hb variants rather than measuring level of Hb in thalassemia diagnosis. It is highly specific in the detection of certain Hb disorders such as sickle cell disease. Even though the electrophoresis in acidic media is quite a powerful technique in separation of many types of Hbs, please keep in mind that not all Hb variants can be separated by electrophoresis in acidic media. For example, Hb Okayama cannot be separated using electrophoresis, but can be done so in HPLC [73].

Capillary electrophoresis is the new format of electrophoresis where separation takes place in a small fused silica capillary. It is rapid, easily automated and consumes low amounts of reagents, as compared to conventional gel electrophoresis. It also offers much higher throughput as compared to HPLC [74]. However, some researchers found that CE has higher instrumentation cost and is less accurate as compared to automated HPLC [75].

4.3. Isoelectric focusing (IEF)

This technique is based on the electrophoresis technique but with a higher degree of separation. Different Hbs migrate in a pH gradient to the point where their net charges are zero.

The order of migration is the same as in alkaline electrophoresis but the narrower bands obtained from this method (IEF) allow for the resolution of HbC, HbE, HbO, HbS, HbD and HbG [76,77].

Two different formats of IEF, thin layer gel and capillary, have been reported [78–81]. Cossu et al. [82] applied the immobilized pH gradient method (IPG) with a thin layer gel that has a pH range of 6.7–7.6 to differentiate heterozygous from homozygous β-thalassemia in newborns. The group suggested the use of umbilical cord blood because it contains only HbF, HbA and acetylated HbF (HbFac) and the ratio of HbA:HbFac or HbF:HbA is used instead of the conventional β:α ratio in the IEF of globin chains.

Capillary IEF showed very promising performance both in qualitative and quantitative aspects. A single IEF run can replace the main tests that normally have to be carried out in combination for qualitative and quantitative analysis of Hbs, for instance, alkaline and acid electrophoresis for major Hb variants, ion exchange chromatography for HbA_2 quantification and alkaline resistant test for HbF [78,81]. It has been proven to have a comparable performance to chromatography or radioactive globin chain methods [79] and can be used for analysis of hemoglobin variants in adult and newborn [83].

4.4. Flow cytometry

Even though acid elution stain test seems to be simple, it is rather time consuming and subject to human error. The more precise and sensitive quantification of HbF can be done using the instrumental based flow cytometric technique [84,85]. The interested component of the cell is bound to a fluorescence label. Light scattering can identify the cell population of interest. Fluorescence intensity is measured to quantify the component of interest. The discovery of monoclonal antibody production has extended the use of flow cytometry. Antibody against HbF tagged with fluorescent dye can be used to specifically determine the amount of HbF. It has been demonstrated that detection of both a fetal cell surface antigen and HbF using two different monoclonal antibodies and two colored dyes is a precise way to identify the fetal cells [86]. The technique called gradient centrifugation has been proved to enrich the fetal cells from the adult blood and can extend the sensitivity of the flow cytometric analysis of HbF [87].

5. Extensive analysis techniques for thalassemia and Hb variants

These are advanced techniques used to detect thalassemia and Hb variants. They are complicated and expensive and therefore are used in the cases for which there are no other ways to accurately confirm or identify the types of thalassemia or Hb variants. They involve DNA technology that can provide in-depth detailed information of gene mutation.

5.1. Polymerase chain reaction (PCR) with different formats of gel electrophoresis

Polymerase chain reaction is a technique that allows a small amount of DNA to be amplified in vitro. The process is composed of cycles of the three following steps: perform heat denaturing to separate the DNA sequence target into two strands, anneal each strand to the specific primers and then extend the polymerase chain from the primer termini [10]. Once there are enough of the DNA target sequences produced, further analysis can be performed. Gel electrophoresis is commonly done following the PCR to separate different DNA fragments. Many additional methods can be coupled with gel electrophoresis and PCR to obtain better information such as those described briefly here.

Direct DNA sequencing of PCR products is quite a straightforward method to indicate the mutation site [88,89].

The restriction fragment length polymorphism (RFLP) technique can differentiate between different DNA sequences based on the length of fragments yielded by a particular enzyme restriction and can indicate the mutation point of a gene in thalassemia patients [90,91].

The amplification refractory mutation system (ARMS-PCR), also known as allele specific PCR, is another technique that has been introduced to be used for thalassemia diagnosis. This technique utilizes two PCR reactions: one contains a primer specific for the normal allele and the other contains one for the mutant allele. Gel electrophoresis is then employed to separate specific DNA bands. Diagnosis of genotyping is based on whether there is amplification in one or both reactions (i.e., the band in normal reaction only indicates normal allele, the band in mutant reaction only indicates mutant allele, and bands in both reactions indicate a heterozygote) [92–94]. ARMS-PCR is more accurate as compared to RFLP.

Single stranded conformation polymorphism (SSCP) is the technique that was developed based on the fact that the mobility in gel electrophoresis of single strands of DNA drastically depends on nucleotide sequence. Single stranded DNA is produced by adding one primer at a concentration higher than another primer in the PCR step. After the primer with lower amount is used up, the reaction will continue producing only the product of the excess primer. The mobilities of single strands are then compared [95]. Single stranded DNA may also be produced by denaturing double stranded DNA, as in the technique called denaturing gradient gel electrophoresis (DGGE). The DGGE technique utilizes the gradient of low to high pH to denature different gene fragments and retard their mobilities in gel. A mobility shift can be detected even with a slight difference in the base pair sequence [92,96,97].

5.2. DNA technology: DNA probe/DNA microchip

Analysis of nucleic acids has led to the understanding of the gene expression that controls Hbs formation. This information is more detailed as compared to information obtained

from protein analysis that normally only suggests type and amount of different Hbs production. The advance of DNA studies and fabrication technology together has led to the development of methods for diagnosis using a DNA microchip. Normally the segment of a gene of interest first has to be amplified by PCR to obtain a sufficient amount prior to hybridization with allele specific oligonucleotide probes that are immobilized on the solid phase or chip [98–100]. The bound target gene can be detected using either labels such as fluorescent substances [101,102] or electronic transducers such as piezoelectronic and ion sensitive field effect transistors (ISFETs) [103]. The attempt to pinpoint the DNA sequences that become over-expressed in a patient has become important because it can lead to the cure or prevention of the disease. In this case the precision and accuracy of detection requires highly sophisticated devices. Therefore, development of a highly sensitive and accurate device or method of detection is currently an important research trend.

One example of devices that has been used commonly is a cytometer. Cytometry is a laser based technique that allows for analysis of physical properties and fluorescence intensity of an individual cell in a heterogeneous environment. The image can differentiate different types of cells or DNA sequences that are labeled with different colors by comparing the ratios of fluorescence of different targets. With the aid of a computer, detection and visualization of many different probes can be done simultaneously [104,105].

6. Indirect studies related to thalassemia indication and treatment follow-up

These studies are not intended to be used for thalassemia diagnosis. However, the relevance of the variable of interest and the existence of thalassemia may help open up a new way to economically test for thalassemia or treatment follow-up.

6.1. Ferritin

Ferritin is the iron storage protein and its level in serum directly relates to the amount of iron stored in the body, which is important for red blood cell production. Normal ranges of ferritin are 12–300 and 12–150 ng ml^{-1} for male and female, respectively [106]. The technique commonly used to quantify ferritin is immunoassay [107,108]. A significantly high level of ferritin is found in patients with iron overload and this may help differentiate thalassemia patients from those with iron deficiency, both of which will have a low red blood cell count [109]. However, please note that iron deficiency does not exclude thalassemia disorder. In addition, any inflammatory disorder can cause a high level of ferritin. Therefore, long term monitoring of ferritin would be necessary, if its level is chosen to be observed, to gain any additional information for thalassemia diagnosis or treatment follow-up [110].

6.2. Skin tissue Fe concentration

Thalassemia patients have an increased amount of iron storage that has been reported to cause an increased risk of cardiovascular disease due to arterial stiffness [111–113]. Evaluating the level of iron in the body over time is one way to obtain additional information for thalassemia diagnosis and to evaluate the efficiency of the treatment. The X-ray spectrometric techniques (diagnostic X-ray and X-ray fluorescence spectrometry) have been employed to rapidly and non-invasively quantify the amount of iron overload in the skin of thalassemia patients [114,115]. Since the amount of iron found in the outer body skin correlates to the amount of iron overloaded in the liver, heart and spleen, the results from the skin can be used as markers for iron-overload organs. However, as mentioned previously, it is necessary to keep in mind that iron deficiency does not exclude thalassemia.

6.3. Magnetic behavior of erythrocytes

A study in physics based on observation of the change in magnetic behavior of Hb at different states (i.e., normal, oxidized and reduced states) has been conducted [116]. The reduced form of Hb induces paramagnetism while the oxidized form of Hb shows diamagnetic behavior. It was found that normal state Hb from β-thalassemia minors has lower diamagnetic response as compared to that of Hb from iron deficiency patients. This may indicate a low oxygen intake of β-thalassemia minor blood.

6.4. Nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance technique is based on the magnetic properties of some nuclei that when placed in the magnetic field, would take up radio frequency energy that matches the magnetic field strength and later re-emit that energy [117]. The phenomenon is known as nuclear magnetic resonance (NMR) because it involves the nucleus in a magnetic field that has its strength in resonance with the applied radio frequency. Originally NMR spectroscopy was used for the study of composition of chemical compounds. Later, the technique was developed into the imaging technology, magnetic resonance imaging (MRI), that became a major breakthrough in medical fields because it can reveal the image of the parts of the body and seems to be the most sensitive means at present. Contrast medium such as gadoteric acid may be introduced to obtain better results [118].

NMR has been widely applied to study body iron overload [119–128]. NMR spectroscopy has been employed mainly for study of iron level in the fraction of tissue in vitro, while NMR imaging has been used mainly for determination of iron in vivo. So far, there has been no report on health hazards directly related to NMR and therefore the NMR technique is considered a safe and non-invasive way to study body iron content.

7. Conclusion

There are many different techniques available for thalassemia diagnosis, but used alone they may not be able to satisfactorily answer every question. Therefore, it is quite common to utilize more than one technique to ensure the diagnostic result. Fig. 1 summarizes techniques commonly used for diagnosis of Hb variants and thalassemia in most laboratories. If MCV, MCH or OFT screening test reveals a normal result, the possibility of having thalassemia can be eliminated, but analysis of Hb variants should be done. If an abnormal result is obtained from the screening test, there is a possibility of having either Hb variants or thalassemia case. If Hb variants tests do not show any abnormal results, thalassemia tests should still be performed. Choices of techniques depend mainly on budget and equipment available. For example, some countries with limited budget such as Thailand, Indonesia and The Philippines utilize OFT rather than MCV and MCH (from automated CBC machine) for thalassemia screening. It should be pointed out that even in laboratories equipped with high technology and many years of experience, quite a few false diagnoses were reported, which resulted in the births of thalassemia children or the abortions of unaffected fetuses [93]. It is very important to take precaution in every step of the diagnostic procedures to ensure the most accurate diagnosis. For example, be sure to use a fresh blood sample, always perform sufficient sample cleaning, and always run duplicate tests with suitable controls. Developments in chemical analysis methodologies are still very useful to this field.

Acknowledgements

We thank Prof. Torpong Sanguansermsri, Thalassemia Laboratories, Pediatrics Department, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand for valuable comments. We also acknowledge the Thailand Research Fund (TRF) and the Post graduate Education and Research in Chemistry (PERCH) for support.

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Spectrochimica Acta Part B 60 (2005) 109–116

SPECTROCHIMICA ACTA PART B

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Size-based speciation of iron in clay mineral particles by gravitational field-flow fractionation with electrothermal atomic absorption spectrometry

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Received 22 January 2004; accepted 8 November 2004

Abstract

Gravitational field-flow fractionation (FFF) coupled to UV and ETAAS detectors has been tested for micron-size particles in the range of 5–20 μm using three Fe-rich clay samples. The iron content estimated after aqua regia extraction was about 20–40 mg kg^{-1}. The ETAAS analysis was performed both off-line from collected fractions and in an online continuous sampling mode using a specially designed flow through vial placed in the autosampler of the ETAAS. Comparison of the direct injection method with total analysis after aqua regia digestion shows that slurry injection of the dilute samples in the gravitational field-flow fractionation (GrFFF) effluent is quite efficient in these samples. In the majority of cases, more than 90% recovery was obtained for the slurry injection method. Fe mass-based particle size distributions and Fe concentration versus particle diameter plots can be generated using certain assumptions. This provides detailed information on size-based speciation of particulate samples. Generally, the Fe concentrations in the particles decreased slightly with an increase in particle size as is often found for soil and sediment samples.
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Keywords: Clay mineral particles; Size-based iron speciation; Micron-size particles; Gravitational field-flow fractionation; Electrothermal atomic absorption spectrometry

1. Introduction

The size of particles influences many properties of solid materials. An important example is the transport and fate of particles in aquatic environments [1] through soil profiles [2] and as atmospheric aerosols [3]. Since many elements and compounds are associated with such particulate material, the study of size-based speciation is very relevant in environmental science. One approach for such work is to use conventional separation methods, such as filtration and settling (gravitational or centrifugal), to prepare subsamples with specified size ranges and to analyze these for the components of interest [4–6]. However, filtration suffers from many artefacts, and settling methods are usually quite time consuming [5,6].

For several decades, various groups have been developing methods involving field-flow fractionation (FFF) for separation purposes [7]. FFF has the advantage of generating a continuous distribution of polydisperse samples which can be either collected as discrete subsamples for analysis [8–10], or in certain cases, the FFF eluent can be fed directly into an analytical device [11]. The data generated by such experiments are often in the form of an analyte-based size distribution [8–11].

Most studies on size-based speciation of environmental samples to date have involved the use of sedimentation FFF (SdFFF) [8,12–15] or flow FFF (FlFFF) [16–22] to characterize submicron colloids. These have been coupled

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doi:10.1016/j.sab.2004.11.007

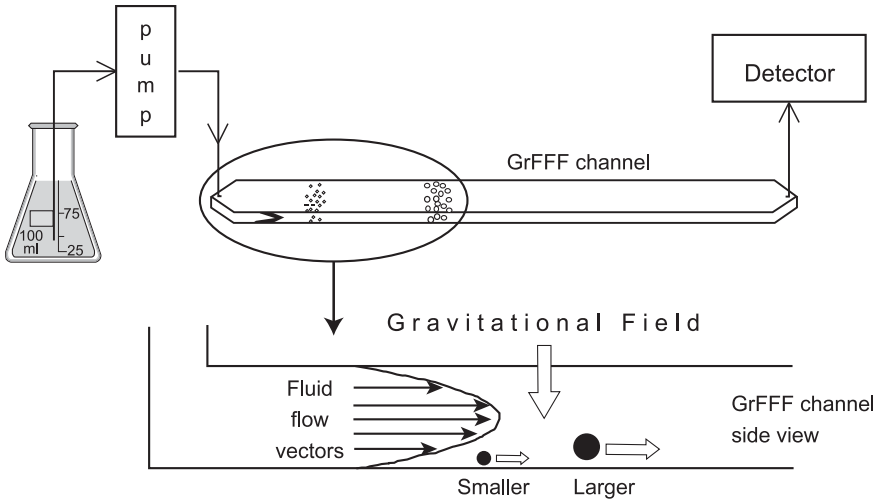

Fig. 1. GrFFF instrument and steric/hyperlayer separation mechanism for micron-size of particles.

both off-line and online with inductively coupled plasma mass spectrometry (ICP-MS) [12–22], and electrothermal atomic absorption spectrometry (ETAAS) is employed as tool [8–11] for element speciation. FFF-ICP-MS has been currently reviewed for some selected applications by Barnes and Siripinyanond [23]. One of the limitations for elemental analysis of particulate slurries is that the efficiency decreases for particles greater than about 5–10 μm. This could be due to trapping of particles in the nebulizer or incomplete atomization/ionization for these larger particles. Recently, we have demonstrated that gravitational FFF (GrFFF) can be combined with ETAAS to achieve size-based speciation data for micron-size silica particles up to 10 μm in diameter [9]. This was done off-line by collecting subfractions for Fe analysis by ETAAS. The digestion efficiency of ETAAS with slurry samples proved to be good at least up to 10-μm diameter particles.

GrFFF is carried out by applying a gravitational field perpendicular to the laminar flow direction in a thin GrFFF separation channel (see Fig. 1). One significant advantage of GrFFF is that the channel can be constructed quite easily and cheaply, and the system can be assembled using widely available components for HPLC or flow injection systems. However, the weak gravitational field restricts the size range that can be fractionated to about 2–50 μm in diameter. Nonetheless, this silt-size range is of importance for sediment transport in rivers and atmospheric aerosols.

As an extension of our work to develop analytical methods for size-based element speciation, we present for the first time data for online GrFFF–ETAAS of micron-size clay particles. This was achieved by continuously feeding the eluent from the GrFFF into a special flow through sampler set in one position of the autosampler. The stream was sampled at regular time intervals, and the Fe content was obtained by slurry introduction into the ETAAS. A major advantage of this method is that samples are withdrawn from a flowing suspension, thus avoiding the problem of settling of sample particles in a vial. This same approach may be useful in other applications where slurry injection is used.

The reason for our focus on Fe, in this and previous publications, is that hydrous Fe oxide coatings are thought to exist on aquatic and soil particles and are generally believed to be involved in the uptake of contaminants such as toxic metals [3] and phosphate [2]. In this paper, we continue with this emphasis, but instead of using pure silica with a coating of synthetic goethite [9], we study the Fe speciation of a series of natural iron-rich silt-size clay minerals.

2. Experimental section

2.1. Instrumentation

2.1.1. GrFFF

The components of the GrFFF instrument have been described in previous work [9,24]. The channel dimensions were 0.095×300×20 mm for the channel thickness, tip-to-tip length and breadth, respectively. The geometric void volume was 0.57 mL. Although the GrFFF channel was only 95 μm thick, no clogging problems were experienced with the dilute (2 mg mL^{-1}) <45-μm diameter sieved samples used in this work. A Milton Roy Constametric III pump (Pennsylvania, USA) was used to deliver the carrier, 10^{-4} M sodium hydroxide solution. Particle suspensions were injected into the carrier stream with a hypodermic syringe (40 μL) through a homemade injection port [24]. The concentration of particles in the FFF eluent was monitored by a UV detector (Model UV8, BAS, USA) at an operating wavelength of 254 nm. The Pocket Sampler (Dick Smith, Australia) with the software supplied was used as the interfacing device for digitizing the GrFFF-UV detection signal.

2.1.2. ETAAS

The ETAAS instrument was a Perkin–Elmer Model 5100 (Norwalk, CT, USA) equipped with Zeeman correction, an HGA-600 graphite furnace and AS-60 autosampler. A pyrolytically coated graphite tube with L'vov platform was used. The fast-heating program for the graphite furnace is summarized in Table 1. The peak area across the 3-s heating segment (step 2) of the program was recorded. This program enables more analysis data to be collected across the GrFFF fractogram. The total analysis time for each replicate was 86 s (including autosampler operating time and cooling step). By using this temperature program, there was no difference for the Fe atomic peak profiles from the slurry, and solution analysis was observed.

2.1.3. GrFFF–ETAAS

Off-line and online determination of iron by GrFFF with ETAAS detection can be carried out by employing the instrument set-ups, as shown in Fig. 2. Off-line GrFFF with ETAAS analysis was done by collecting fractions (ISCO, Model RETRIEVER 500 fraction collector, Lincoln, USA) of the eluent after passing through the GrFFF separation device, as shown in Fig. 2(a). Analysis of the iron content in each fraction was obtained through slurry introduction into an electrothermal atomic absorption spectrometer (ETAAS). For online GrFFF–ETAAS analysis, the eluent from FFF unit was continuously fed into the bottom of a specially designed flow through sampler vial (see Fig. 2(b)) which was placed in the autosampler of the ETAAS. Sample suspensions (10–20 μL of 2.01–2.07 mg mL^{-1} depending on the sample) were introduced into the GrFFF, and the eluted particle suspension was continuously introduced through the bottom of the sampler vial. The overflow was directed to waste (Fig. 2(b)). The autosampler arm was set always to sample from this same position so that it introduced discrete samples of the eluent into the ETAAS. A photograph of the online GrFFF–ETAAS instrumentation is shown in Fig. 2(c).

2.2. Chemicals and samples

2.2.1. Chromatographic silica particles (5 and 10 μm)

Chromatographic silica was obtained from used HPLC columns (CN packing from PARTISPHERE RTF Columns, Whatman, UK). Particle suspensions of about 2 mg mL^{-1} in water were prepared. The 5-μm particles were spherical, and the 10-μm particles had more irregular shapes. These were used as particle size standards.

Table 1
Fast heating graphite furnace temperature program

Step	Furnace temperature(°C)	Ramp time (s)	Hold time (s)	Internal Ar gas flow (mL min^{-1})
1	140	1	15	300
2[a]	2400	3	10	0
3	2600	1	5	300

[a] Peak area measurement.

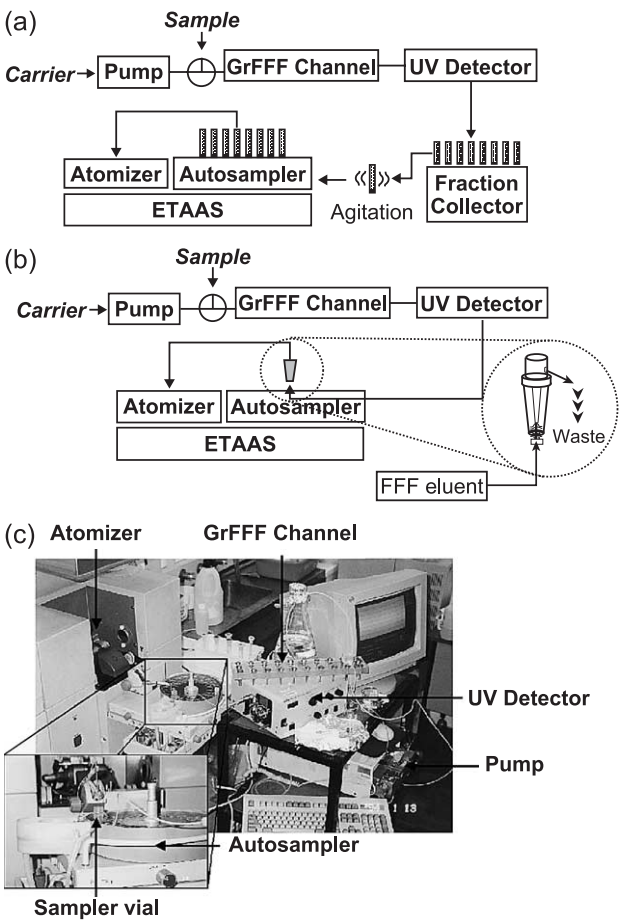

Fig. 2. Instrumental set-up for GrFFF coupled with ETAAS. (a) Off-line GrFFF–ETAAS, (b) online GrFFF–ETAAS showing the flow through sampler vial and (c) photo of the online GrFFF–ETAAS instrumentation.

2.2.2. Clay samples

All clay samples were of natural origin and were obtained from different provinces in Thailand. The clays are referred to as red clay (Kanchanaburi province), ball clay 1 and ball clay 2 (sampled from different sites in Payao province). They were dry sieved through a 45-μm sieve, then dried further at 60 °C for 12 h in an oven and stored in a desiccator until required. Each sample was suspended in water (2 mg mL^{-1}).

2.2.3. Reagents

All reagents were prepared using water obtained from a Milli-Q system (Millipore, Milford, MA, USA). Carrier of sodium hydroxide (10^{-4} M) was prepared using a reagent from Aka Chemicals (Sweden). A standard iron solution (1000 mg L^{-1}) was SpectrosoL® grade from BDH. The desired standard concentrations (10, 20, 40, 60 and 80 μg L^{-1}) were diluted appropriately from the stock standard and used to obtain the calibration line: Y=0.0049×, r^2=0.9947.

3. Results and discussion

3.1. Mass- and Fe-based fractograms obtained by GrFFF with ETAAS

Fractograms of the three clays generated with off-line and online GrFFF–ETAAS analysis for iron are given in Fig. 3. It was found that the UV and Fe profiles obtained by off-line and online operations from all samples agreed reasonably well with each other. The largest discrepancy was between the off-line and online UV fractograms for ball clay 1, which might have been caused by specific errors of the runs. In all subsequent plots, the online fractogram data were used for the calculations. The fact that the sample peak was not resolved from the void peak indicated the presence of large particles of about 20 μm or more.

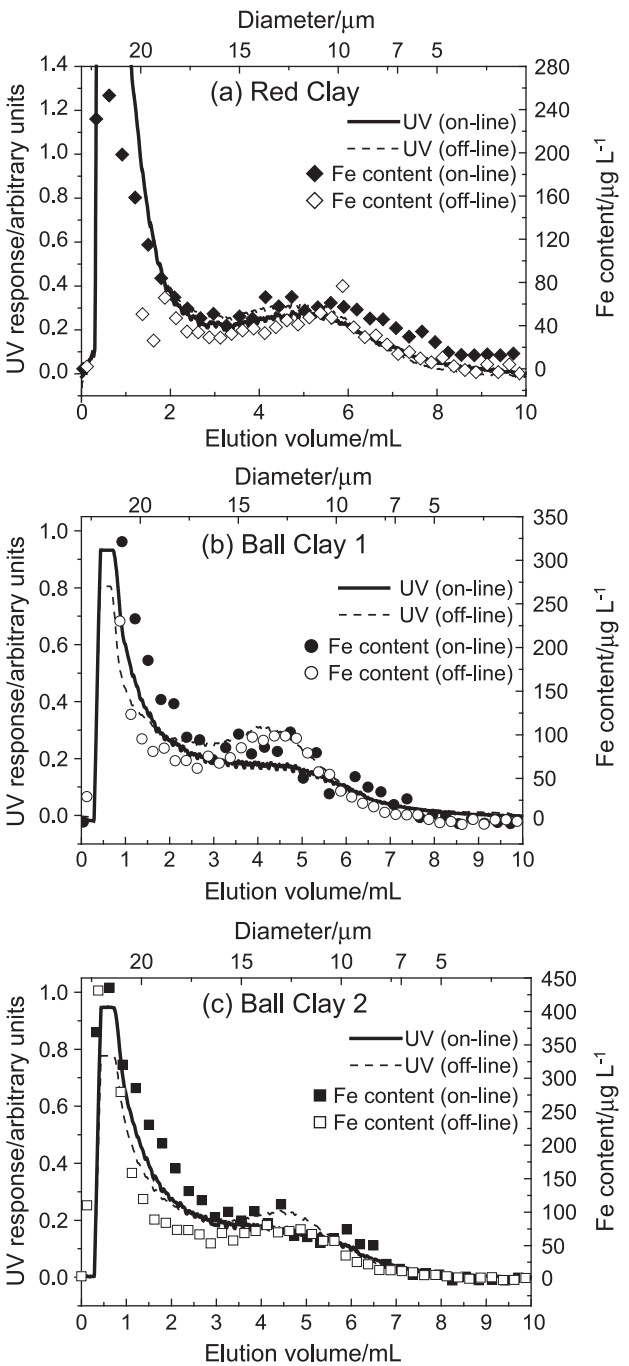

Fig. 3. UV and Fe-based fractograms of clay samples obtained by off-line/online GrFFF–ETAAS (a) red clay, (b) ball clay 1 and (c) ball clay 2.

3.2. Conversion from elution time to diameter

Due to the complications in the steric/hyperlayer model, the conversion of the elution time or volume to diameter must be done empirically via calibration with suitable standard particles. By assuming that the standard particles have the same density as the clay particles, the conversion of the diameter scale can be made employing the empirical formula [25],

$$\log t_r = -S_d \log d + \log t_{r1} \quad (1)$$

where t_r is measured retention time, d is the diameter of the particles, S_d the size selectivity, and t_{r1} is a constant equal to the extrapolated value of t_r corresponding to particles of unit diameter.

For the purposes of illustrating the general approach, a calibration line was obtained using only two chromatographic silica samples (5 and 10 μm) as particle size standards. Although it is not adequate for accurate size determination, the errors are not likely to be excessive as many previous studies have found that the calibration graph is linear [7,26]. Fig. 4 shows fractograms of the 5- and 10-μm silica particles and the plot of log t_r versus log d. This plot was used to generate the equation for converting the x-axis to a diameter scale as described above. In this case, the calculated selectivity using the data for the 5- and 10-μm silica was found to be 0.92, and t_{r1} was 98.

It should be noted that there may be a systematic error in the calibration performed because the 5-μm silica standard particles are spherical, but the 10-μm silica standard and clay particles are more platey in shape. It is known that platey particles experience higher lift forces than spheres of the same volume and hence elute at shorter times [27].

A rough estimate of the magnitude of the error introduced by shape can be deduced as follows. A survey of the literature shows that the selectivity for sedimentation FFF (including the gravitational version) usually falls between 0.7 and 0.8. The gradient for our data in Fig. 4(b) is higher (0.92) because t_r for the 10-μm nonspherical particles is smaller than would be expected for a spherical particle of the same volume. If we use a gradient of 0.75 and the single 5-μm spherical particle calibration point, we can estimate that, for a 10-μm spherical sample particle, we would obtain a diameter of

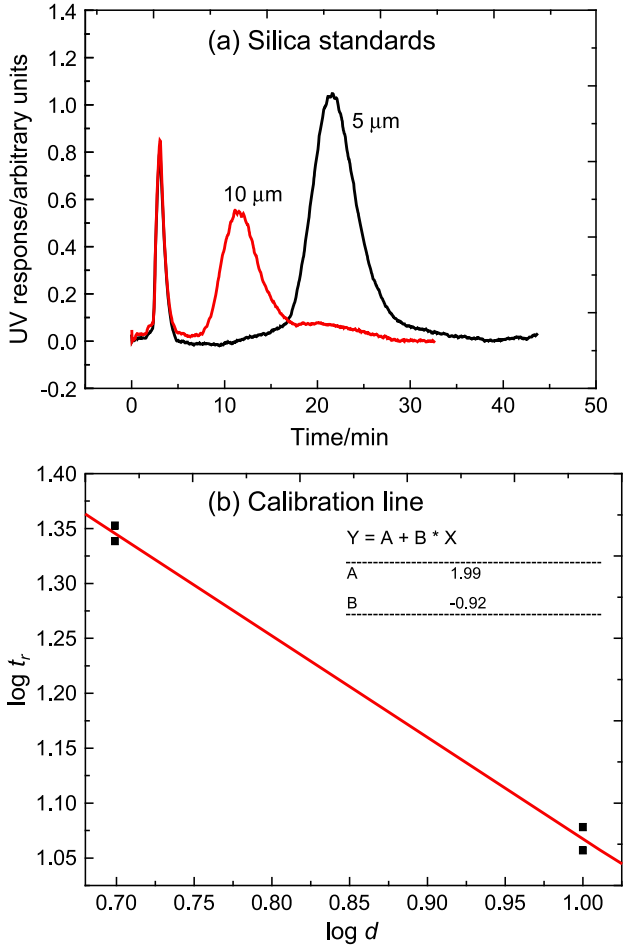

Fig. 4. (a) GrFFF Fractograms of 5- and 10-µm silica particles and (b) plot of log t_r versus log d.

8.8 µm or thus incurring an error of 12%. This simple calculation illustrates the magnitude of the shape error to be expected but should not be taken as the error in our results. Indeed, the situation here is even more complicated as the 5 µm standard was spherical, the 10 µm standard appeared to be somewhat irregular in shape, and the ball clay samples were very platey.

The density of the silica particles may also differ from the clays although this is likely to be less than 20%. Ideally, the calibration standards and samples should have the same shape and density. The calibration error encountered here are of no importance in this paper which is intended just to outline the concept of GrFFF–ETAAS approach for size-based speciation.

3.3. Conversion from UV detector signal to eluted mass

For submicron particles, the UV detector response is usually assumed to be directly proportional to the mass concentration of particles ($\mathrm{d}m^{\mathrm{c}}_{\mathrm{pi}}/\mathrm{d}V_{\mathrm{i}}$). However, according to the approach of Reschiglian et al. [28,29] for micron-size particles, the general relationship for the suspension concentration for large particles (10-fold bigger than the incident wavelength), which comes from Mie theory, can be expressed as

$$\frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{pi}}}{\mathrm{dV_i}} \propto UV_i d_i \tag{2}$$

where UV_i; is the UV detector response at point i along the FFF elution profile; $m^{\mathrm{c}}_{\mathrm{pi}}$ is the mass of sample eluted up to elution volume V_i, and d_i is the particle diameter eluting at V_i. It should be noted that the superscript c in these quantities signifies that it is the cumulative amount eluted up to point i on the fractogram.

3.4. Mass- and Fe-based particle size distributions of clay samples

The appropriate y-axis for a particle size distribution ($\mathrm{d}m^{\mathrm{c}}_{\mathrm{pi}}/\mathrm{d}d_i$) is given by [7]

$$\frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{pi}}}{\mathrm{dd_i}} = \frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{p}}}{\mathrm{dV_i}}\left|\frac{\delta V_i}{\delta d_i}\right| \propto \mathrm{UV_i d_i}\left|\frac{\delta \mathrm{V_i}}{\delta \mathrm{d_i}}\right| \tag{3}$$

where δd_i is the increment in d_i corresponding to increment δV_i in V at point i along the fractogram. The mass-based size distribution is thus a plot of $\mathrm{d}m^{\mathrm{c}}_{\mathrm{pi}}/\mathrm{d}d_i$ versus d_i.

When the GrFFF was connected to the ETAAS system, the Fe content can be evaluated. The mass concentration of the Fe present in the eluent ($\mathrm{d}m^{\mathrm{c}}_{\mathrm{Fe}_i}/\mathrm{d}V_i$) is used to plot the Fe fractogram. This is then converted to an Fe-based particle size distribution using the equation,

$$\frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{Fe_i}}}{\mathrm{dd_i}} = \frac{\mathrm{dm}^{\mathrm{c}}_{\mathrm{Fe_i}}}{\mathrm{dV_i}}\left|\frac{\delta V_i}{\delta d_i}\right| \tag{4}$$

where $m^{\mathrm{c}}_{\mathrm{Fe}_i}$ represents the cumulative mass of Fe eluted up to digitized point i on the fractogram. The Fe-based particle size distribution is obtained by plotting $d^{\mathrm{c}}_{\mathrm{Fe}_i}/\mathrm{d}d_i$ against particle diameter d_i.

Fig. 5 shows the particle mass and Fe distributions of the three clay samples. It was found that the samples contained particles in a broad size range starting from about 2 µm and extending beyond 20 µm. However, in these GrFFF runs, the larger particles (>20 µm) are eluted with the void peak where there is no resolution of particle size. Again, we caution that extrapolation beyond the 10-µm upper limit of the calibration standards is not recommended, but in this illustration of the method, it is tolerated because of the expected linear calibration graph.

In all samples, the size distributions decrease rapidly in the range from 4 to 2 µm. This truncation of the distributions is expected in these runs since no stop flow relaxation step was used. Stop flow relaxation is sometimes required in order that the particles travel across the channel to the accumulation wall under the influence gravity. Thus, some of the particles <2 µm would be eluted in the void peak as their relaxation time is >35 s compared to the mean residence time of the carrier in the channel of 171 s [7]. The

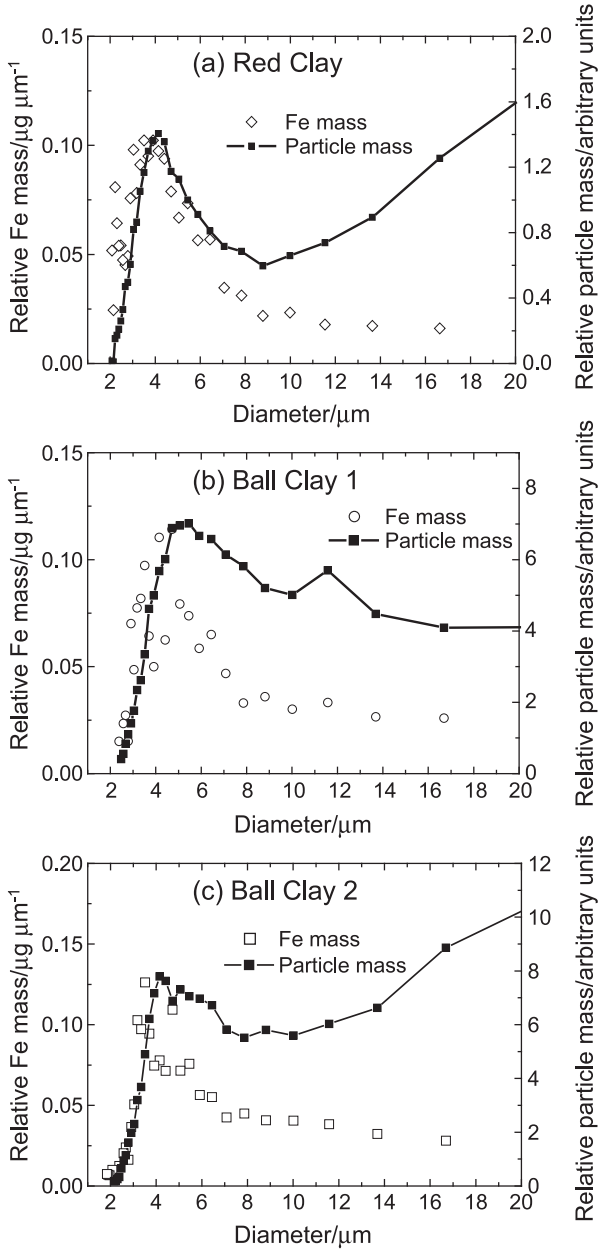

Fig. 5. Mass- and Fe-based particle size distributions (a) red clay, (b) ball clay 1 and (c) ball clay 2.

relaxation time is estimated as the time for the smallest particle of interest to settle across the channel width, w. The relaxation time (t_{relax}) is calculated by transposing the Stokes settling velocity equation to give $t_{relax}=18\eta w/g\Delta\rho d^2$, where η is the carrier liquid viscosity, w is the channel thickness, d is the particle diameter, g is the gravitational acceleration and $\Delta\rho$ is the density difference between the particle and the carrier liquid. However, optical microscope observation of these samples revealed that only small amounts of material were present with diameter <2 μm. The lack of sample relaxation will cause some spreading of the smaller particles but should be insignificant for particles greater than about 4 μm which have relaxation times <9 s.

3.5. Fe content distributions

The Fe concentration in the particles is given by

$$\frac{dm^c_{Fe_i}}{dm^c_{p_i}} = \frac{dm^c_{Fe_i}/dV_i}{dm^c_{p_i}/dV_i} \propto \frac{dm^c_{Fe_i}}{dV_i}\frac{1}{UV_i d_i} \quad (5)$$

The Fe concentration distributions were obtained by plotting $\frac{dm^c_{Fe_i}/dV_i}{UV_i d_i}$ i.e $\frac{[Fe_i]}{UVd}$ against particle diameter.

The distribution of the Fe concentration in the particles is plotted in Fig. 6. The data are only plotted between 5 and

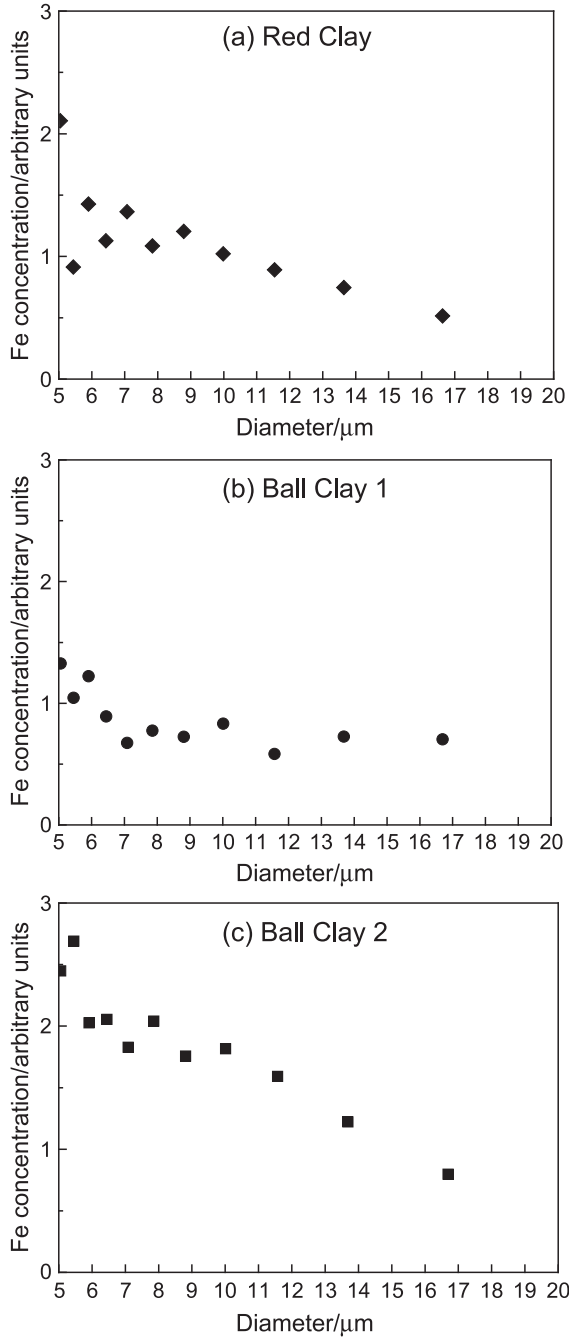

Fig. 6. Distribution of Fe concentrations in the particles as a function of diameter (a) red clay, (b) ball clay 1 and (c) ball clay 2.

Table 2
Total Fe concentrations in the three clay samples

Samples	Fe content (mg g^{-1})		
	Digested	Online	Off-line
Red clay	41.7	32.6	38.6
Ball clay 1	19.9	18.2	18.3
Ball clay 2	21.7	23.9	21.7

20 μm. For >20-μm particles, no resolution from the void peak is obtained at the run conditions employed. The data below 5 μm are not shown as both the UV and Fe concentration values are small, which can result in a large swing in the graph due to errors in setting the correct baselines in the fractograms.

The Fe content increases in the smaller particles, particularly for ball clay 2. This is commonly found for soil and sediment samples [1,11,14]. This may indicate that a significant proportion of the Fe is present as a surface coating on the particles, or that there is an increase in the proportion of Fe containing particles in the smaller size range.

3.6. Efficiency of Fe analysis by GrFFF–ETAAS

An evaluation was carried out on the effectiveness of the slurry ETAAS method for Fe analysis after GrFFF separation. Summation of the iron amounts in each fraction across the entire Fe-based fractogram (including the void peak) provides an estimate of the total mass of Fe injected into the GrFFF with the samples. This was done by integration of the area under the Fe-based fractograms obtained by both off-line and online operations (Fig. 3).

The results were compared with the total iron contents of the original samples analyzed by flame atomic absorption spectrometry after being digested with aqua regia. This comparison is presented in Table 2. It can be seen that the total Fe contents obtained by the GrFFF–ETAAS methods (both off-line and online slurry injections) agreed reasonably well with the digestion analysis. Thus, it would appear that the slurry ETAAS analysis for Fe does not suffer from the same loss of efficiency. This agrees with some previous studies [30].

Using the mean value of all three methods for a given sample, the percentage deviations of each method from this mean can be calculated, as shown in Table 3. It was found that the percent deviations from the mean for the digestion, online and off-line methods were 3–11%, 3–13% and 3%, respectively.

Assuming that the digestion method gave the most accurate estimate of the total Fe content of the sample, the percentage deviations from this value for the slurry methods were found to be 8–22% for the online method and 0–8% for the off-line method. The mean deviation (discounting the sign) was 9%, and only one value was greater than 10%. By far, the most significant outlier was the online red clay result (22% deviation). One contributing factor may be the fact that only 10 μL of sample was injected compared to 20 μL for the ball clays.

It was found that for ball clay 2, the slurry results were higher than those obtained from the digestion analysis. This suggested that there could be other errors in the analysis in addition to inefficient slurry atomization. Thus, it is reasonable to conclude from the results in Tables 2 and 3 that the slurry efficiency is quite good. However, optimization of the slurry method is required to improve the overall accuracy of the Fe analysis.

4. Conclusion

GrFFF–ETAAS was demonstrated to be an effective method for investigating the Fe size-based speciation of micron-size particles. Direct injection of the dilute suspensions of GrFFF eluent into the graphite furnace was shown to be quite efficient. The average deviation of the Fe contents from the values determined after aqua regia digestion was 9%.

The method was illustrated using some Fe-rich clay samples. The Fe concentration in the particles increased slightly with decreased particle size, perhaps indicating that significant amounts of the Fe exists as surface hydroxy oxide coatings. An alternative explanation is an increase in the proportion of Fe-rich minerals in the smaller particles.

GrFFF is the most cost effective of the FFF suite of separation techniques and could be constructed in the simplest workshops. However, it is only applicable over a size range of about 2–50 μm. The ETAAS can be operated

Table 3
Percentage deviations of the total mass of Fe in the three clay samples obtained by various ETAAS methods from either the mean Fe content value or the Fe content of the total digested sample

Sample	%Deviation of Fe content from the mean value[a]			%Deviation of Fe content from the digested value[b]	
	Digested	Online	Off-line	Online	Off-line
Red clay	11	13	3	22	7
Ball clay 1	6	3	3	8	8
Ball clay 2	3	6	3	10	0

The mean value was for the three methods.
"methodi" refers to either the digest, online or off-line method for ETAAS analysis.
[a] %Deviation of Fe content the from mean value=(Total $Fe_{(methodi)}$−Total $Fe_{(mean)}$×100/Total $Fe_{(mean)}$).
[b] %Deviation of Fe content from digest value=(Total $Fe_{(methodi)}$−Total $Fe_{(digest)}$×100/Total $Fe_{(digest)}$).

online using a specially designed sampler vial which was used in the autosampler. GrFFF–ETAAS has been demonstrated for Fe speciation here but should also be useful for a range of other elements.

Acknowledgments

Thanks are due to the Thailand Research Fund for the grants including the Royal Golden Jubilee (RGJ) PhD scholarship for RC and the Postgraduate Education and Research Program in Chemistry (PERCH) for support. The Institute for Science and Technology Research and Development, Chiang Mai University, for the support in a visit by RB to Chiang Mai University. Thanks are due to Dr. Ponlayuth Sooksamiti for the provision of the clay samples.

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Novel approach for mono-segmented flow micro-titration with sequential injection using a lab-on-valve system: a model study for the assay of acidity in fruit juices

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Received 3rd September 2004, Accepted 30th November 2004
First published as an Advance Article on the web 10th January 2005
DOI: 10.1039/b413585g

A new concept for micro-titration using a "lab-on-valve" (LOV) system with sequential injection of mono-segmented flow is proposed. The performance of the system was demonstrated by the assay of acidity in fruit juices which is based on acid–base neutralization. A standard/sample solution containing citric acid, indicator, sodium hydroxide, were sandwiched between air segments and were aspirated in microliter volumes through a selection valve into a holding coil. The acid, indicator, and base were mixed by flow reversal. After removing air segments, the solution was pushed to the detector for monitoring of the change in absorbance of the indicator color, which depended on the concentration of the remaining base. With LOV, microliter volumes of the solution can be detected without dispersion of the color zone. A calibration graph (plot of absorbance *vs.* acidity value) in the range of 0.2–1.2% (w/v) as citric acid was established. Sample throughput of 30 sample h^{-1} and good reproducibility (RSD = 1.2%, n = 11 for 0.6% acidity) were achieved. The procedure has been applied to determine acidity in fruit juices.

Introduction

Titration is an analytical technique for measuring the concentration of the analyte based on the reaction of known stoichiometric ratio between analyte and standard reagent of known concentration (titrant). Apart from visual detection, there are various ways of end point estimation including electrochemical ones which are more precise and accurate[1–3] but these applications are only limited to analytes that have electrochemical properties. All batch titrations normally involve quite high volume of reagents consumption and manually operations which are subjected to personal error.

The development of the flow injection technique has opened a new way for titration.[4–8] However, there has been a debate whether the technique of "flow injection titration" really fits the definition of titration.[9,10] This is because it is based on different principles compared to normal titration. When the sample plug, for example an acid, is injected into a carrier stream of titrant, for example a base, the dispersed zones at the two interfaces will become neutralized. The distance between the two boundaries increases with the increase of sample concentration, resulting in increasing of peak width of the FIA gram.[11,12] The calibration graph can be constructed from the peak widths (or peak area) of the standard solution of various concentrations. Then, the concentration of sample can be estimated from this calibration graph without the need for knowledge on stoichiometric ratio of sample and titrant. Alternatively, the mixing chamber can be placed in line to mix sample and titrant. The end point can be observed from the titration curve obtained after suitable sequential addition of the titrant, similar to normal batch titration where there's no need for a calibration graph.[10,13–16]

Despite the debate over this "flow injection titration" technique and whether it is really a titration, this technique has been applied to determine various analytes and parameters.[17–26] The flow injection technique offers many advantages over batch titration. The continuous flow of reagents at constant flow rate makes it possible to record the signal at constant time at the early stage of titration rather than titration until reaching the end point. This system helps to shorten the analysis time and increase sampling rate. The system can be fully automated and therefore personal error can be reduced. As a closed system, it also helps to prevent sample contamination from the outside environment and *vice versa* it helps to prevent the operator from direct contact with the chemicals. Unstable reagents can be used in the flow injection technique because they will not be exposed to air and light as much as in the batch procedure. The development of third generation FIA or sequential injection analysis (SIA) offers a higher degree of automation using computer software control and a more feasible system for many applications using bi-directional pump with micro volume manipulation.[27–30]

A mono-segmented flow is a system where air segments are used to sandwich the sample–reagent plug to eliminate dispersion between the sample–reagent zone and the carrier stream. Thus, longer resident time and better sensitivity with low reagent consumption can be achieved.[31–35]

We propose the new concept of mono-segmented flow micro-titration using a sequential injection analysis-lab-on-valve (SIA-LOV) system. The LOV is a newly developed device that is integrated as part of the selection valve of the SIA system. Since the first report by Ruzicka,[36] the applications of

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the LOV has been extended.[37–44] Reagents can be drawn, reacted and detected right on the valve.

The performance of the proposed mono-segmented flow with the SIA-LOV system was demonstrated by acid–base titration. The acid–base balance can be very important even at the cell level.[45] Studies of some substances of limited amount will also benefit from micro-volume titration. For example, determination of enzyme activity (*e.g.* pectinmethylesterase) may involve a reaction that can produce or consume an acid or base (*e.g.* when reacting with pectin, the methyl group on pectin will be changed to a carboxylic group that can be detected with an acid–base indicator)[46] and it should be possible to employ the proposed system for this purpose. Therefore, determination of acidity at small volumes has important potential. In this work, the determination of citric acid in fruit juices at the micro volume level was chosen as a model study because of the simplicity of the process and wide availability of samples and reagents.

Experimental

Apparatus

The sequential injection with the lab-on-valve system (FIAlab 3000, FIAlab, USA[47]) was composed of a syringe pump (with 2500 μl syringe), a peristaltic pump, a 6 port selection valve with lab-on-valve (LOV-SIA, FIAlab, USA) on the top, a fibre optic spectrophotometer (USB2000 UV/VIS, Ocean Optic, USA) with a tungsten halogen light source (LS-1, Ocean Optic, USA) and a computer. The first optical fibre guided light from the light source to the LOV, while the second one carried light from the LOV to the spectrophotometer. The two optical fibres were placed facing each other on a channel of LOV, forming a flow cell of 1 cm path length. All connections were made with 0.8 mm id PTFE tubing. The system was controlled by a computer with FIAlab software for windows (FIAlab, USA). Fig. 1 illustrates various components of the manifold used in this system.

Reagents and sample

Deionized water (Milli Q, Millipore) was used in all the experiments. All reagents were of analytical grade, unless otherwise stated. Citric acid (Carlo Erba) was used to prepare standard solutions of various acidities. A stock standard solution of 10.0% (w/v) citric acid was prepared by dissolving 27.3420 g of citric acid in 250 ml of 10% (w/v) sucrose solution. The working solutions were prepared by diluting the stock solution with 10% (w/v) sucrose solution. Sodium hydroxide solution was prepared by dissolving 2.00 g of sodium hydroxide (Lab-scan) in 250.00 ml deionized water and then diluting to 0.10 M. It was standardized with 0.10 M potassium hydrogen phthalate (Univar) if the exact concentration was required.

Indicator solution (0.1% (w/v)) was prepared by dissolving 0.10 g of Indigo carmine (BDH) in 100.00 ml deionized water. Other concentrations were prepared in the same manner.

Fruit juice samples were collected from local convenience stores. They all contain real fruit juices, 25–40%. They were filtered through Whatman #1 filter paper before being analysed. The clear sample solutions were kept in the refrigerator and were used within one week.

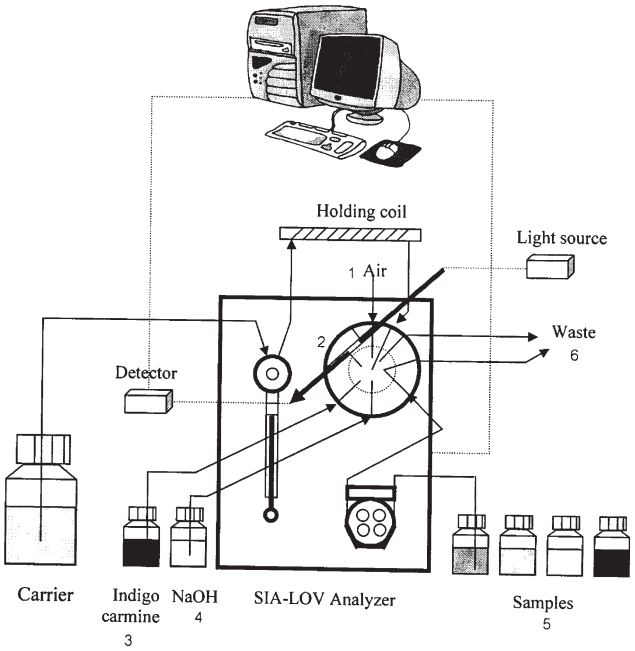

Fig. 1 A schematic diagram of the SIA-LOV manifold using a FIALab 3000 system for the mono-segmented flow titration system.

Procedure

An operation cycle of the system shown in Fig. 1 was controlled by a computer program (FIAlab 5.0). First, the carrier (H_2O) was drawn passing all the lines to clean them and to remove air from the system. The sample or standard citric acid solutions and reagents were drawn to fill up their lines. Next, 100 μl of water were aspirated into the multi-purpose flow cell at the flow rate of 0.5 ml min^{-1}. The reference absorbance of the carrier solution at this condition was detected and memorized. Then, air (15 μl), 0.0–1.2% (w/v) standard solution or samples (20 μl), 0.1% (w/v) indigo carmine solution (6 μl), 0.12 M sodium hydroxide solution (40 μl) and finally, another air segment (200 μl) were drawn in that order into the holding coil at the flow rate of 1 ml min^{-1} through port 1, 5, 3, 4 and 1 respectively. The mono-segment reagent zone was formed as shown in Fig. 2. After mixing the reagents in the mono-segment zone by reversing the flow once, the last air segment was removed through port 6. The solution zone was propelled at the flow rate of 1 ml min^{-1} through the multi-purpose flow cell *via* port 2. The change in absorbance of the indicator was detected at 608.9 nm. The first air segment was also discarded. Finally, the system was cleaned by

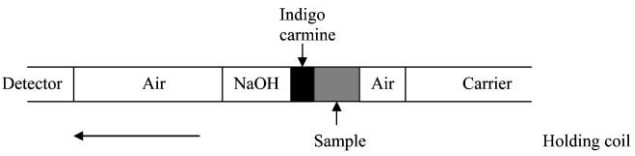

Fig. 2 The sequential drawing of regents and air to form a mono-segment.

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pumping water through the flow cell. A calibration graph was constructed by plotting the peak heights (maximum absorbance) obtained *vs*. acidity values (as % w/v of citric acid) using a Microsoft® Excel program. In all experiments, triplicate measurements were done.

Standard AOAC method

All samples were analysed by the AOAC official method 942.15 for acidity (titratable) of fruit products[48] and the results were compared with those obtained from the proposed SIA-LOV mono-segmented titration. For the colorless or slightly colored juices, a 25 ml juice was diluted to 250 ml with recently boiled water and about 0.8 ml of phenolphthalein was added. Then it was titrated with 0.1 M NaOH until the indicator turned pink. For highly colored juices, more dilution of samples was needed. First, juices were diluted the same way as in the case of colorless samples. Then the solution was titrated with 0.1 M NaOH to just before the end point and after that about 0.8 ml of phenolphthalein was added. Next, a 2 or 3 ml precisely measured volume of solution was further diluted to 20 ml with water. This extra dilution helps to dilute the color of fruit juices so that the end point was easily seen. In the case where the end point is not reached after a long titration, this dilute portion should be poured back into the original solution and more titrant should be added until the end point can be observed.

Results and discussion

Preliminary study

The concept of this novel approach for mono-segmented flow micro-titration involves a fixed amount of NaOH used as a titrant to titrate citric acid in samples. Sample, titrant and indicator were aspirated into the system and formed a stack zone that was separated from the carrier by air segments. After the titrant and a suitable volume of acidic sample in a mono-segment were well mixed, the remaining amount of NaOH, which had not been used in the reaction at the time when the detection took place, would define pH of the final solution. For different concentrations of acid samples, different amounts of NaOH were left leading to various pHs of the final solutions which could be followed by the change in color of a suitable acid–base indicator. The change can be observed in a short time and there is no need to reach the equivalent point.

Fig. 3 illustrates the pH change with volume of titrant obtained from calculation of the excess amount of NaOH left after completion of neutralization with citric acid. This was done to consider the preliminary condition to be used in the experiment. The change in color of the indicator was observed at the region shown at the beginning of the curve. According to the results obtained from the standard batch titration method, acidities found in fruit juice samples were in the range of 0.2–1.1% (w/v) expressed as citric acid, therefore the citric acid concentration in this range was selected for the calculation. 0.12 M NaOH (40 μl) was chosen to ensure a complete protolysis of citric acid which would occur at pH higher than 8.5. A volume ratio of NaOH : acid sample solution of 2 : 1 was selected because it gave a good discrimination among

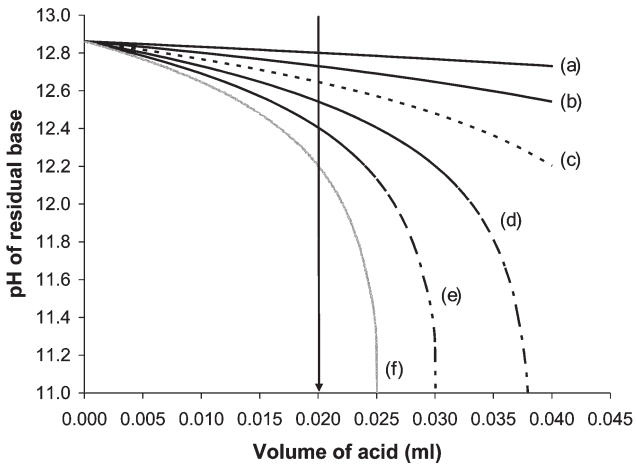

Fig. 3 Calculated pH changes in the titration between NaOH *versus* citric acid. The concentration of NaOH is 0.100 M and concentrations of citric acid are (a) 0.2, (b) 0.4, (c) 0.6, (d) 0.8, (e) 1.0, and (f) 1.2% (w/v). The arrow marks the pH values that were observed in the experiments when using different acid concentrations.

various concentrations of acidity. In this work, indigo carmine was selected because its color intensity is linearly proportional to NaOH concentration in the pH range of 11.4–13.0[49] which is the range of pH change in this particular titration. It is yellow in basic solution and blue in acidic solution. The decrease in the blue acidic form was selected to be monitored because the blue color was less interfered by colored substances in the fruit juice samples. Maximum absorption wavelengths of the indigo carmine indicator are at 400 nm (yellow) and 609 nm (blue).

The combined benefits of the mono-segmented flow technique and those of the SIA-LOV system have led this proposed system to have high sensitivity and sample throughput with less reagents and time consumption. Concentration of sample can be determined without any complicated calculations.

Optimization

Flow rate for aspiration of air segment and other reagents. The effect on flow rate for drawing air segment was investigated. The flow rate for aspiration of the air segment may not be the most obvious parameter to be optimized but it is very important. Since unsuitable flow rate for air led to small bubbles and the experiment could not be carried out, we studied this parameter at the beginning to ensure the proper handling of the air segment in the system for further experiments.

A flow rate of 1 ml min^{-1} was selected as it provided a steady air segment and short analysis time. With higher flow rate, the air segment was broken into a number of small air bubbles which may be difficult to be completely removed from the system.

For aspiration of other reagents, the flow rate of 1 ml min^{-1} was also chosen to compromise the sensitivity and sample throughput.

Concentration of an indicator. Calibration graphs were constructed using different concentrations of indigo carmine which varied from 0.05, 0.10, 0.20, to 0.40% (w/v). It was found that the slopes and R^2 values of the calibration graphs

decreased when the concentration of indigo carmine was increased. Therefore, the 0.05% (w/v) indigo carmine indicator was selected for further studies because it gave a greater slope and R^2 values.

Volumes of acid, base, indicator and air. Volumes of acid sample, base and indicator were chosen to find a compromise condition according to reagent consumption and sensitivity. The amounts chosen were 20, 40 and 6 μl for sample, base and indicator, respectively.

The air segment of 15 μl effectively separated the sample–reagent mono-segment from the carrier. However, at the beginning of the zone, 200 μl air was used just to save the carrier.

Number of flow reversals. It was found that one reversal flow was enough to mix the sample and reagents in the mono-segmented zone. The calibration signal profiles, obtained in the range of 0.0–1.2% (w/v) citric acid, are shown in Fig. 4.

Sample matrix. Fruit juices normally contain various organic matters and the most common one is sugar which is either added or is naturally present in juices. Sugar severely interfered with the determination of citric acid at low concentration. This might be due to the light refraction of high density and high viscosity sugar solution. The interference from sugar in samples could be overcome by adding sugar into the standard solutions to adjust the density and viscosity of the standard solutions to be similar to those of sample solutions as much as possible. An improved optical cell might be useful in reducing this problem.

Table 1 Summary of selected operation condition for determination of 0.0–1.2% (w/v) citric acid

Parameter	Condition
Reagent solution	40 μl of 0.12 M sodium hydroxide 6 μl of 0.05% (w/v) indigo carmine indicator
Sample volume	20 μl
Volume of air	15 μl for the end of zone 200 μl for the beginning of zone
Blank solution	10% (w/v) of sugar solution
Flow rate	0.5 ml min^{-1} for aspiration 1.0 ml min^{-1} for dispensation to detector
Volume of detection	80 μl
Wavelength	608.9 nm

Normally, the amounts of sugar in samples are approximately 10% (w/v) as indicated on the product labels. Therefore, the series of standard citric acid solutions was prepared in 10% (w/v) sugar solution to compensate for any effect of sugar in real samples.

Selected conditions. The selected conditions for the SIA-LOV determination of acidity in the range of 0.0–1.2% (w/v) as citric acid are summarized in Table 1.

Analytical characteristics

The experiment was carried out as described previously. The corresponding calibration graph, $Y = 0.4347X + 0.3414$ (where Y is absorbance and X is concentration of citric acid) with $R^2 = 0.9980$, was obtained from the signal profiles, as shown in Fig. 4.

A solution containing 0.6% (w/v) citric acid and 10% (w/v) sugar was used to study precision of the proposed method. The analysis of 11 replicates was done under the selected condition. The relative standard deviation (RSD) was found to be 1.2%. This indicates that the proposed system has good precision. Sample throughput was found to be 30 samples h^{-1}.

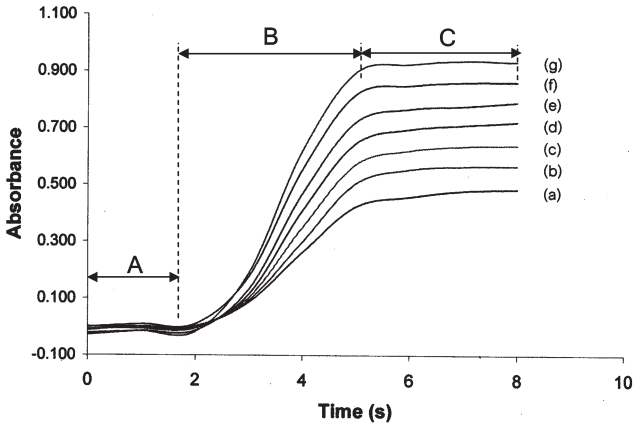

Fig. 4 Signal profiles of the standard solution. (a) 0.0, (b) 0.2, (c) 0.4, (d) 0.6, (e) 0.8, (f) 1.0 and (g) 1.2% acidity. Part A is absorbance due to carrier (water). Part B is absorbance due to the interface region between water and mixing zone (air segment has been prior discarded). Part C is absorbance due to the final pH of the mixing zone which depends on amount of remaining NaOH.

Table 2 Comparison of % (w/v) acidity found by the mono-segmented flow SIA-LOV system and the standard AOAC titration method

Sample no.	Type of fruit juice	% (v/v) real fruit juice (as labeled)	Citric acid found (% (w/v))[a]	
			Standard AOAC method	SIA-LOV
1	Lychee	30	0.30 ± 0.01	0.33 ± 0.01
2	Lime	25	0.37 ± 0.02	0.39 ± 0.03
3	Lychee and pineapple	40	0.41 ± 0.01	0.44 ± 0.03
4	Pineapple	25	0.42 ± 0.01	0.43 ± 0.01
5	Lime	15	0.44 ± 0.01	0.47 ± 0.01
6	Apple and pineapple	40	0.45 ± 0.01	0.49 ± 0.02
7	Orange	25	0.45 ± 0.01	0.48 ± 0.04
8	Pineapple	25	0.50 ± 0.01	0.53 ± 0.02
9	Apple	25	0.52 ± 0.01	0.58 ± 0.02
10	Pineapple	100	0.91 ± 0.03	0.94 ± 0.01
11	Pineapple	100	1.02 ± 0.03	1.08 ± 0.02

[a] Mean of triplicate results.

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Analysis of fruit juice samples

The accuracy of the proposed method was determined by comparing results obtained from SIA-LOV with those obtained from a standard AOAC titration method. Comparative analyses of the same samples were carried out on the same day and the results are shown in Table 2.

From Table 2, the acidity found in fruit juice samples (expressed as citric acid concentration) using the SIA-LOV method agrees well with those found by the standard AOAC titration method. The correlation of the two methods are linear: $Y = 1.03X + 0.02$ with $R^2 = 0.9965$ where the Y axis represents the results from the LOV mono-segmented micro-titration method and the X axis represents the results from the AOAC standard titration method. The slope and intercept of the correlation graphs of the fruit juices are close to 1 and 0, respectively. This indicated that both methods correlate well.

The results imply that if the matrices of sample are known and standard solution can be prepared in matrices matching those in sample solution, the proposed method would be very useful for routine analysis. Alternatively, the standard addition technique could be applied to the analysis of samples with unknown matrices.

Conclusion

A new concept of mono-segmented flow micro-titration with SI-LOV was proposed and demonstrated by acid–base titration for determination of acidity of fruit juices. The analysis by this system helps to reduce amounts of sample, reagents and time consumption. Micro-volume of reagents used make it possible to be applied to rare samples. This system provides good reproducibility and it is convenient for in-line dilution of sample. The proposed system should be able to be adapted to study other acids or other substances involving reactions that produce or consume acid or base. It can be coupled to other types of detection.

Acknowledgements

We thank the Thailand Research Fund (TRF) and the Postgraduate Education and Research Program in Chemistry (PERCH) for support.

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Evaluation of on-line preconcentration and flow-injection amperometry for phosphate determination in fresh and marine waters

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Received 16 September 2004; received in revised form 4 December 2004; accepted 6 December 2004
Available online 11 February 2005

Abstract

Dissolved reactive phosphorus (DRP) was determined as orthophosphate (PO_4-P) in fresh and saline water samples by flow-injection (FI) amperometry, without and with in-valve column preconcentration. Detection is based on reduction of the product formed from the reaction of DRP with acidic molybdate at a glassy carbon working electrode (GCE) at 220 mV versus the Ag/AgCl reference electrode. A 0.1 M potassium chloride solution was used as both supporting electrolyte and eluent in the preconcentration system. For the FI configuration without preconcentration, a detection limit of 3.4 μg P l^{-1} and sample throughput of 70 samples h^{-1} were achieved. The relative standard deviations for 50 and 500 μg P l^{-1} orthophosphate standards were 5.2 and 5.9%, respectively. By incorporating an ion exchange preconcentration column, a detection limit of 0.18 μg P l^{-1} was obtained for a 2-min preconcentration time (R.S.D.s for 0.1 and 1 μg P l^{-1} standards were 22 and 1.0%, respectively). Potential interference from silicate, sulfide, organic phosphates and sodium chloride were investigated. Both the systems were applied to the analysis of certified reference materials and water samples.
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Keywords: Phosphate; Flow-injection amperometry; Preconcentration; Bio-Rad AG-X8; DRP; Schlieren effect

1. Introduction

Phosphorus is an essential plant and animal nutrient. However, due to human activities, inadvertent addition of phosphates to watersheds has caused eutrophication, which is commonly manifested by algal bloom formation [1]. Analytical methods for determination of phosphorus species, especially readily bioavailable dissolved reactive phosphorus (DRP) are essential for investigating the sources, cycling and fate of phosphorus (P) in aquatic ecosystems.

Spectrophotometric methods for determination of DRP are commonly based on the reduction of the phosphomolybdate complex (formed from reaction between reactive phosphate and acidic molybdate) to form the intensely coloured "molybdenum blue" product. Both batch [2] and flow-based [3,4] procedures have been reported. Although the spectrophotometric method provides good sensitivity, it suffers both from interferences, e.g. silicate, turbidity [3] and refractive index (Schlieren) effects in estuarine and marine samples, which can cause large errors in quantitation [5]. Electroanalytical methods are more tolerant of these interferences, and do not suffer from the Schlieren effect. Voltammetric or amperometric determination of phosphate has been performed using a range of different electrochemical reactions including, (1) reduction of 12-molybdophosphate to molybdenum blue [6–12], (2) oxidation of molybdenum blue, which was electrochemically prereduced and adsorbed on the electrode [13,14], (3) reduction of molybdovanadophosphate [15] and (4) oxidation of $FePO_4$ after reductive accumulation of Fe(II) in the presence of phosphate [16]. Flow-injection has also been applied for electrochemical determination of

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doi:10.1016/j.talanta.2004.12.064

phosphate in order to improve performance of the technique. Some electrochemical methods involving the use of enzymes for determination of DRP have also been reported [17,18]. An FI amperometric system incorporating inorganic pyrophosphatase and nucleoside phosphorylase–xanthine oxidase reactors was used for simultaneous determination of phosphate and pyrophosphate [17]. An FI amperometric system with a nucleoside phosphorylase–xanthine oxidase column was also used for determination of phosphate with a detection limit of 1.25 μM [18]. The analytical characteristics of these electroanalytical techniques are summarized in Table 1.

The three major objectives of this work, based on the reduction of 12-molybdophosphate on a glassy carbon working electrode (GCE), were to evaluate amperometric detection of phosphate in fresh and marine waters, investigate the use of an in-valve preconcentration column and develop a simple manifold suitable for application in field instruments.

2. Experimental

2.1. Instrumentation

The FIA manifolds used in this study are illustrated in Fig. 1. A laboratory-made FIA workstation, consisting of two peristaltic pumps (Ismatec CA5E, Switzerland), an

Table 1
Summary of electroanalytical techniques and their analytical characteristics for determination of phosphate

Technique	Electrochemical reactions	Working electrode	Linear range ($\mu g\,P\,l^{-1}$)	Detection limit ($\mu g\,P\,l^{-1}$)	Reference
Batch injection analysis—amperometry	Reduction of molybdophosphate complex	Carbon paste	30–600	10	[6]
Batch/FI—differential pulse voltammetry (DPV)	Reduction of 12-molybdophosphate in acetone–water mixture	–	–	–	[7]
Voltammetry/amperometry	Reduction of molybdophosphate complexes	Gold microdisk	30–30000	–	[8]
Voltammetry	Reduction of 12-molybdophosphate in a capillary fill device	Screen-printed carbon	150–60000	150	[9]
FI-voltammetry	Reduction of 12-molybdophosphate in a reverse FI system	Glassy carbon	150–15000	–	[10]
Batch/FI—DPV	Reduction of 12-molybdophosphate	–	–	–	[11]
FI-voltammetry	Reduction of 12-molybdophosphate	Glassy carbon	30–15000	–	[12]
Adsorptive stripping voltammetry (AdSV)	Reoxidised of the preadsorbed hetoropoly blue	Glassy carbon	0.3–30	0.3	[14]
Voltammetry (DPASV)	ASV of β-heteropolymolybdates stabilized by acetone	Glassy carbon	–	–	[13]
Cyclic voltammetry	Reduction of 12-molybdophosphate	Carbon paste	400–25000	40	[22]
SIA-amperometry	Detection of molybdophosphate	Glassy carbon	100–3000	100	[23]
Voltammetry	Reduction of molybdovanadophosphate	Hanging Hg drop	0.06–300 mg P l^{-1}	0.15	[24]
FI-voltammetry	Reduction of molybdovanadophosphate	Glassy carbon	–	30	[15]
Cathodic stripping voltammetry (CSV)	Reduction of Fe(III) phosphate	Glassy carbon	–	100	[16]
FI-amperometry	Reduction of H_2O_2 produced from enzyme reaction (pyrophosphatase, nucleoside phosphorylase, xanthine oxidase)	–	–	–	[17]
FI-amperometry	Reduction of H_2O_2 produced from enzyme reaction (nucleoside phosphorylase, xanthine oxidase)	Platinum	–	40	[18]
FI-amperometry	Reduction of 12-molybdophosphate	Platinum	– 1550	0.6	[25]
FI-amperometry	Reduction of 12-molybdophosphate	Glassy carbon	50–1000	3.4	This work
FI-amperometry, with preconcentration	Reduction of 12-molybdophosphate	Glassy carbon	0.1–10	0.18	This work

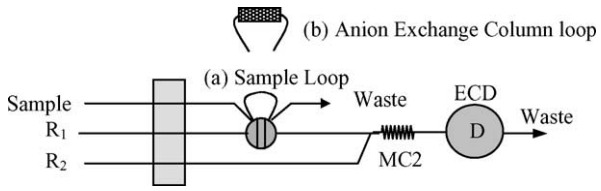

Fig. 1. FIA manifolds for the direct determination of orthophosphate: 60 cm mixing coil (MC2), $R_1 = 0.1\,\mathrm{mol\,l^{-1}}$ potassium chloride, $R_2 = 0.5\%$ ammonium molybdate in 2.5% (v/v) sulfuric acid, (a) 50 μl sample loop; (b) in-valve anion exchange column for preconcentration.

injection valve (Rheodyne 5041, USA) actuated by pressure from a compressed air-gas cylinder, and an electrochemical detector (Princeton Applied Research model 400, USA) was employed. The amperometric signal (μA) was converted to voltage (mV) within the detector, where 1 V full scale corresponds to 100 μA. PTFE tubing (0.5 mm i.d.) was used for mixing coils, and all manifold lines. The FCS computer program (A-Chem Technologies, Australia) was used to control the system and collect the data from the detector. The thin-layer flow-through cell (5 mm × 15.6 mm × 0.09 mm) consisted of a GCE (3 mm diameter, Princeton Applied Research, MP1305), a Ag/AgCl reference electrode and stainless steel cell body auxiliary electrode. The GCE was polished daily and rinsed with deionized water.

For the manifold in Fig. 1, standard/sample was injected into a stream of potassium chloride before merging with the acidic molybdate reagent. This manifold was also modified by replacing the sample loop with an ion exchange minicolumn to preconcentrate the analyte before injecting into the system. The potassium chloride served both as an electrolyte to promote electrical conduction and decrease the difference in electrical conductivity between sample and acidic molybdate and as the eluant to elute the phosphate from the ion-exchange column. The volume of standard/sample loaded onto the column was defined by the loading flow-rate and time.

2.2. Reagents

All solutions were prepared from analytical grade reagents and high purity, deionized water (Continental). The 500 ml of acidic molybdate solution (R_2 in Fig. 1) was prepared from 2.5 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (BDH) and 12.5 ml of concentrated H_2SO_4 (BDH). A 0.1 M potassium chloride solution was prepared from oven-dried KCl (BDH). Standard phosphate solutions in the concentration range of 10–1000 $\mu\mathrm{g\,P\,l^{-1}}$ were prepared daily from an intermediate solution (5 $\mathrm{mg\,P\,l^{-1}}$), diluted from a 100 $\mathrm{mg\,P\,l^{-1}}$ stock solution (0.2197 g oven-dried KH_2PO_4 (BDH) in 500 ml water). A 200 $\mathrm{mg\,l^{-1}}$ sulfide stock solution was prepared from $Na_2S{\cdot}9H_2O$ (Sigma–Aldrich).

The anion-exchange mini-column was prepared by placing Bio-Rad AG1-X8 resin (Bio-Rad, USA, 200-400 mesh, chloride form) in a perspex column (5 mm × 1.5 mm i.d.), and plugging both ends with nylon mesh. The following certified reference materials were purchased from the Queensland Health Scientific Services, Australia, natural waters NLLNCT-Round 7: bottle 1, bottle 3, bottle 5 and bottle 7.

2.3. FI procedure

Potassium chloride and molybdate flow-rates in the FI system shown in Fig. 1 were 2.0 and 0.9 $\mathrm{ml\,min^{-1}}$, respectively. The potential applied to the GCE was fixed at 220 mV versus the Ag/AgCl reference electrode. The sensitivity range of the detector was 0.2 and 0.1 μA for the preconcentration system. Orthophosphate standard solutions (10–1000 $\mu\mathrm{g\,P\,l^{-1}}$) were measured in triplicate. Potential interferences including sodium chloride, sulfide, silicate and some organic phosphates were studied. The developed methods were validated using four certified reference materials. Eight water samples, which were collected from the water overlying the sediment in laboratory bioreactors were also analysed.

3. Results and discussion

3.1. Manifold without preconcentration

DRP concentrations, measured as orthophosphate (PO_4-P), were determined using the manifold depicted in Fig. 1a). The detection limit of this FI system, calculated using the criterion of 3× the standard deviation of the blank signal [19] was 3.4 $\mu\mathrm{g\,P\,l^{-1}}$. Linear calibration was obtained over the concentration range 50–1000 $\mu\mathrm{g\,P\,l^{-1}}$; the equation was: $y = 0.7834x - 2.4996$, $R^2 = 0.9995$. There was no curvature evident in the calibration curve, suggesting that even higher orthophosphate concentrations could be determined, if wastewater analysis was required. Attempts to simplify the manifold by using a single manifold line with injection of the sample into the acidic molybdate reagent resulted in a high negative blank peak due to the electrical conductivity difference between the reagent and sample streams. The detection limit in this case was 22 $\mu\mathrm{g\,P\,l^{-1}}$.

3.1.1. Signal reproducibility

The stability of the FI system was examined by measuring 50 replicate injections of 50 and 500 $\mu\mathrm{g\,P\,l^{-1}}$ phosphate standards. Fig. 2 shows that at a lower concentration of phosphate (50 $\mu\mathrm{g\,P\,l^{-1}}$), the peak-height response was very reproducible. The mean and R.S.D. of peak heights were 21.3 mV and 1.3%, respectively. More variability was observed with the higher phosphate concentration, where the mean and R.S.D. of peak heights were 182.6 mV and 9.5%, respectively. The poor reproducibility of these results at higher phosphate concentrations suggests that the reduced molybdenum blue complex may have precipitated in the flow cell.

3.1.2. Interference study

Several potentially interfering species have been studied. Quintana et al. [6], as well as many others, have reported

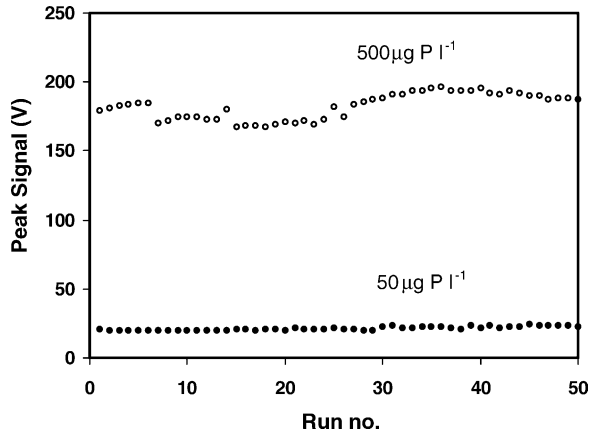

Fig. 2. Reproducibility of orthophosphate standard peak heights (50 and 500 $\mu g\,P\,l^{-1}$). Fifty replicate injections using the FI system 1a.

that silicate can cause major problems with phosphate determinations [2,6,15]. It was found here with silicate solutions of concentrations lower than 50 $mg\,l^{-1}$, no peak signal was obtained. Given that silicate is usually less than 20 $mg\,l^{-1}$ in natural waters, silicate presents no significant interference for the determination of phosphate by this method.

Sulfide is another interferent in spectrophotometric phosphate determination [20]. Sulfide can be reduced at 220 mV (versus Ag/AgCl) at the working electrode and gives a negative peak. It was found that 5 $mg\,l^{-1}$ of sulfide affected the peak-height responses of phosphate (50–100 $\mu g\,P\,l^{-1}$), but no interference was detected for 2 $mg\,l^{-1}$ sulfide.

Potential interference by five organic phosphate species: adenosine-5-monophosphate, 2-aminoethyl phosphonic acid, glycerophosphate, phenyl phosphate and phytic acid, was also investigated. It was found that 100 $\mu g\,P\,l^{-1}$ of each organic phosphate did not produce FIA peaks using the same conditions and manifolds investigated here for the determination of DRP. In natural waters, total organic phosphate concentrations do not normally exceed 50 $\mu g\,P\,l^{-1}$, although this may not be the case in sediment pore waters.

It was found that sodium chloride concentrations up to 4% (40 $mg\,l^{-1}$) did not affect peak height responses of phosphate over the range 50–1000 $\mu g\,P\,l^{-1}$. This method is therefore directly applicable to sea water samples without the need for salinity compensation. This represents a major advantage of this approach compared with the spectrophotometric FI determination of phosphate where adjustments are required to overcome the Schlieren (RI) effect [5].

3.1.3. Method validation and applications

Four reference materials: fresh waters, NLLNCT-Round 7: bottle 1 and bottle 3, and sea waters, NLLNCT-Round 7: bottle 5 and bottle 7, were analysed. The results shown in Table 2, indicate good agreement between expected and measured P concentrations, in both fresh and marine waters.

The method was also applied to eight saline water samples collected from the Gippsland lakes in eastern Victoria. The results, shown in Table 3, agreed well with the FI-spectrophotometric method (paired *t*-test, $t_7 = -0.10$, $p = 0.923$).

Table 2
Analysis of the certified reference materials (CRM)

Water type	CRM NLLNCT-round 7	Certified value ($\mu g\,P\,l^{-1}$)	Found[a] ($\mu g\,P\,l^{-1}$)
Fresh water	Bottle 1	27.0 ± 0.8	29.4 ± 1.4
	Bottle 3	97.9 ± 1.1	86.8 ± 1.1
Sea water	Bottle 5	27.7 ± 1.2	23.1 ± 0.3
	Bottle 7	11.8 ± 0.9	13.6 ± 1.7

[a] Mean of triplicate injections.

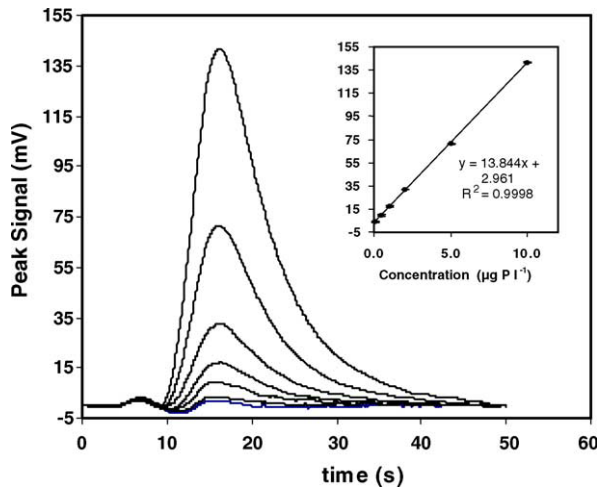

Fig. 3. FI graph and calibration graph of standard orthophosphate concentrations of 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 $\mu g\,P\,l^{-1}$: using the FI system 1b and preconcentration time = 2 min, detector range = 0.1 µA.

3.2. The FI system with preconcentration

The anion exchange minicolumn replaced the sample loop in the manifold shown in Fig. 1(b) and was used for preconcentration and subsequent determination of orthophosphate concentrations below 10 $\mu g\,P\,l^{-1}$. The calibration graph (Fig. 3) was linear between 0.1 and 10 $\mu g\,P\,l^{-1}$. Using a 2 min preconcentration time, at a loading flow-rate of 0.9 $ml\,min^{-1}$, the detection limit was 0.18 $\mu g\,P\,l^{-1}$. An

Table 3
Analysis of phosphate concentrations in sea water samples

Sample	Found ($\mu g\,P\,l^{-1}$)	
	Electroanalytical procedure	Conventional spectrophotometric FIA
BB11	17	39
BB12	146	138
BB13	319	337
BB14	408	389
BB15	393	380
BB16	417	419
BB17	417	418
BB18	435	436

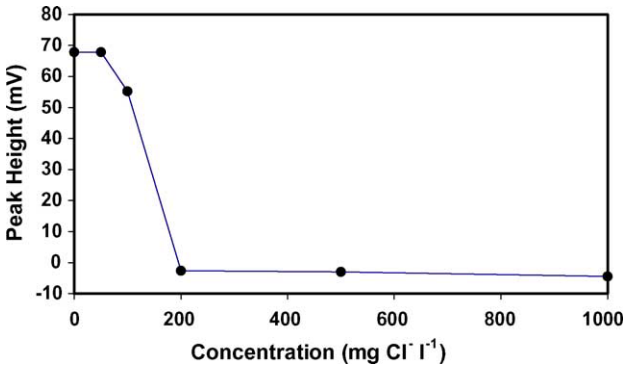

Fig. 4. The effect of chloride on peak signal response of 5 μg P l^{-1}. Preconcentration time: 2 min.

enrichment factor of 18, determined as the ratio of sensitivities with and without the preconcentration, was achieved.

Potential competition for ion-exchange sites by chloride ions present in the sample was investigated by analysing a series of 5 μg P l^{-1} standards containing chloride at a range of concentrations (0–1000 mg l^{-1}). Results indicate that chloride ion concentrations higher than 50 mg l^{-1} reduce the peak height response of 5 μg P l^{-1} (Fig. 4). At 200 mg l^{-1} of Cl^-, the phosphorus peak height response was indistinguishable from the blank signal. Similar interferences might be expected with other anions, especially divalently charged species such as sulfate with higher affinity for ion exchange sites on the column.

The preconcentration method was then applied to tap water samples. Recoveries for spiked orthophosphate standards were in the range of 92–126% (Table 4).

The viability of a single standard calibration using the preconcentration system was investigated using 5 and 10 μg P l^{-1} phosphate standards. By varying the preconcentration time (or loading time) for a sample, a linear relationship between μg P and peak height was obtained. The μg P was calculated by flow-rate (ml min^{-1}) × loading time (min) × standard solution concentration (μg P l^{-1}) × 10^{-3}. The data points using the 5 μg P l^{-1} solution coincided with the 10 μg P l^{-1} data (equation $y = 7345x + 7.47$, $R^2 = 0.996$), hence demonstrating the validity of the single standard calibration method.

Table 4
Spike recovery of phosphate by adding phosphate to tap water samples, using the preconcentration manifold (FI system 1b)

Sample	Concentration (μg P l^{-1})		% Recovery[a]
	Added	Found	
A	–	0.74[a]	–
	3.00[b]	3.50[c]	92
B	–	1.05[a]	–
	5.00[b]	6.83[c]	116
C	–	0.66[a]	–
	1.00[b]	1.92[c]	125
D	–	0.50[a]	–
	0.50[b]	1.09[c]	118

These data were calculated from the calibration equation: $y = 12.989x - 0.4932$, $R^2 = 0.999$.
[a] %Recovery = $[(c - a)/b] \times 100$.

4. Conclusion

An FI method using electrochemical detection has been developed for sensitive and rapid determination of orthophosphate. The efficacy of this method is demonstrated by a linear range (50–1000 μg P l^{-1}), covering orthophosphate concentrations commonly found in natural waters, with a detection limit of 3.4 μg P l^{-1} and sample throughput of 70 samples h^{-1}. The method is applicable to both fresh and saline samples and totally avoids the refractive index problems typically found with spectrophotometric methods. Analysis of oligotrophic freshwater samples is facilitated using the preconcentration system, where a detection-limit of 0.18 μg P l^{-1} was achieved using a 2 min preconcentration time. This method was only applicable to water samples with low chloride concentrations (<50 mg l^{-1}). It was demonstrated that a convenient, single standard calibration could be used with this preconcentration system. The analytical performance of these methods compares extremely favorably with the other studies listed in Table 1. The successful application of this simple, two line manifold to DRP determination in fresh and marine waters indicates great potential for further development into an instrument capable of field use, where artifacts engendered by sample preservation and storage can be avoided [21].

Acknowledgements

The authors acknowledge the support of the Commission on Higher Education (CHE), Chiang Mai University (through the Postgraduate Education Development scheme), Naresuan University and the Postgraduate Education and Research Program in Chemistry (PERCH) for the scholarship to YU and of the Water Studies Centre, Monash University and the Thailand Research Fund (TRF).

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Determination of linear alkylbenzene sulfonates in water samples by liquid chromatography–UV detection and confirmation by liquid chromatography–mass spectrometry

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Received 4 September 2004; received in revised form 7 March 2005; accepted 7 March 2005
Available online 19 April 2005

Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the separation and determination of individual (C_{10}–C_{13}) linear alkylbenzene sulfonates (LAS). New sets of conditions have been established for routine analysis of individual chemical forms of four LAS surfactants, i.e. C_{10}–C_{13} LAS. Under a condition set using a mobile phase containing 1.5 mM ammonium acetate in methanol/water 80:20 (v/v) mixture, detection limits obtained were in the range 1.5 ppb (for C_{10} LAS) to 11.5 ppb (for C_{13} LAS). This offers the advantages of significant improvement in resolution, short separation time and using less amount of common salt under isocratic condition. In addition, the use of simple mobile phase containing a simple low amount of salt cannot deposit at the entrance of mass spectrometric detector. The method is applicable to the simultaneous determination of LAS surfactants in various water samples. LAS surfactants presented in these samples were also successfully confirmed by using electrospray mass spectrometry.
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Keywords: Anionic surfactant; Linear alkylbenzene sulfonates; HPLC; Mass spectrometry; Water

1. Introduction

Linear alkylbenzene sulfonates (LAS), synthetic anionic surfactants, have been used in household laundry and dish-washing detergents [1]. The commercial product is a mixture of homologues containing carbon atoms between 10 and 13 atoms. Each of these homologues consists of positional isomers resulting from the attachment of the phenyl ring to the carbon atoms of the linear alkyl chain (Fig. 1) [2,3]. They are rapidly biodegraded under aerobic conditions. LAS-containing detergents are used in large quantities, and are therefore, released in the environment. LAS homologues with alkyl chain lengths from C_{10} to C_{13} have been found in municipal wastewaters and sediments at the ppm levels [4–6]. It has been reported that LAS and their degradation products can affect membrane permeability, enzyme and lysosomal activity [7,8]. The toxicity of the LAS containing 13 carbon atoms to the microalgae, namely *Chaetoceros gracitis* was found to be greater than that of the C_{11} LAS [9]. For these reasons, the identification and quantification of individual LAS species are invaluable for estimating the environmental impact and potential health effects of LAS species.

The standard methylene blue method has long been used for determining total amounts of sulfonate- and sulfate-based anionic surfactants in wastewater. Although the method cannot differentiate individual anionic surfactant, it is normally expressed as methylene blue-active substances (MBAS) concentrations down to 25 ppb [10]. It is time consuming and is often interfered by sample matrix, i.e. organic sulfonates, sulfates, carboxylates and phenol. This method also requires a large quantity of the toxic solvent for extraction, such as chloroform.

A number of methods have been developed for identifying and quantifying individual chemical forms of anionic surfactants. Chromatographic techniques like gas chromatography

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doi:10.1016/j.talanta.2005.03.011

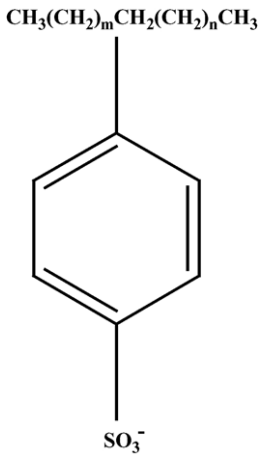

Fig. 1. General chemical structure of LAS.

(GC) [4,11–15], electrophoresis [1] and high-performance liquid chromatography (HPLC) [16–18] are efficient separation methods for the analysis of LAS mixture. Due to their low volatility and anionic form, derivatization of these compounds is necessary, when GC-based method is used [19].

HPLC is currently a suitable method for the determination of LAS. Reversed-phase HPLC provides a good separation of LAS mixture when using various chromatographic detectors, e.g. ultraviolet (UV) [16–18], fluorescence [2,20,21] and mass spectrometry (MS) [22–24]. However, most existing methods and procedures are still far from being considered suitable for the routine determination of individual chemical forms of LAS. Most HPLC methods with UV detector require the mobile phase containing either sodium perchlorate [22] or additive mixture, such as triethylamine and acetic acid [23,24], trifluoroacetic acid (TFA) and tetrabutylammonium dihydrogenphosphate (TBA–H_2PO_4) [25] or cetyltrimethylammonium ($CTMA^+$) ions [26] in order to resolve LAS homologues under gradient conditions. In practical application of the mobile phase containing high amount of those compounds, particularly sodium perchlorate ($10\,g\,l^{-1}$) can shorten the column life and can also clog the capillary, when mass spectrometric detector is used. In addition, the complicated mass spectra of LAS homologues would be obtained. This makes the identification of individual LAS in environmental samples so difficult.

As reported earlier by other workers, LAS compounds containing 10–13 carbon atoms are used in large amounts and are, therefore, released in the environment [1,4–6]. The toxicity of surfactant to aquatic organisms increases with increasing of carbon atoms [9].

The main purpose of this study was to develop HPLC method that would allow for routine analysis of LAS mixture, particularly in respect to reducing analysis time, improving separation efficiency for all the four LAS surfactants, precision and accuracy under isocratic condition. As mentioned earlier, several publications have been reported to use complicated mixtures as mobile phase under gradient conditions for separating some LAS surfactants. These approaches can also cause either capillary blockage or additional spectral interferences, when a mass spectrometric detector is used for confirmation results. Therefore, common salts, i.e. sodium chloride, sodium acetate and ammonium acetate added into mobile phase were chosen because of their suitability of identifying LAS in water samples using mass spectrometric detection.

2. Experimental

2.1. Chemicals

Linear alkylbenzene sulfonates in the forms of sodium salts were obtained from Henkel (Germany). HPLC-grade methanol was purchased from BDH (Poole, England). Sodium chloride, sodium acetate and ammonium acetate were analytical grade and purchased from Carlo Erba (Barcelona, Spain). Milli-Q water was used in this study.

2.2. Instrumentation

A HP 1100 high-performance liquid chromatograph (Agilent Corp., Wilmington, USA) consisting of an Agilent 1100 quaternary pump and an Agilent 1100 UV detector (224 nm) was employed. An inlet frit with 2 μm pore size was placed between the injector and HPLC column. A Zorbax Eclipse XDB C_8 column (Agilent Corp., USA), 15 cm × 4.6 mm i.d., containing 5 μm diameter packing material was used.

Samples were injected onto this column via an injection valve filled with 20 μl loop. The mobile-phase system was the mixture of methanol/water containing various amounts of sodium chloride, sodium acetate or ammonium acetate at flow rate $1.0\,ml\,min^{-1}$. All chromatographic elutions were isocratic and carried out at room temperature.

A HP 1100 series mass-selective detector single quadrupole instrument equipped with the orthogonal spray–ESI (Agilent, USA) interface was used for these investigations. The fragmentor voltage, nebulizer pressure, drying gas flow rate, drying gas temperature and capillary voltage were set to 150 V, 20 psi, $10\,l\,min^{-1}$, 350 °C and 3500 V, respectively.

2.3. Sample preparation

Prior to HPLC analysis, water samples were subjected to purification and preconcentration on the Sep-Pak C_{18} cartridge (Waters, USA). The cartridge was preconditioned with 7 ml methanol, followed by 7 ml of deionised water and then the sample was passed through the cartridge. The cartridge was washed with 6 ml of the mixture of MeOH–H_2O (30:70, v/v) and was eluted with 3 ml of methanol.

3. Results and discussion

The optimization of LAS separation using reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection at 224 nm was achieved, employing Eclipse XDB C_8 column with 15 cm × 4.6 mm dimension and 5 μm diameter packing material. Optimum separation of LAS homologues containing 10–13 carbon atoms was obtained by appropriately adjusting the composition of mobile phase, type and the concentration of salt. The emphasis was placed on the use of common salts (sodium chloride, sodium acetate and ammonium acetate), instead of using sodium perchlorate, in order to avoid capillary blockage and high background signal when using mass spectrometric detection.

3.1. Effect of mobile-phase composition

Mobile-phase compositions in the range of 70–80% (v/v) methanol in water were investigated. Separation of a mixture of C_{10} LAS, C_{11} LAS, C_{12} LAS and C_{13} LAS was carried out as depicted in Fig. 2. LAS compounds were separated using 70% methanol in water as mobile phase. However, most peaks obtained were broad, particularly C_{13} LAS. It was also observed that the LAS compounds containing 10 and 11 carbon atoms were not resolved completely when using 75% methanol. No separation was observed when using the amounts of methanol exceeding 80%. It is evident from these chromatograms that the composition of mobile phase affects peak resolution and peak shape significantly. It was noticed that peak resolution deteriorated with increasing methanol content. This could be explained that surfactants

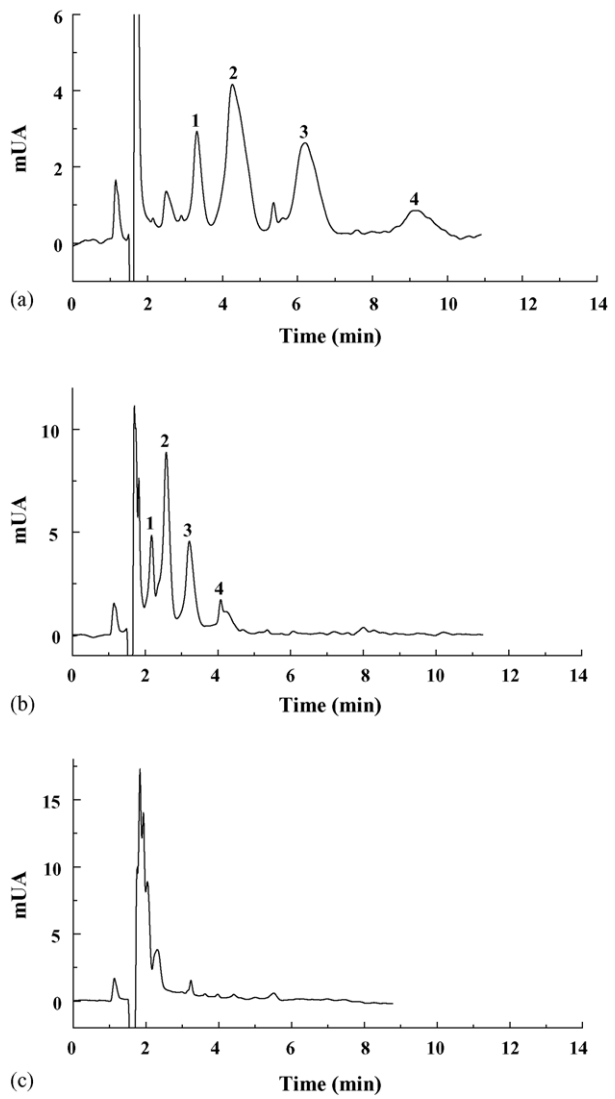

Fig. 2. Chromatograms of mixture of four LAS compounds obtained using various mobile-phase compositions of MeOH–H_2O: (a) 70:30; (b) 75:25; (c) 80:20. Peak identification: (1) C_{10} LAS; (2) C_{11} LAS; (3) C_{12} LAS; (4) C_{13} LAS.

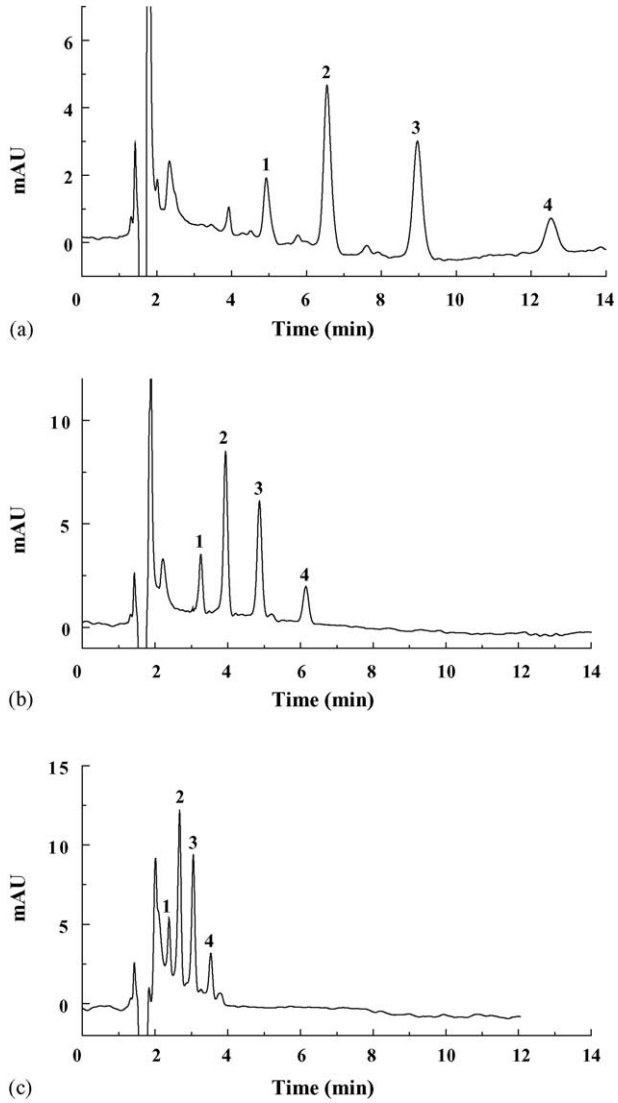

Fig. 3. Chromatograms of mixture of four LAS compounds obtained using various mobile-phase compositions in the presence of 3.5 mM NaCl and MeOH–H_2O: (a) 75:25; (b) 80:20; (c) 85:15. Peak identification: (1) C_{10} LAS; (2) C_{11} LAS; (3) C_{12} LAS; (4) C_{13} LAS.

are hydrophobic in nature. The hydrophobic characteristic of long-chain surfactants is suppressed by increasing methanol resulting in reducing retention time [27].

3.2. Effect of type and concentration of salt

Preliminary experiments were undertaken in an attempt to find suitable salt adding into mobile phase for improving LAS separation. Sodium chloride was common salt used for separations of LAS mixture as shown in Fig. 3. Four LAS compounds were successfully resolved within 6 min when using the 80:20 (v/v) mixture of methanol and water containing 3.5 mM NaCl. When using the 75:25 (v/v) mixture of methanol and water containing 3.5 mM NaCl the same four LAS compounds were separated in over 12 min. It was also observed that C_{10} LAS and C_{11} LAS were partially resolved when using the amounts of methanol exceeding 85%. The mixture of methanol/water (80:20, v/v) was, therefore, selected for further method development.

As demonstrated earlier, the selection of a suitable common salt is a critical factor in obtaining optimum resolution and short separation times. Three types of common salts, i.e. sodium chloride, sodium acetate and ammonium acetate were investigated at the concentrations ranging from 1 to 10 mM adding into the mixture of methanol/water (80:20, v/v) along with a mobile-phase flow rate 1.0 ml min^{-1}. It was observed that the resolution and their retention time increased with the concentration of salt (Figs. 4–6). The minimum concentrations of sodium chloride, sodium acetate and ammonium acetate that could be used to separate the four LAS compounds (resolution ≥ 1.5) in approximately 5 min under isocratic condition were 1, 2 and 1.5 mM, respectively. In comparing the data obtained in this study to the data reported elsewhere [28] indicated that the developed method provided a significantly improved resolution, peak shape, particularly C_{12} LAS, short analysis time and simple approach for confirmation results by mass spectrometric detector.

3.3. Linearity, accuracy and detection limit

Linearity, accuracy and detection limit for individual LAS compounds were studied (Table 1). The mobile phase

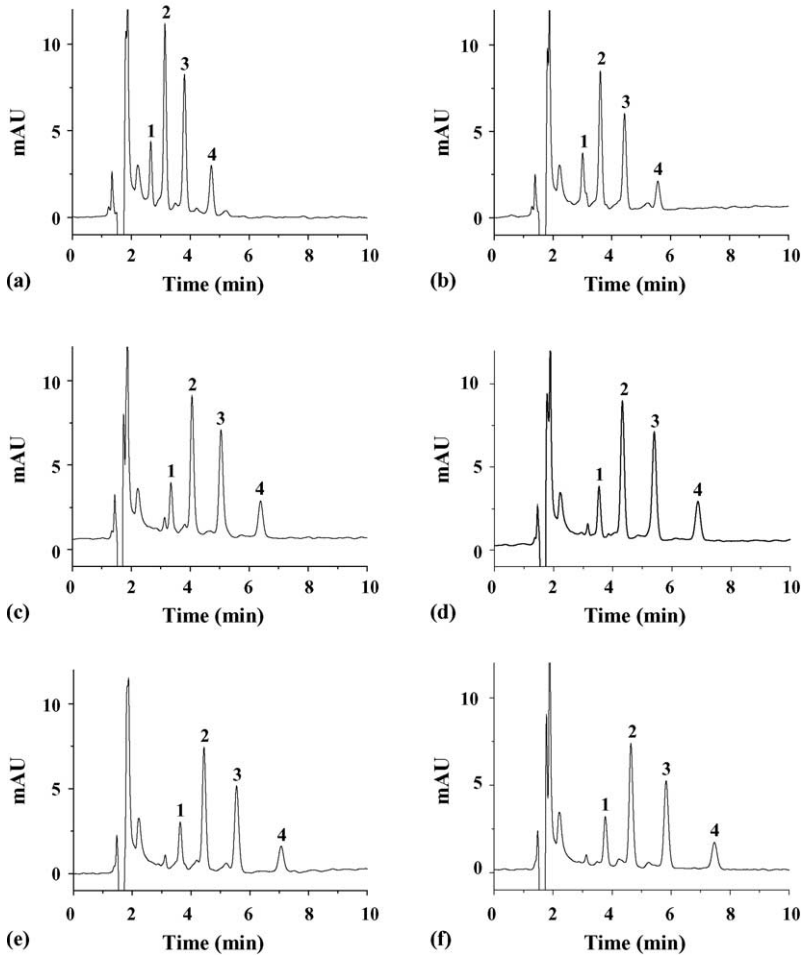

Fig. 4. Chromatograms of mixture of four LAS compounds obtained using 80% (v/v) methanol in water containing various concentrations of sodium chloride: (a) 1 mM; (b) 2 mM; (c) 4 mM; (d) 6 mM; (e) 8 mM; (f) 10 mM. Peak identification: (1) C_{10} LAS; (2) C_{11} LAS; (3) C_{12} LAS; (4) C_{13} LAS.

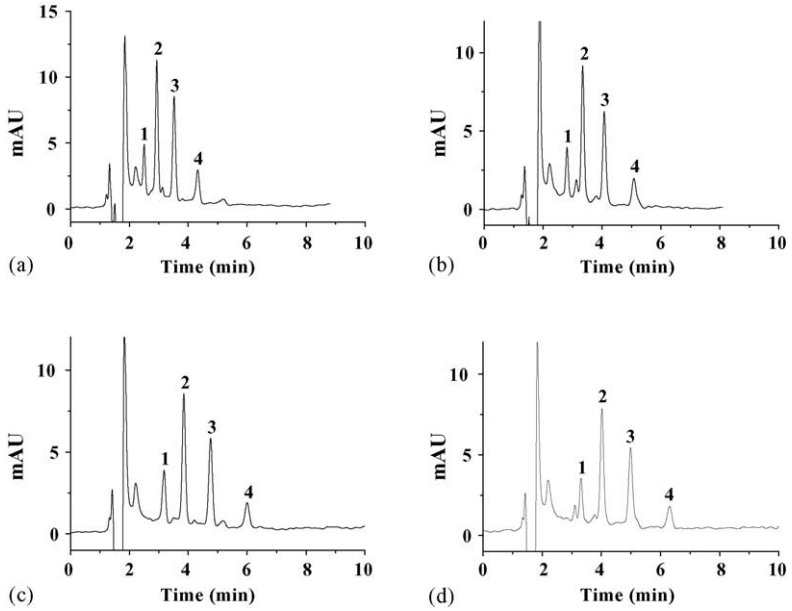

Fig. 5. Chromatograms of mixture of four LAS compounds obtained using 80% (v/v) methanol in water containing various concentrations of sodium acetate: (a) 2 mM; (b) 4 mM; (c) 8 mM; (d) 10 mM. Peak identification: (1) C_{10} LAS; (2) C_{11} LAS; (3) C_{12} LAS; (4) C_{13} LAS.

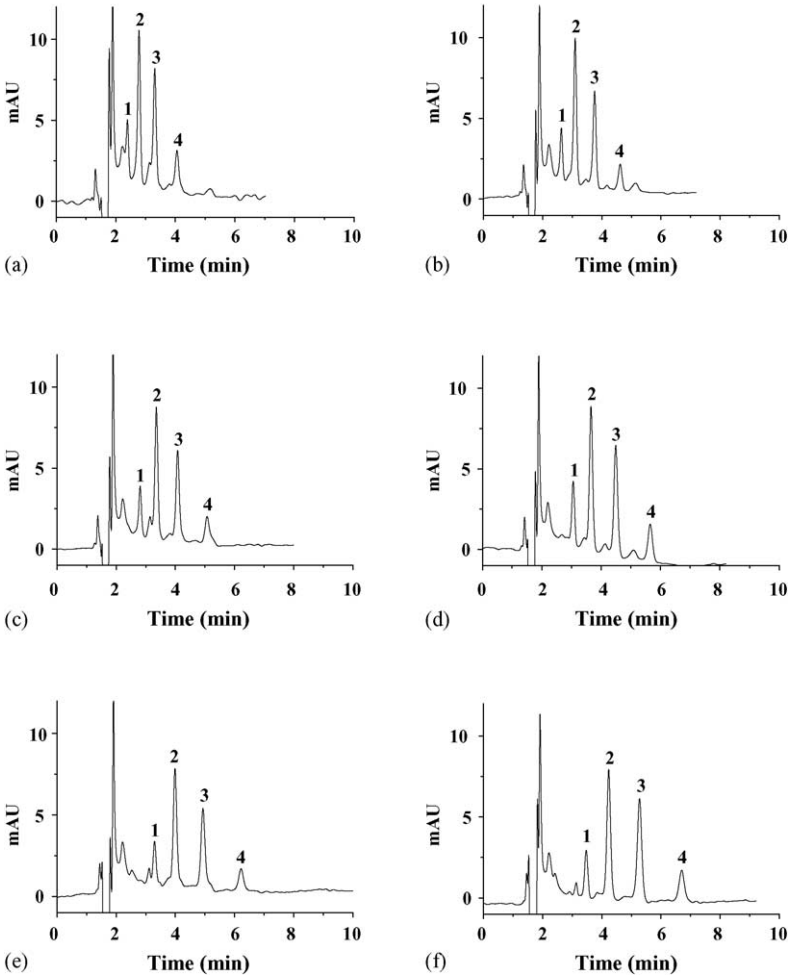

Fig. 6. Chromatograms of mixture of four LAS compounds obtained using 80% (v/v) methanol in water containing various concentrations of ammonium acetate: (a) 1 mM; (b) 1.5 mM; (c) 2 mM; (d) 4 mM; (e) 6 mM; (f) 8 mM. Peak identification: (1) C_{10} LAS; (2) C_{11} LAS; (3) C_{12} LAS; (4) C_{13} LAS.

Table 1
Analytical merits of the proposed method (linearity, accuracy and detection limit)

Compound	Concentration range (ppb)	R^2	Linearity cut-off (ppb) × 10^3	%Recovery	Detection limit[a] (ppb)
C_{10} LAS	6–310	0.9984	41	91–101	1.5
C_{11} LAS	20–1030	0.9997	137	92–99	8.0
C_{12} LAS	20–1070	0.9994	143	95–99	7.0
C_{13} LAS	20–590	0.9979	78	94–102	11.5

[a] Calculation based on three times the background standard deviation.

containing 80% methanol and 1.5 mM ammonium acetate in water were selected for this study due to the results obtained previously in compromising between resolution ($R_s \geq 1.5$) and analysis time (within 5 min). The accuracy expressed in terms of percentage recovery was done by spiking various amounts of LAS standard into the water samples collected from wastewater in Chiang Mai and Utraradit, Thailand. The percentage recoveries of this method for C_{10} LAS, C_{11} LAS, C_{12} LAS and C_{13} LAS were found to be between 91 and 101 ($n=3$), 92 and 99 ($n=3$), 95 and 99 ($n=3$) and 94 and 102 ($n=3$), respectively. Satisfactory recovery was obtained. The limit of detection for each LAS compound was calculated as three times the background standard deviation [29]. The C_{10} LAS had the lowest detection limit of 1.5 ppb. The C_{13} LAS, which is the last compound to elute under the conditions employed, had the highest detection limit value of 11.5 ppb. From the observation, the peak shape of C_{13} LAS is rather broader than that of C_{10} LAS.

3.4. Analysis of LAS surfactant in real water samples using HPLC–UV

Prior to real sample analysis, results obtained with the four LAS standards indicated that an increase of methanol from 75 to 80% is expected to cause C_{10} LAS and C_{11} LAS to elute close to some probable interferences (Fig. 6b). The mobile phase containing 1.5 mM ammonium acetate in methanol/water mixture of 78:22 (v/v), instead of the ratio of 80:20 (v/v) methanol/water, was used in order to avoid matrix effects arising from the water extracts. These effects cause approximately 2 min increase in the separation time of LAS compounds. As for water extracts, the chromatograms in Fig. 7 illustrate the separation of the four compounds for various water samples. Under the condition, the LAS compound concentrations in various water samples determined using a Zorbax Eclipse XDB C_8 column in combination with the methanol/water mixture containing ammonium acetate, are presented in Table 2.

3.5. Identification of LAS surfactants in water extract using LC–ES–MS

It is well known that the identification of LAS compounds using chromatographic techniques is based solely on retention-time matching. As a consequence, errors can result from using this approach, especially in the case of co-eluting compounds. Also of particular interest is the numerous unknown anionic surfactants that have been found in environmental samples when analysing them using HPLC–UV [17–19].

To overcome such problems, the negative-ion electrospray (ES)–mass spectrometry was used for confirmation of LAS compounds in water samples. The mass spectra of water extracts (Fig. 8) show the *m/z* 183 ion common to LAS compounds. In addition, the high intensity of the molecular ions observed at *m/z* 297, 311, 325 and 339 originating from the water extracts are similar to those originating from the LAS

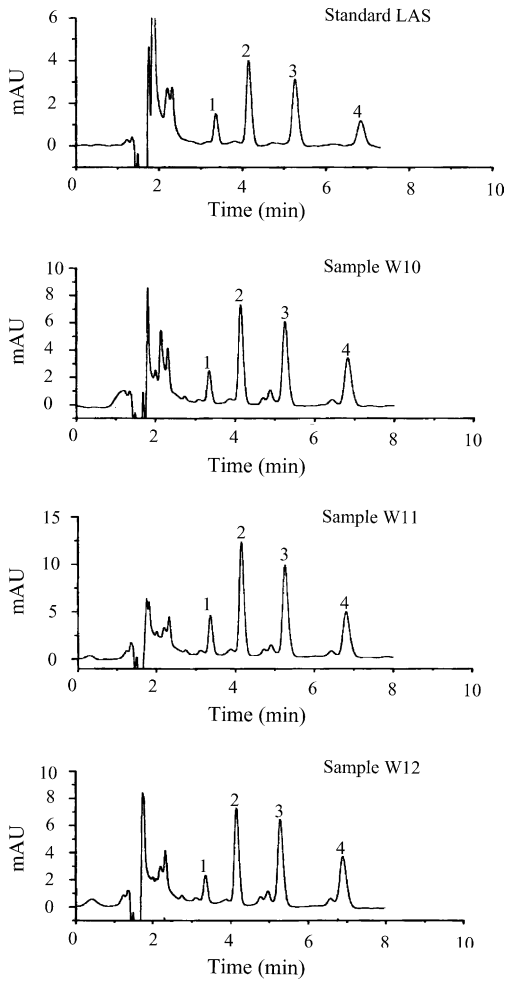

Fig. 7. Chromatograms of mixture of four LAS compounds in standard solution and water extracts obtained using 78% (v/v) methanol in water containing 1.5 mM ammonium acetate. Peak identification: (1) C_{10} LAS; (2) C_{11} LAS; (3) C_{12} LAS; (4) C_{13} LAS.

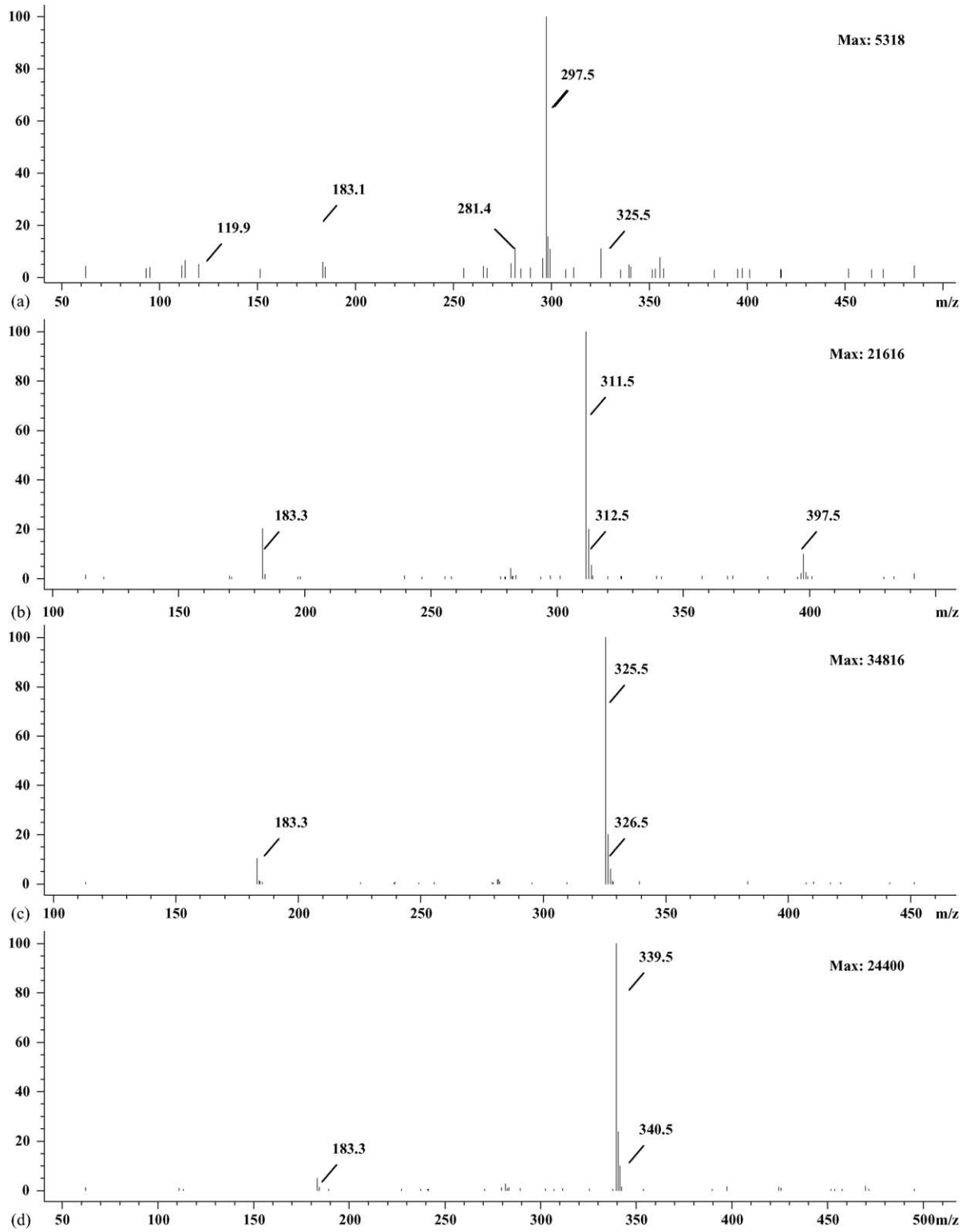

Fig. 8. (a–d) Negative-ion ESI mass spectra of LAS compounds originating from water extract (W10).

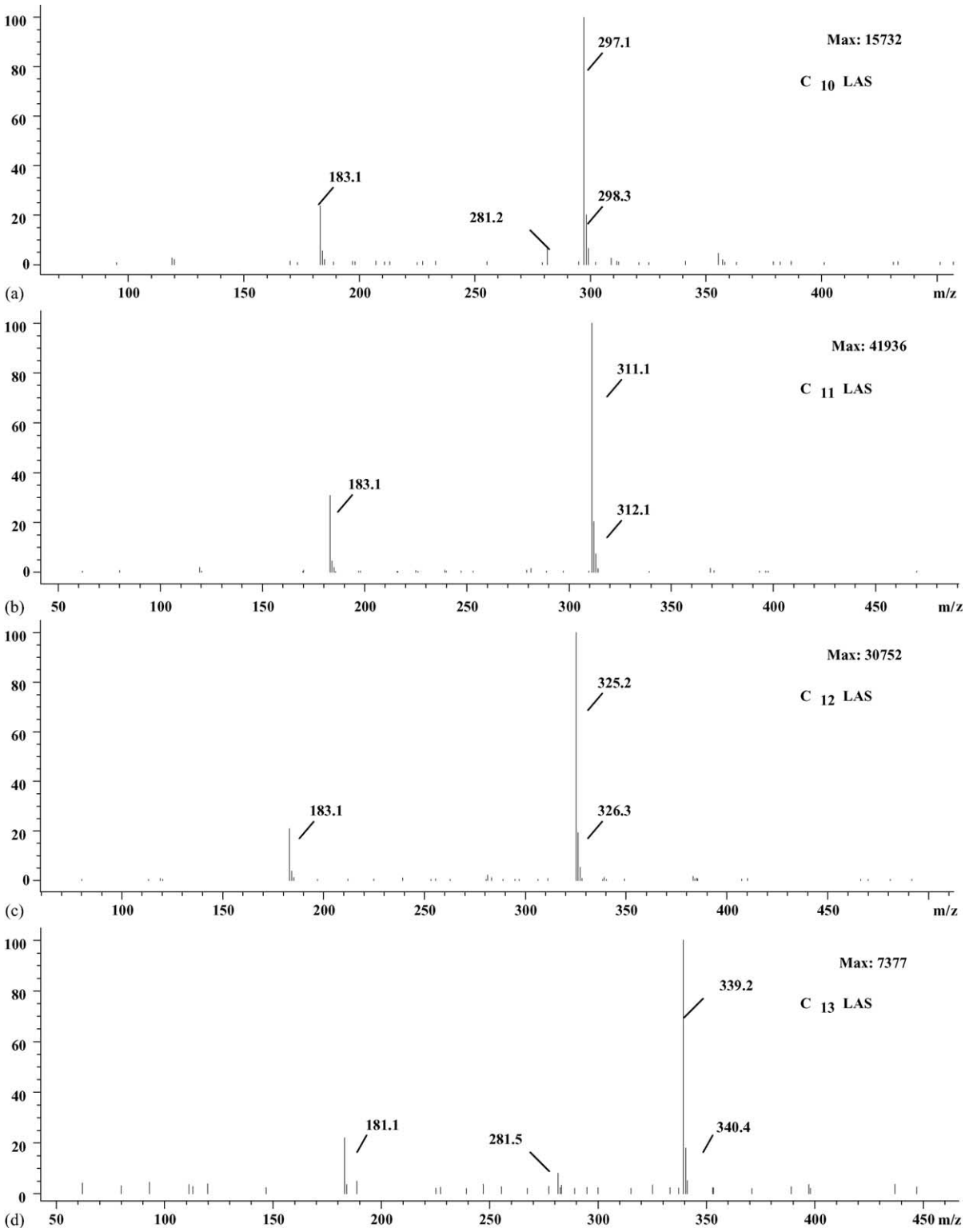

Fig. 9. (a–d) Negative-ion ESI mass spectra of the molecular ion originating from the mixture of LAS standard.

Table 2
Determination of LAS compounds in water samples (mean ± S.D.; $n = 3$)

Type of water	Concentration (ppb)			
	C_{10} LAS	C_{11} LAS	C_{12} LAS	C_{13} LAS
W1	115.3 ± 2.7	303.2 ± 7.9	184.4 ± 8.0	81.9 ± 2.2
W2	310.0 ± 0.3	1173.4 ± 4.6	1145.0 ± 12.9	424.4 ± 5.0
W3	5.0 ± 0.1[a]	23.4 ± 0.5	26.5 ± 1.1	21.1 ± 1.5
W4	n.d.	n.d.	13.0 ± 0.9	14.8 ± 0.5
W5	n.d.	n.d.	n.d.	n.d.
W6	3.4 ± 0.1[a]	12.8 ± 0.9	15.1 ± 1.3	16.7 ± 1.6
W7	54.1 ± 1.9	120.1 ± 5.5	69.1 ± 4.8	36.6 ± 5.2
W8	n.d.	n.d.	n.d.	n.d.
W9	n.d.	n.d.	n.d.	n.d.
W10	8.8 ± 0.3[a]	30.9 ± 0.7	33.5 ± 1.7	25.2 ± 1.7
W11	14.5 ± 0.8[a]	48.0 ± 2.4	46.9 ± 3.5	28.9 ± 2.8
W12	6.5 ± 0.4[a]	27.8 ± 1.9	30.3 ± 2.1	22.6 ± 1.3

n.d., not detected (less than the detection limit value); W1, drainage water from student dormitory, Chiang Mai University; W2, wastewater from Center of Medical Sciences, Ministry of Public Health; W3, wastewater from Khuy Hospital, Utraradit Province; W4, domestic wastewater released into Thorn canal, Utraradit Province; W5, natural water in Mae-Ping River, Chiang Mai Province; W7, wastewater in Mae-Kha canal, Chiang Mai Province; W8, natural water in Ang-Kaew reservoir, Chiang Mai University; W9, water in Chiang Mai Moat, Chiang Mai Province; W6 and W10–W13, natural water from irrigation canal, Chiang Mai Province.

[a] Preconcentration as described in Section 2.3.

standards (Fig. 9). These ions correspond to C_{10} LAS, C_{11} LAS, C_{12} LAS and C_{13} LAS, respectively.

4. Conclusion

The developed method offers superior performance characteristics, i.e. a simple method, significant improvement in resolution, short analysis time and using less amount of common salt (1.5 mM ammonium acetate) under isocratic condition. With this regard, it is easy to use this method with a mass spectrometric detector without any blockage of MS capillary. In addition, the use of low amounts of salt also increases the column's life and only requires very short re-equilibration time between each injection. Overall, these features demonstrate that the method is suitable to be used for routine analysis for both the identification and quantification of individuals of C_{10}–C_{13} LAS surfactants in various water samples.

Acknowledgements

The authors thank the Thailand Research Fund (TRF) for its support, the Development and Promotion for Science and Technology Talents Project of Thailand (DPST) for the scholarship to P.S. and the Postgraduate Education and Research Program in Chemistry Program (PERCH) of Thailand for the partial support.

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A novel stopped flow injection—amperometric procedure for the determination of chlorate☆

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Received 13 July 2005; received in revised form 20 September 2005; accepted 20 September 2005
Available online 17 October 2005

Abstract

A novel stopped flow injection—amperometric (sFI-Amp) procedure for determination of chlorate has been developed. The reaction of chlorate with excess potassium iodide and hydrochloric acid, forming iodine/triiodide that is further electrochemically reduced at a glassy carbon electrode at +200 mV versus Ag/AgCl electrode is employed. In order to increase sensitivity without using of too high acid concentration, promoting of the reaction by increasing reaction time and temperature can be carried out. This can be done without increase of dispersion of the product zone by stopping the flow while the injected zone is being in a mixing coil which is immersed in a water bath of $55 \pm 0.5\,^{\circ}C$. In a closed system of FIA, a side reaction of oxygen with iodide is also minimized. Under a set of conditions, linear calibration graphs were in the ranges of 1.2×10^{-6}–6.0×10^{-5} mol l^{-1}and 6.0×10^{-5}–6.0×10^{-4} mol l^{-1}. A sample throughput of 25 h^{-1} was accomplished. Relative standard deviation was 2% ($n = 21$, 1.2×10^{-4} mol l^{-1} chlorate). The proposed sFI-Amp procedure was successfully applied to the determination of chlorate in soil samples from longan plantation area.
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Keywords: Stopped flow injection; Chlorate; Amperometry

1. Introduction

One major source of chlorate discharge to the environment is pulp mill effluent where chlorine dioxide is used for bleaching [1]. Minor amounts of chlorate and chlorite are also produced when chlorine dioxide is used to disinfect supply water [2]. Potassium chlorate ($KClO_3$) is used as an oxidant in fireworks and matches [3]. Chlorate compounds have also been widely applied to soil in order to stimulate flowering of longan trees [4], especially at longan plantation in the north of Thailand [5]. Although chlorate is not very toxic to human, it has been shown to cause haemolytic anemia [6]. Chlorate is very toxic to marine brown algae, i.e. Macrocystis and Fucus, which are vital components of the coastal ecosystem [2].

Ion chromatography (IC) has been reported for simultaneous determination of chlorate and other species, such as, IC/mass spectrometry for bromate, chlorate, iodate, and chlorine dioxide determination [7], IC/spectrophotometry using post-column reaction with osmate-catalyzed for chlorate, chlorite, bromate, and nitrite determination by detection the triiodide at 288 nm [8]. A high capacity anion exchange column with suppressed conductivity detection was also used by the U.S. EPA in Method 300.1 for the determination of bromate, bromide, chlorite, and chlorate by direct injection [9]. However, this technique requires relatively expensive instrument and rather long analysis time, so it is not suitable for determination of only one single analyte, when only chlorate is needed.

The iodometric method employing the amplification reaction of chlorate with iodide ion and acidic medium ($ClO_3^- + 6I^- + 6H^+ \rightarrow 3I_2 + Cl^- + 3H_2O$) [10–12] has been commonly used for chlorate determination, but the reaction of chlorate ion with iodide ion is slow under mildly acidic condition. The rate law of the reaction, $-d[ClO_3^-]/dt = k[ClO_3^-][I^-]^{1.5}[H^+]^2$ [10] indicates that the reaction could be accelerated under a high acid concentration. However, under this condition a readily oxidation

☆ Presented at the 13th International Conference on Flow Injection Analysis (ICFIA), 24–29 April 2005, Las Vegas, NV, USA.

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doi:10.1016/j.talanta.2005.09.023

of iodide ion by oxygen in air would occur. In a potentiometric titration using sodium thiosulfate as titrant [13], 6 M hydrochloric acid was employed with hexane as oxygen shielding agent. Utilizing a FI system, which prevents direct exposure to air, 12 M hydrochloric acid can be used to accelerate the reaction. This leads to more sensitive methods based on spectrophotometric detection of triiodide at 370 nm [11,12,14]. A similar FI system has been applied to the determination of hypochlorite and chlorate [15]. The former species can react with iodide at room temperature while the latter requires an elevated temperature. A potentiometric flow injection procedure for the determination of oxychlorine species such as chlorate, chlorite and hypochlorite using a redox electrode detection and a Fe(III)–Fe(II) potential buffer solution containing chloride has also been reported [16]. A FI-amperometric method for the determination of chlorate and hypochlorite based on formation of chlorine by the reaction of the analytes with chloride in 8 mol l^{-1} sulfuric acid has also been described [17].

In this work, we developed a sFI-Amp procedure based on the reaction of chlorate with an excess iodide ion in an acidic condition to produce triiodide which is further electrochemically reduced at a glassy carbon electrode. In order to achieve high sensitivity without the use of too high acid concentration, a novel sFI procedure was introduced to promote the reaction by increasing a reaction time and a temperature. This can be done by stopping the flow while the injected zone was being in a mixing coil which was immersed in a water bath. In this way, dispersion of the product zone would be minimized. A simple homemade amperometric analyzer with a lab built flow through electrochemical cell can be used as a detector. A closed system of such a FI also minimizes any side reaction of oxygen with iodide. Moreover, the amperometric detection minimizes interferences from substances usually found in a soil sample which would interfere seriously in a spectrophotometric detection. Application of the proposed sFI-Amp procedure was demonstrated to the determination of chlorate in soil samples from longan plantation area.

2. Experimental

2.1. Apparatus

The sFI-Amp manifold for chlorate determination is illustrated in Fig. 1(a). It was a modified FIAstar system (5010 Analyzer, Tecator, Sweden), consisting of a peristaltic pump (P), a 6-port injection valve (I) and timing controlled unit. A

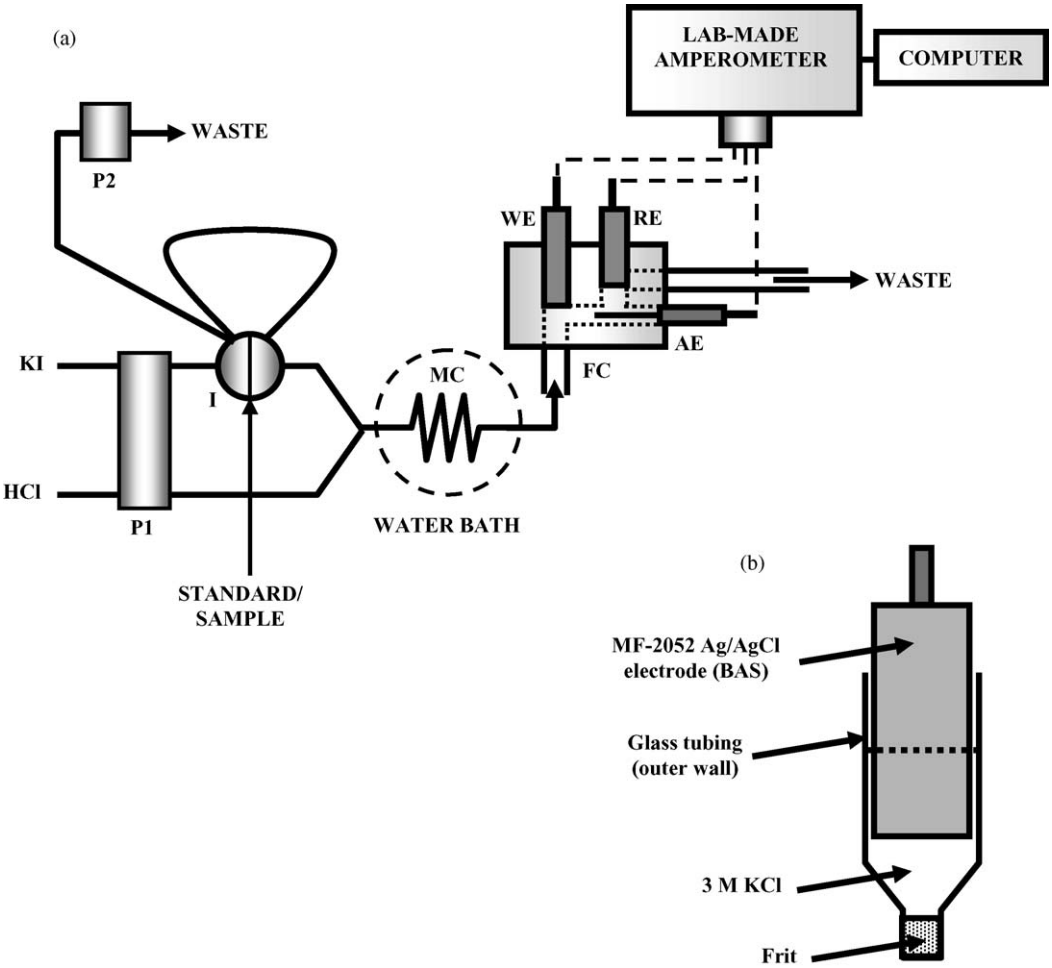

Fig. 1. (a) The sFI-Amp manifold used for determination of chlorate: P1 and P2: peristaltic pump 1 and 2; I: injection valve; MC: mixing coil; AE: auxiliary electrode; WE: working electrode; and RE: reference electrode. (b) A double junction design reference electrode.

lab-made amperometric analyzer with lab built flow through electrochemical cell (FC) was used as a detector. A personal computer with an in-house built software with Basic Stamp 2SX interface unit (Parallex, USA) was employed for recording data. Temperature was controlled by using a water bath (Gallenkamp, England).

A wall jet FC was assembled as shown in Fig. 1(a) (as side view). A Perspex plastic block was drilled to insert electrodes with *o*-ring sealing. Electrolyte carrier solution was entered the cell from the bottom and passed sequentially through a glassy carbon (MF-2012, 3.0 mm diameter, Bioanalytical System (BAS), USA) working electrode (WE), a platinum wire (MW-1032, BAS, USA) auxiliary electrode (AE) and an Ag/AgCl (MF-2052, BAS, USA) reference electrode (RE), respectively. A double junction design of RE (Fig. 1(b)) was used in order to prolong the lifetime of the MF-2052 RE.

All tubings for assembling the FI system were Teflon tube of 0.8 mm i.d., except pump tubes.

2.2. Reagents

Potassium iodide (AnalaR, BDH) and hydrochloric acid (Reagenti, CARLO ERBA) were used to prepare reagent solutions of concentration 0.07 mol l^{-1} and 7.0 mol l^{-1}, respectively. The potassium iodide solution was freshly prepared by dissolving 2.92 g KI in 250 ml water and kept in a dark bottle.

Potassium chlorate (99.5 %, w/w, AJAK), after standardization by standard iodometric procedures [18], was used to prepare stock standard solution of 12×10^{-3} mol l^{-1} chlorate. A stock solution was stored in a cool and dark place. Working standard solutions were daily prepared by appropriate dilution of the stock solution with water.

All solutions were prepared using Ultrapure water (Milli-Q, Millipore).

2.3. Collection and preparation of soil sample

Soil samples were collected from the longan plantation field in Chiang Mai, northern of Thailand. A soil sample was taken from 15 points at around the rim of the longan tree, at the depth of 15 cm. The sample was dried and ground before a portion of about 100 g was taken for further treatment.

A 25 g of dried-soil sample was extracted by shaking for 2 h with 50 ml of water. It was filtered with filter paper (Whatman, No. 42) and rinsed with water. The filtrate was made up to 100 ml in a volumetric flask with water.

2.4. sFI-Amp manifold and procedure

The sFI-Amp manifold (Fig. 1(a)) was designed to inject a standard or sample (100 μl) into a 0.07 mol l^{-1} potassium iodide stream before merging with 7.0 mol l^{-1} hydrochloric acid stream. This would prevent high concentration of hydrochloric acid entering/destroying the injection valve. The closed FI system also helps to reduce an oxidation of iodide ion by oxygen in air which is an extremely fast reaction in high concentration of hydrochloric acid ($4I^- + 4H^+ + O_2 \rightarrow 3I_2 + 2H_2O$) [10]. A period (called "travelling time") after injection was made until the pump was stopped was set to be 12 s. By doing this, the sample zone was being inside a mixing coil (MC), which was immersed into a water bath of 55 ± 0.5 °C. This temperature would promote the reaction of chlorate with iodide and acid to form triiodide. After a period of stopping (called "stopping time") of 90–120 s, the sample zone was flowed further through a FC and the reduction current of the triiodide was monitored by applying a constant potential of +200 mV at a glassy carbon working electrode versus Ag/AgCl reference electrode. The current was converted to potential by the amperometric analyzer and then recorded by the computer as a FI peak. A calibration graph was plotted between the peak height (mV) and chlorate concentration. Chlorate concentrations in sample was calculated using the calibration equation.

3. Results and discussion

3.1. Optimization of the FI-Amp system for the determination of chlorate

3.1.1. Amperometric instrument parameters

The sFI-Amp procedure for determination of chlorate is based on the conversion of chlorate to triiodide by reacting with an excess iodide and hydrochloric acid. A previous report on the detection of triiodide by amperometry based on the reduction of triiodide at a glassy carbon working electrode with an applied potential of +200 mV versus Ag/AgCl reference electrode was followed [19]. The home-made FC was designed to allow the solution passing to WE, AE and RE, respectively, in order to prevent the reaction product at AE to pass over the WE, so this kind of interference could be avoided.

3.1.2. Effect of concentration of potassium iodide

Concentrations of potassium iodide were studied. From the preliminary study, it was found that HCl concentration of at least 7 mol $l^{-1}$and a temperature of 45 °C should be used in order obtained a signal of chlorate in concentration range of 6.0×10^{-5}–6.0×10^{-4} mol l^{-1}. The concentrations of KI in range of 0.01–0.3 mol l^{-1} was investigated, while the following conditions were selected and kept constant: injection volume, 100 μl; MC length, 300 cm; flow rate of both of potassium iodide and hydrochloric acid streams, 2.9 ml min^{-1}; water bath temperature, 45 °C; travelling time, 12 s; and stopping time, 120 s. A series of chlorate standard solutions (6.0×10^{-5}–6.0×10^{-4} mol l^{-1}) was injected into the system. The obtained peak heights (mV) were plotted versus chlorate concentration (mol l^{-1}) yielding a calibration graph. Linear calibration graph was obtained for each concentration of KI used. A plot of the slopes of the calibration graphs versus KI concentrations is depicted in Fig. 2. It should be noted that at a higher concentration of KI, higher baseline signals were obtained which might be due to air oxidation of iodide or some iodate impurity presented in KI and this may lead to decrease of sensitivity (slope). Too low concentration of KI is not sufficient

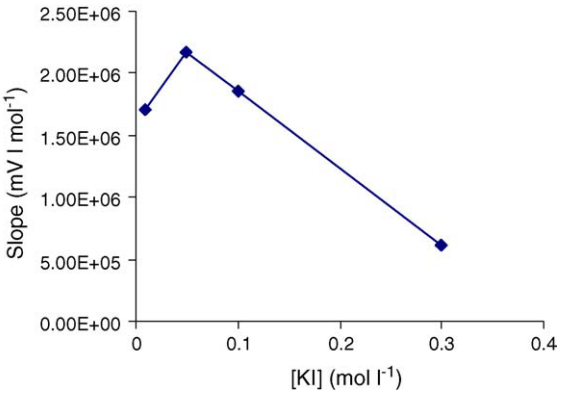

Fig. 2. Effect of concentration of potassium iodide (stopping time = 120 s, temperature = 45 °C, HCl concentration = 7 mol l^{-1}, other conditions see text).

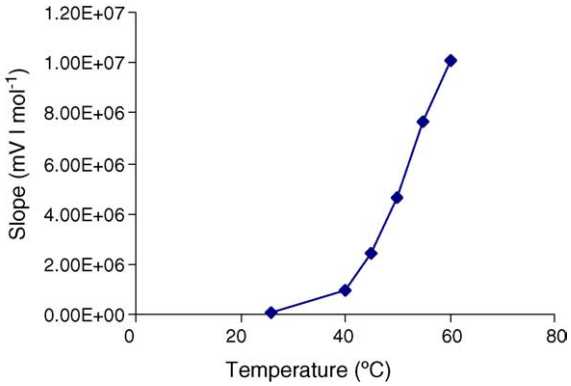

Fig. 4. Effect of temperature (stopping time = 120 s, HCl concentration = 7 M, KI concentration = 0.07 mol l^{-1}, other conditions see text).

in the reaction with the selected concentration range of chlorate and lead to a less sensitivity as well. KI of concentration 0.07 mol l^{-1} was chosen for further study.

3.1.3. Effect of stopping time and temperature

The stopping time in a range of 0–300 s was studied while the above selected condition, with 0.07 mol l^{-1} KI and temperature of 45 °C was used. The results in Fig. 3 show that the slope of calibration graph increased linearly with the increasing of the stopping time. The stopping time of 90–120 s was chosen in order to compromise between sensitivity and rapidity of the analysis.

With a 120 s stopping time, the effect of temperature was then studied. A plot of slope of calibration graph versus temperature is shown in Fig. 4. It can be noticed that sensitivity increased linearly with the increase of temperature in a range of 40–60 °C. The temperature of 55 °C was selected in order to avoid the evolution of bubble in the line when using a higher temperature. It should be noted that although the longer stopping time or higher temperature lead to high sensitivity, the higher peak height of blank solution was also observed. This may be due to the air oxidation of iodide which can be minimized by purging the reagent and sample solutions with oxygen-free nitrogen gas.

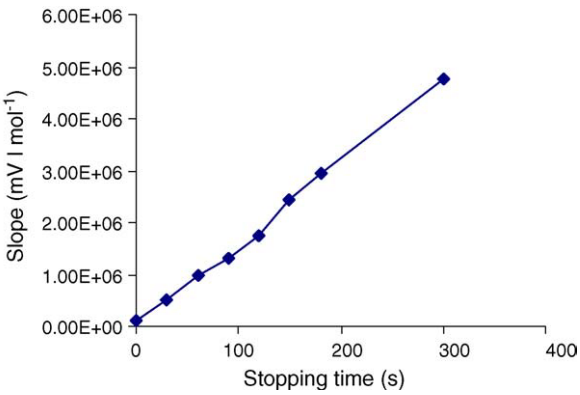

Fig. 3. Effect of stopped time (temperature = 45 °C, HCl concentration = 7 mol l^{-1}, KI concentration = 0.07 mol l^{-1}, other conditions see text).

3.1.4. Effect of HCl concentration

Attempts have been made to reduce concentration of the HCl used. Under the above selected conditions, it was found that sensitivity rapidly decreased when a concentration of HCl was reduced less than 7 mol l^{-1}. So 7 mol l^{-1} HCl should be recommended. However, a lower acid concentration may be employed but a longer stopping time should be applied so that a desired sensitivity would be obtained. This would then affect the sample throughput.

3.2. Analytical characteristics

Employing the above selected conditions as shown in Table 1, a series of concentration of chlorate standard solution was injected into the system. FI peaks similar to those of normal FI were obtained as shown in Fig. 5. A calibration graph was constructed by plotting peak height (mV) versus chlorate concentration (mol l^{-1}). Calibration graphs were linear in the ranges of 1.2×10^{-6}–6.0×10^{-5} mol l^{-1} ($y = (1.217 \times 10^{7})x + 14.3$, $r^2 = 0.9977$) and 6.0×10^{-5}–6.0×10^{-4} mol l^{-1} chlorate ($y = (6.421 \times 10^{6})x + 451.5$, $r^2 = 0.9964$). With a 120 s stopping time, a detection limit of 1.2×10^{-6} mol l^{-1} chlorate was

Table 1
Selected conditions of the proposed sFI-Amp procedure

Parameter	Analytical characteristics
Injection volume (μl)	100
Mixing coil length (cm)	300
Reagent concentration (mol l^{-1})	
KI	0.07
HCl	7
Flow rate (ml min^{-1})	
KI	2.9
HCl	2.9
Travelling time (s)[a]	12
Stopping time (s)[b]	90–120
Temperture of water bath (°C)	55 ± 0.5

[a] Travelling time: the period between the point of injection to the point at which the flow is stopped.
[b] Stopping time: the period during the flow is stopped.

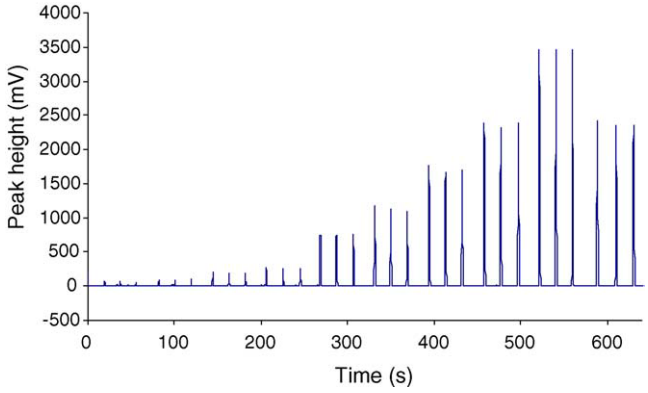

Fig. 5. FIgram obtained from sFI-Amp system for chlorate determination: peaks from left to right were blank, 1.2, 6.0, 12, 60, 120, 240, 360 and 600 μmol l^{-1} of chlorate standard and sample 1, respectively. Conditions as in Table 1 were employed, with stopping time 120 s. Signal was recorded only at the period after the flow was restarted to push the reaction product into the detector.

obtained. A sample throughput was 25 h^{-1}. Relative standard deviation was 2% (1.2×10^{-4} mol l^{-1} chlorate, $n = 21$).

Another merit of sFI is the less consumption of reagent, especially when a high concentration of reagent solution is concerned. In this procedure, the consumption of about 1.5 ml each of 7 mol l^{-1} HCl and 0.07 mol l^{-1} KI per injection should be noted.

3.3. Interference study

The species that can react easily with iodide ion to produce triiodide, e.g. iodate, hypochlorite, chlorite, could be seriously interfere in this system. These ions could be determined first by employing low acid concentration and room temperature and chlorate concentration could be obtained later using elevated conditions [15]. However, these ions are expected to present in some selected soil samples with concentrations of much lower than that of chlorate.

The common ions (CO_3^{2-}, NO_3^-, SiO_3^{2-}, Ca^{2+}, Cu^{2+}, Fe^{3+}, $Mn^{2+}$and Zn^{2+}), which may be found in soil sample, were studied. The effects of these ions were investigated, by adding the ions to the 1.2×10^{-4} mol l^{-1} chlorate standard solution. The relative error in concentration of the standard chlorate solution of less than 5% was obtained for the interfering ion/chlorate mole ratios of 3 for Cu^{2+}, 4 for Fe^{3+}, and 50 for CO_3^{2-}, NO_3^-, SiO_3^{2-}, Ca^{2+}, Mn^{2+} and Zn^{2+}. $Cu^{2+}$and Fe^{3+} exhibited positive interference because the $Cu^{2+}$and Fe^{3+} can reduce the iodide ion. Fortunately, copper and iron in soil-extracted solution (using water) were at very low concentrations (less than 2.4×10^{-5} mol l^{-1} of both ions, determined by flame-atomic absorption spectrophotometry) and did not interfere in this system.

3.4. Analysis of soil samples

The developed sFI-Amp procedure was applied to the determination of chlorate in soil samples from longan orchards in Chiang Mai, Thailand. Potassium/sodium chlorates are applied to the soil to stimulate flowering of the fruit. Due to a good solubility of the chlorate compounds in water, the soil was extracted with deionized water to have a chlorate concentration in the extract in a suitable range of the calibration graphs (1.2×10^{-6}–6.0×10^{-5} and 6.0×10^{-5}–6.0×10^{-4} mol l^{-1} chlorate). The extracted solution was injected into the system and chlorate content was obtained from the calibration graphs as presented in Table 2. The iodometric titration [18] was performed for comparison. The results of the batch titration method were slightly different from the sFI-Amp method, which may come from interfering of oxygen in air and slow reaction of chlorate with iodide and hydrochloric acid, which was difficult to control in batch titration method. However, the results obtained from both methods are in agreement (*t*-test, 95% confidence).

Table 2
Chlorate contents in soil samples from longan orchards determined by the proposed sFI-Amp procedure and the iodometric titration method [18] (triplicate results)

Sample number[a]	Chlorate (μg/g) of dried soil	
	sFI-Amp	Iodometric titration
1	335 ± 3	341 ± 6
2	108 ± 6	118 ± 5
3	88 ± 5	100 ± 4
4	20 ± 4	16 ± 5
5	27 ± 1	34 ± 7
6	15.1 ± 0.4	11 ± 5
7	3.9 ± 0.4	ND[b]
8	28 ± 1	21 ± 4
9	15 ± 2	10 ± 4
10	5.9 ± 0.4	ND
11	140 ± 1	147 ± 6

[a] Dried soil samples were obtained from Assoc. Prof. Dr. Somchai Ongprasert, Department of Soil Resource and Environment, Faculty of Agricultural Production, Maejo University, Chiang Mai, Thailand.
[b] ND: not detected.

4. Conclusion

A novel stopped flow injection amperometric procedure was developed for the determination of chlorate in soil sample. Chlorate in micro-molar concentration can be easily determined with this simple procedure even using a home-made amperometric analyzer and a home-made flow through electrochemical cell. High sensitivity can be obtained without conditions employing too high acid concentration, by promoting the reaction using longer reaction time and higher temperature by stopping the flow while the reaction zone being in a mixing coil which is immersed in a water bath. In a closed FI system, air oxidation of iodide reagent is also minimized. The amperometric detection also reduced the interferences from the substances (e.g. colored species) usually found in soil sample which may interfere very much in spectrophotometric detection. The proposed system provided a sample throughput of 25–35 h^{-1} with less reagent consumption and successfully demonstrated for application to soil samples.

Acknowledgements

We thank the Thailand Research Fund (TRF) and the Postgraduate Education and Research Program in Chemistry (PERCH) for financial support. O.T. thanks the Commission on higher Education (CHE) of Thailand and Naresurn University for providing her scholarship. Assoc. Prof. Somchai Ongprasert is gratefully acknowledged for providing of soil samples. Thanks also to Dr. Ponlayuth Sooksamiti for AAS determination of copper and iron contents in the samples.

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Exploiting sequential injection analysis with lab-at-valve (LAV) approach for on-line liquid–liquid micro-extraction spectrophotometry☆

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Available online 10 October 2005

Abstract

Sequential injection analysis (SIA) with lab-at-valve (LAV) approach for on-line liquid–liquid micro-extraction has been exploited. Sample, reagent and organic solvent were sequentially aspirated into a coil attached to a central port of a conventional multiposition selection valve, where the extraction process was performed. The aqueous and organic phases were separated in a conical separating chamber LAV unit attached at one port of the valve. The organic phase containing extracted product was then monitored spectrophotometrically. The system offers a novel alternative on-line automated extraction in a micro-scale and has been successfully demonstrated for the assays of diphenhydramine hydrochloride (DPHH) in pharmaceutical preparations and anionic surfactant in water samples.
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Keywords: Sequential injection analysis; Lab-at-valve; On-line liquid–liquid micro-extraction

1. Introduction

Liquid–liquid extraction is one of the most versatile techniques for sample matrix separation and/or analyte preconcentration. It has been applied to various analytical fields. For pharmaceutical applications, liquid–liquid extraction involves in the analytical processes for determination of active ingredient compounds such as cinnarizine [1], diphenhydramine hydrochloride (DPHH) [2,3] and paracetamol [4]. For environmental applications, it also exists in the determination of various compounds such as iron(II) and iron(III) [5], phenolic compounds [6] and surfactant [7]. However, manual extractions present a series of drawbacks such as high consumption of sample and toxic organic solvent, low sampling frequency, loss of analyte through manipulation and contamination of atmosphere by organic vapor. Many efforts have been made to overcome these inherent drawbacks. Among them, the successful techniques are probably the on-line liquid–liquid extraction using flow systems [8–10].

A variety of flow-based systems have been reported for the on-line liquid–liquid extraction. Flow injection liquid–liquid extraction was proposed in 1978 by Karlberg and Thelander [11] and Bergamin et al. [12]. Consumption of reagents and organic solvent are lower than those in manual procedures, consequently reducing the waste generated.

Based on this feature, different flow injection systems for liquid–liquid extraction were developed for a large number of analytical applications [7,13–18].

As another generation, the technique called sequential injection analysis (SIA) [19–22] has also been employed for on-line liquid–liquid extraction by sequential aspiration of a small volume of organic solvent and aqueous sample into contact before either resolve them using phase separator [23] or performing back extraction [24].

In the recent years, SIA has been modified into miniaturization with different concepts. Ruzicka proposed SIA with lab-on-valve (LOV) format in which a very precisely specific LOV module was mounted atop a selection valve to perform chemical reaction and monitor for a change in a conduit on such a modified multiposition valve [25]. SIA with a simple approach called lab-at-valve (LAV), firstly introduced by our group [26], is another approach of SIA which becomes an alternative cost effective micro total analysis system. SIA-LAV uses a designed LAV unit that can be fabricated using an ordinary less precise

☆ Paper presented at the 13th International Conference on Flow Injection Analysis (ICFIA), Las Vegas, USA, 24–29 April 2005.

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0039-9140/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.talanta.2005.09.002

machine tool, to have a suitable function for chemistry of interest and can be easily attached at a port of the conventional selection valve in a usual way. This simpler approach was successfully demonstrated for potentiometric determination of chloride [27].

In the present work, SIA-LAV approach was investigated as a novel alternative for simple on-line liquid–liquid microextraction. A desired component, a separating chamber, was attached at one port of a conventional multiposition selection valve. Sample, reagents, and organic solvent were sequentially aspirated into an extraction coil (EC). By flow reversal, good extraction efficiency can be achieved. After that, the aqueous and organic phases were separated in a conical separating chamber attached at one port of a conventional multiposition selection valve ("Lab-at-Valve" concept). The organic phase containing extracted product was then monitored spectrophotometrically. Applications to the assays of diphenhydramine hydrochloride in pharmaceutical preparations and anionic surfactant in water samples were selected as models.

2. Experimental

2.1. Chemicals and reagents

All of reagents used were analytical reagent grade. Deionized water was used throughout the experiments. Diphenhydramine hydrochloride reference standard of 100% purity (Ministry of Public Health, Thailand) was used. Stock solution (1000 mg/l) of diphenhydramine standard was prepared by dissolving 0.1000 g of the reference standard in water and diluting to the mark of 100 ml volumetric flask. Working standards were freshly prepared by diluting the stock solution with water to obtain appropriate concentrations. A bromocresol green (BCG) solution (5×10^{-4} mol/l) was prepared by dissolving 0.0349 g BCG powder in water with addition of 0.2 ml of 0.1 mol/l sodium hydroxide and diluting to 100 ml with water. The solution was filtered before use. A phthalate buffer (pH 3) was prepared using 100 ml of 0.1 mol/l potassium hydrogenphthalate, 40.6 ml of 0.1 mol/l hydrochloric acid and diluting to 200 ml with water.

Stock solution (1000 mg/l) of sodium dodecylsulphate (SDS) was prepared by dissolving 0.1087 g of the standard in water and made up to a volume of 100.00 ml.

Stock solution (0.10%, w/v) of methylene blue was prepared by dissolving 0.10 g of the methylene blue in 100 ml of water.

2.2. Apparatus

The SI system used is schematically depicted in Fig. 1. It consisted of a modified autoburette Dosimat 765 (Metrohm, Switzerland) equipped with a 10 ml exchange unit, for a pumping system, and connected to a personal computer via RS232C interface, a 10-position selection valve VICI with a microelectric actuator (Valco Instruments, USA) and a Spectronic21 (Bausch & Lomb, USA) detector with a flow through cell (Hellma, Germany) of 1 cm light path. The autoburette was connected to the center of the selection valve via the EC (0.79 mm i.d. × 400 cm PTFE tubing) and a separating chamber was placed at port-1 of the selection valve. Both instrumental control and data acquisition were manipulated via a software using LabVIEW, developed in house and using a CYDAS ULV interfacing board (CyberResearch, USA). This software provided control of the volume to be dispensed or aspirated by the autoburette, flow rate, selection of the different valve positions and performed data acquisition. The data processing was computed by using Microcal Origin 6.0.

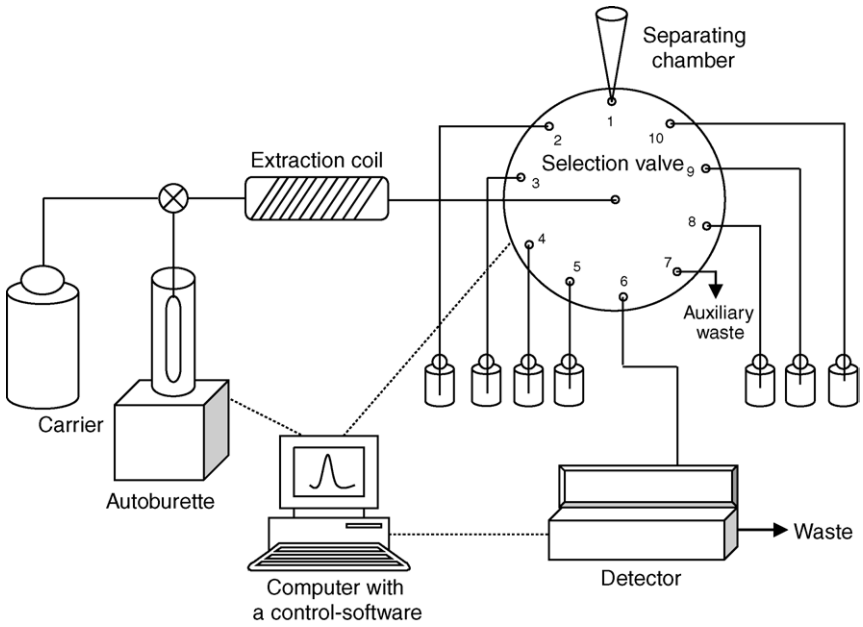

Fig. 1. Schematic diagram of SIA system.

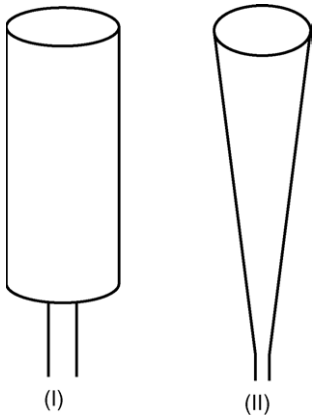

Fig. 2. Two types of separating chamber; I: cylindrical shape (8 mm i.d. × 5 cm long) and (II): conical shape (8 mm i.d. of the wider end × 7 cm long, modified from a 1 ml pipette tip (Eppendoft, Germany)).

3. Results and discussion

3.1. The assay of diphenhydramine hydrochloride

3.1.1. Optimization of the operational sequence

By using the separating chamber type I, as shown in Fig. 2, a sequence order of aspiration was firstly optimized. Three sequence orders (Fig. 3) were examined and a suitable one, as demonstrated in Fig. 3(c), which provided better sensitivity, was selected. BCG solution was firstly aspirated into the EC, then the standard/sample solution was introduced, an ion-association formed. A phthalate buffer (pH 3) and chloroform were then aspirated. At pH 3, a high extraction efficiency of ion-pair compound was obtained [2]. The extraction step was performed in the EC by programming the autoburette to aspiration and dispensed modes. This process determines the time and efficiency that the two phases are in contact and is a key parameter for better extraction efficiency. The solution was then dispensed into the separating chamber where the aqueous and organic phases were separated. The organic phase containing ion-association product was transported into the EC and transported into a flow through cell of the spectrophotometer. An absorbance at 415 nm was followed.

It should be noted that although the exchange unit of the autoburette was made from glass and furnished with PTFE plunger, manipulation of the organic extractant was accomplished by filling the syringe of the exchange unit with water. Before the detection step, the organic solvent was aspirated and stored in EC for use. It is necessary to avoid the introducing of aqueous into the flow cell of detector to prevent the change of baseline signal due to aqueous droplet.

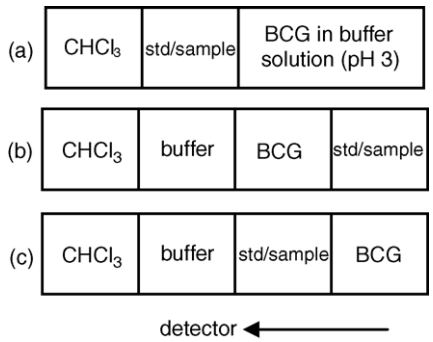

Fig. 3. Sequence orders of the SIA system for the determination of diphenhydramine hydrochloride.

3.1.2. Influence of the separating chamber shape

Two types of the separating chambers, as shown in Fig. 2, were examined to reach the best separation between the organic and aqueous phases. The results show that the better separation between two phases was obtained by using the conical shaped separating chamber. Therefore, the separating chamber type II was adopted.

3.1.3. Optimization of the chemical parameters

The concentration and volume of the reagents concerning in the reaction were optimized. According to the preliminary study, the concentration of BCG of 5×10^{-4} mol/l was excess to form the ion-association with diphenhydramine hydrochloride in the working range of 10–40 mg/l [2].

The selected conditions and operational sequence of the proposed system were summarized in Table 1. By fixing the aque-

Table 1
Selected conditions and operational sequence of the SIA system for the determination of diphenhydramine hydrochloride

Sequence	Valve position	Mode[a]	Volume (μl)	Description
1	5	PIP	170	Aspirate 5×10^{-4} mol/l BCG into EC
2	3–4, 8–9	PIP	170	Aspirate standard/sample into EC
3	2	PIP	150	Aspirate 5×10^{-4} mol/l buffer (pH 3) into EC
4	10	PIP	400	Aspirate chloroform into EC
5	1	PIP	1300	Extract
6	1	DIS C	2000	Extract
7	1	PIP	1100	Extract
8	1	DIS C	2000	Extract and propel to the separating chamber[b]
9	7	DIS C	3000	Clean EC
10	10	PIP	400	Aspirate chloroform into EC
11	1	PIP	250	Aspirate chloroform phase containing ion-association compound into EC
12	6	DIS C	40	Dispense chloroform phase containing ion-association compound through the selection valve
13	7	DIS C	3000	Clean EC
14	10	PIP	2900	Aspirate chloroform into EC
15	6	DIS C	2000	Dispense to detector with flow rate of 10 ml/min

[a] PIP = pipetting/aspiration, DIS C = cumulative dispensing.
[b] No standing time for phase separation.

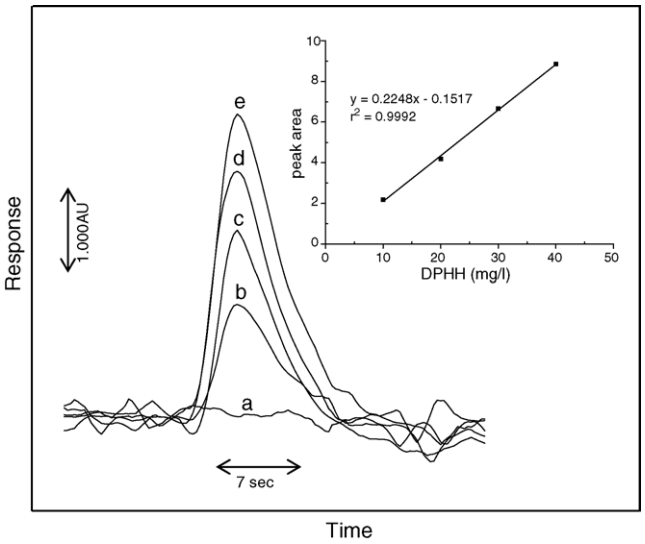

Fig. 4. Typical SI-grams and calibration graph of diphenhydramine hydrochloride; a = blank, b = 10, c = 20, d = 30, e = 40 mg/l.

ous volume (see Table 1), the volume ratio between aqueous to organic phases is of great importance in solvent extraction because it determines enrichment factor in the preconcentration of the analyte. The volume of chloroform used for the extraction was varied. The slopes of the calibration equation indicated that the sensitivity increased with decreasing the volume of chloroform: $y = 0.2248x - 0.1517$, $y = 0.1922x - 1.7877$ and $y = 0.0833x + 1.0726$ for 400, 450 and 470 μl, respectively. Using chloroform less than 400 μl, it was difficult to reach the complete separation between the two phases, i.e. aqueous droplets being entrapped within the organic phase. Better enrichment factor may be obtained by increasing the volume of the standard/sample solutions for a fixed volume of chloroform, but a larger separating chamber must be used.

3.1.4. Analytical characteristics

Fig. 4 shows the calibration graph and typical SI-grams of diphenhydramine hydrochloride. Calibration graph of diphenhydramine hydrochloride versus peak area was found to be linear in the range of 10–40 mg/l with the linear equation and correlation coefficient (r^2) of $y = 0.2248x - 0.1517$ and 0.9992, respectively. The limit of detection (LOD) calculated as three times the standard deviation was 1.90 mg/l. The relative standard deviation (%RSD) was less than 3.6 ($n = 11$, 20 mg/l diphenhydramine hydrochloride). The recovery was found to be 102%. Sample throughput of 5 h^{-1} can be achieved. A higher throughput may be possible if the modified autoburette would be replaced by another type of syringe pump, although it could be more expensive.

According to our previous study [2], using this reaction, the amount of the foreign compounds commonly presented together with diphenhydramine hydrochloride in preparations containing single tertiary alkylamine drug would not interfere in the determination.

3.1.5. Application to samples

The proposed SI method was applied to the determination of diphenhydramine hydrochloride in some pharmaceutical preparations (containing single tertiary alkylamine). Some locally commercial pharmaceutical preparations were taken as samples to be assayed. A weighed quantity of a sample was dissolved with deionized water, shaken for 15 min and adjusted to a volume with water to obtain a solution having a concentration in the range of a calibration graph. The solution was then filtered before analysis. The results were compared to those obtained by the standard method using high performance liquid chromatography [28], as summarized in Table 2. Evaluation by t-test at 95% confidence level indicates that there is no significant difference in the results obtained by the proposed SIA and the standard methods.

3.2. The assay of anionic surfactant

3.2.1. Optimization of the experimental parameters

The same SIA setup was used for the determination of anionic surfactant in water samples. The anionic surfactant reacts with methylene blue to form ion-association compound, which can be extracted into chloroform. The concentration of anionic surfactant can be determined by measuring the absorbance of the chloroform phase at 650 nm [29]. Some experimental parameters, i.e. operational sequence, volume and concentration of reagents were optimized. Zone sequence was chosen: standard/sample-methylene blue-chloroform. The aqueous sample containing anionic surfactant was firstly aspirated into the EC. Next, the methylene blue solution was introduced, the ion-association compound formed. Then, chloroform was aspirated and the extraction was done by programming the autoburette to aspiration and dispensed modes. After extraction in the EC, the solution was propelled to the separating chamber, where the separation between aqueous and chloroform phases occurred. The chloroform phase containing ion-association compound was re-aspirated into the EC for transportation to a detector.

The concentration of methylene blue solution was varied from 0.0038 to 0.02%. The sensitivity increased with increasing the methylene blue concentration and reached the maximum at

Table 2
Determination of diphenhydramine hydrochloride in pharmaceutical preparations by the proposed and standard methods

Sample	Label	Proposed method		Standard method [28]	
	(mg/5 ml)	Amount found (mg/5 ml)	% label	Amount found (mg/5 ml)	% label
1	12.50	12.57 ± 0.27	101	13.55 ± 0.49	108
2	12.50	12.81 ± 0.55	102	13.91 ± 0.12	111

Table 3
Selected conditions and operational sequence of the SIA system for the determination of anionic surfactant in water samples

Sequence	Valve position	Mode[a]	Volume (μl)	Description
1	2–4, 8–9	PIP	250	Aspirate standard/sample into EC
2	5	PIP	250	Aspirate methylene blue into EC
3	10	PIP	400	Aspirate chloroform into EC
4	1	PIP	1300	Extract
5	1	DIS C	2000	Extract
6	1	PIP	1100	Extract
7	1	DIS C	2000	Extract and propel to the separating chamber[b]
8	7	DIS C	3000	Clean EC
9	10	PIP	400	Aspirate chloroform into EC
10	1	PIP	250	Aspirate chloroform phase containing ion-association compound into EC
11	6	DIS C	40	Dispense chloroform phase containing ion-association compound through the selection valve
12	7	DIS C	3000	Clean EC
13	10	PIP	2900	Aspirate chloroform into EC
14	6	DIS C	2000	Dispense to detector with flow rate of 10 ml/min

[a] PIP = pipetting/aspiration, DIS C = cumulative dispensing.
[b] No standing time for phase separation.

0.01%. Using a methylene blue solution of higher concentration than 0.01% can cause higher background level due to methylene blue partition into chloroform phase. Therefore, methylene blue concentration of 0.01% was selected. The selected conditions and operational sequence of the SIA system for the determination of anionic surfactant are summarized in Table 3.

3.2.2. Analytical characteristics

Using the SIA setup shown in Fig. 1 and under the above-mentioned selected conditions, the linear calibration graph was obtained for SDS in the concentration range of 1–10 mg/l with the regression equation of $y = 1.0711x - 0.1356$ and the correlation coefficient of 0.9995, as shown in Fig. 5. The LOD of 0.48 mg/l was obtained. The RSD was less than 5% ($n = 11$, 5.0 mg/l SDS). A five samples per hour assay can be obtained. Increase of the throughput may be obtained by employing another type of pump.

3.2.3. Application to samples

The applicability of the proposed procedure was demonstrated for the determination of anionic surfactant in drainage water samples. The results were compared to those obtained by the methylene blue standard method [29], as summarized in Table 4. There is no significant difference in the results obtained from the proposed and the standard procedures.

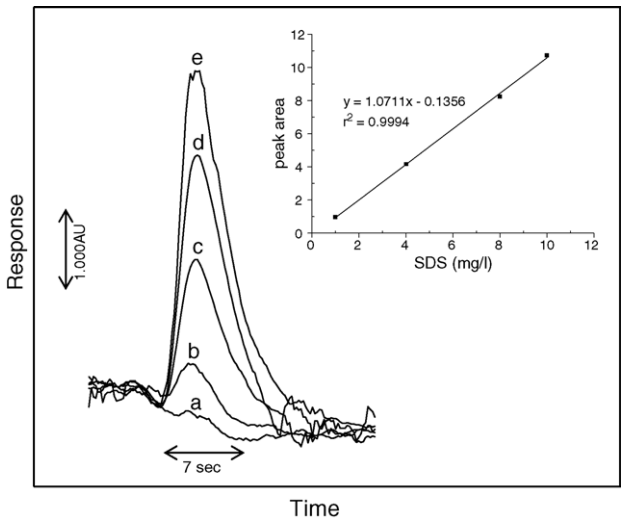

Fig. 5. Typical SI-grams and calibration graph of standard SDS; a = blank, b = 1, c = 4, d = 8, e = 10 mg/l.

Table 4
Determination of anionic surfactant in drainage water samples by the proposed and standard methods

Sample	Found (mg/l)	
	Proposed method	Standard method [29]
1	3.38 ± 0.01	3.42
2	2.76 ± 0.02	2.77

4. Conclusion

Sequential injection analysis with lab-at-valve approach for alternative simple on-line liquid–liquid micro-extraction was exploited. A simple fabricated LAV unit, a separating chamber, attached at a port of a conventional multiposition selection valve offers an on-line automated extraction in a micro-scale. Therefore, consumption of sample, reagent and organic solvent, also waste generation, is tremendously reduced. A cost effective LAV apparatus can be easily fabricated using a less precise machine tools. Applications for the assays of diphenhydramine hydrochloride in pharmaceutical preparations and anionic surfactant in water samples have been successfully demonstrated. Instead of using Spectronic 21 detector, investigation to plug a fiber optic spectrophotometric detection system to the conical separation chamber has been in progress to make such a system become a real SIA-LAV liquid–liquid extraction.

Acknowledgements

The Staff Development Project of the Ministry University Affairs, the Commission on Higher Education, is gratefully acknowledged for the scholarship to R.B. during the Ph.D. study at Chiang Mai University. This work was supported by the Thailand Research Fund (TRF), the Postgraduate Education and Research Program in Chemistry (PERCH) and the Graduate School, Chiang Mai University. Metrohm Siam and Metrohm AG are acknowledged for the on-loan autoburette.

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Exploiting guava leaf extract as an alternative natural reagent for flow injection determination of iron

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Received 26 May 2005; received in revised form 16 July 2005; accepted 16 July 2005
Available online 29 August 2005

Abstract

Guava leaf extract is utilized as an alternative natural reagent for quantification of iron. The flow injection technique enables the use of the extract in acetate buffer solution without the need of further purification. Some properties of the extract such as its stability and ability to form a colored complex with iron were studied. The proposed system is an environmentally friendly method for determination of iron with less toxic chemical wastes.
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Keywords: Guava leaf extract; Natural reagent; Flow injection; Iron

1. Introduction

Many scientific studies involve the use of chemicals that cause contamination in the environment. For example, water studies to determine the amount of different ions in natural water may need chemical reagents that are not normally present in the environment. If wastes from such studies are not properly stored or treated, these foreign substances could contaminate soil or water sources. Gaining popularity, the research area known as green chemistry aims to explore the use of alternative reagents or alternative synthetic methods that minimize the use of toxic chemicals.

In this study, a natural guava (*Psidium guajava* L., Myrtaceae family) leaf extract has been investigated as an alternative natural indicator for iron quantification by the flow injection technique. Reports have indicated that guava leaves contain chemicals that are useful for whitening [1], anti-pigmentation [2], anti-bacterial treatment [3], leather tanning [4], and diet food and beverage components [5], and in the prevention of diabetes. [6] In Thailand, there has been some local usage of guava leaves as an indicator of the presence of iron in ground water. A change in color of water to a darker color helps local villagers in making pre-treatment decisions such as adding alum to precipitate the iron. This use has never been published and no scientific explanation is available.

To determine the concentration of iron by a FI system, a water sample is introduced into a closed flow system and mixed with reagent that can form a colored complex, which can be detected by a colorimeter. Examples of some reagents [7] that have been used in determination of iron include 1,10-phenanthroline, 2,4,6-tri(2′-pyridyl)-1,3,5-triazine, azo-dye derivatives, mercaptoquinoline, thiocyanate and salicylate. [8] In this study, we investigate the use of guava leaf extract as an alternative reagent for quantification of iron using a FI system. Although the active chemical species in the extract is unknown and the extract may not be pure, the use of this guava leaf extract to determine the amount of iron in solution is made possible by the FI system. One of the features of FI systems is that they can be employed with reagents, which are not necessarily pure. This is because in the flow injection technique, an analyte to be determined goes through the

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doi:10.1016/j.talanta.2005.07.039

system in exactly the same condition as that of the standard. [9]

The use of a natural and easily available material in place of a toxic and expensive reagent should be beneficial from both environmental and economic aspects. In this report, the authors conduct preliminary studies of the potential use of guava leaf extract as an alternative reagent to quantify iron using a simple FI system. Some properties of the extract, including its stability, ability to form a complex with iron (II) and (III), spectral properties, suitable extraction medium, and its potential to be used for quantification of iron in water samples have been investigated.

2. Experimental

2.1. Reagents

A 100 mL volume of 1000 ppm stock standard Fe (II) solution was prepared by dissolving 0.7022 g of $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ (Carlo Erba) in water containing 1% (v/v) concentrated H_2SO_4 (BDH). A 1000 ppm stock standard Fe (III) solution was prepared similarly but with 0.7240 g of $Fe(NO_3)_3{\cdot}9H_2O$ (Carlo Erba). A 250 mL volume of 0.2 M sodium acetate buffer pH 4.8 solution was prepared by dissolving 6.87 g $CH_3COONa{\cdot}3H_2O$ (Merck) in water containing 1.16% (v/v) acetic acid (Carlo Erba). HCl solution of the same pH 4.8 was prepared by diluting concentrated HCl (Merck) with deionized water to the desired pH. Ascorbic acid (Merck) was prepared at the concentration of 2% (w/v). Interference studies were done by spiking some cations, Ca^{2+} ($CaCl_2$, Merck), Mg^{2+} ($Mg(NO_3)$, Merck), Co^{2+} ($CoCl_2$, BDH), Ni^{2+} ($NiCl_2$, BDH) and Cr^{3+} ($CrCl_3$, BDH) into Fe (III) 10 ppm solutions at the final concentration of 1, 5 and 10 ppm (10:1, 2:1 and 1:1 concentration ratios of Fe (III):interference ion).

2.2. Guava leaf extraction

Fresh guava leaves of 10.0 g were ground in 150 mL water (pH 7.0), diluted HCl (pH 4.8) or acetate buffer (pH 4.8) with a blending machine for 5 min. Then the suspension was filtered through filter paper no. 1 (Whatman) and the filtrate was kept at room temperature for further use. The extract was prepared daily.

2.3. Study of the conditions for guava leaf extract–iron complex formation

Both Fe (II) and Fe (III) solutions were prepared in three sets; in water, in HCl solution and in acetate buffer. Two iron concentrations of 10 and 100 ppm were used. Guava leaf extracts in the three media were mixed with iron solutions prepared in the matching medium at the ratio of 1:1 (v/v) (10 mL extract:10 mL Fe solution). Guava leaf extract without iron solution was used as a blank solution. Mixtures of guava leaf extract and iron solution were scanned for absorption spectra, measured against each medium, in the visible region 400–700 nm using a UV–vis spectrophotometer (Lambda 25, Perkin-Elmer).

2.4. Study of stability of guava leaf extract

After selection of the suitable medium for the extract from the previous experiment, guava leaf extract was prepared in that medium and allowed to stand in an uncovered beaker for 1, 2, 3, and 4 h. Mixtures of the extract and Fe (II and III) 10 and 100 ppm solutions were prepared as previously described. Mixtures were then scanned for absorption spectra to investigate absorption characteristics.

2.5. Apparatus

Peristaltic pumps (Ismatec) were used for reagent drawing. Pump tubing was Tygon and all the other tubing was PTFE (1/16 in. i.d.). Samples were introduced via a six-port injection valve (FIAlab). A Spectronic 21 spectrophotometer (Spectronic Instruments) with a flow through cell (80 μL, Hellma, Germany) was set at 570 nm and connected to a computer to record the FIA-grams using Stamp (Parallax, USA) and Microsoft Excel (Microsoft Corp., USA) software programs.

2.6. Manifold designs

2.6.1. Normal FI system

The simple one line normal FI manifold, where a sample is injected into the stream of reagent, is shown in Fig. 1(a).

2.6.2. Reverse FI system

In order to save reagent, the reverse FI manifold with two lines was tried where the reagent was injected into the stream of buffer that was later mixed with the stream of sample solution (Fig. 1(b)).

2.6.3. Column FI system

To shorten the extraction process, a column packed with ground guava leaves was connected in-line with the FI system, see Fig. 1(c). This column is made from a small Perspex glass tube with 3 mm i.d. and 2.5 cm length. Both ends were plugged with Teflon wool.

All three types of manifolds have an injection loop of 60 μL.

3. Results and discussion

3.1. Optimum condition of guava leaf extract-iron complex formation

The aim of this study was to find a suitable acidic solution for guava leaf extract–iron complex formation. Buffers

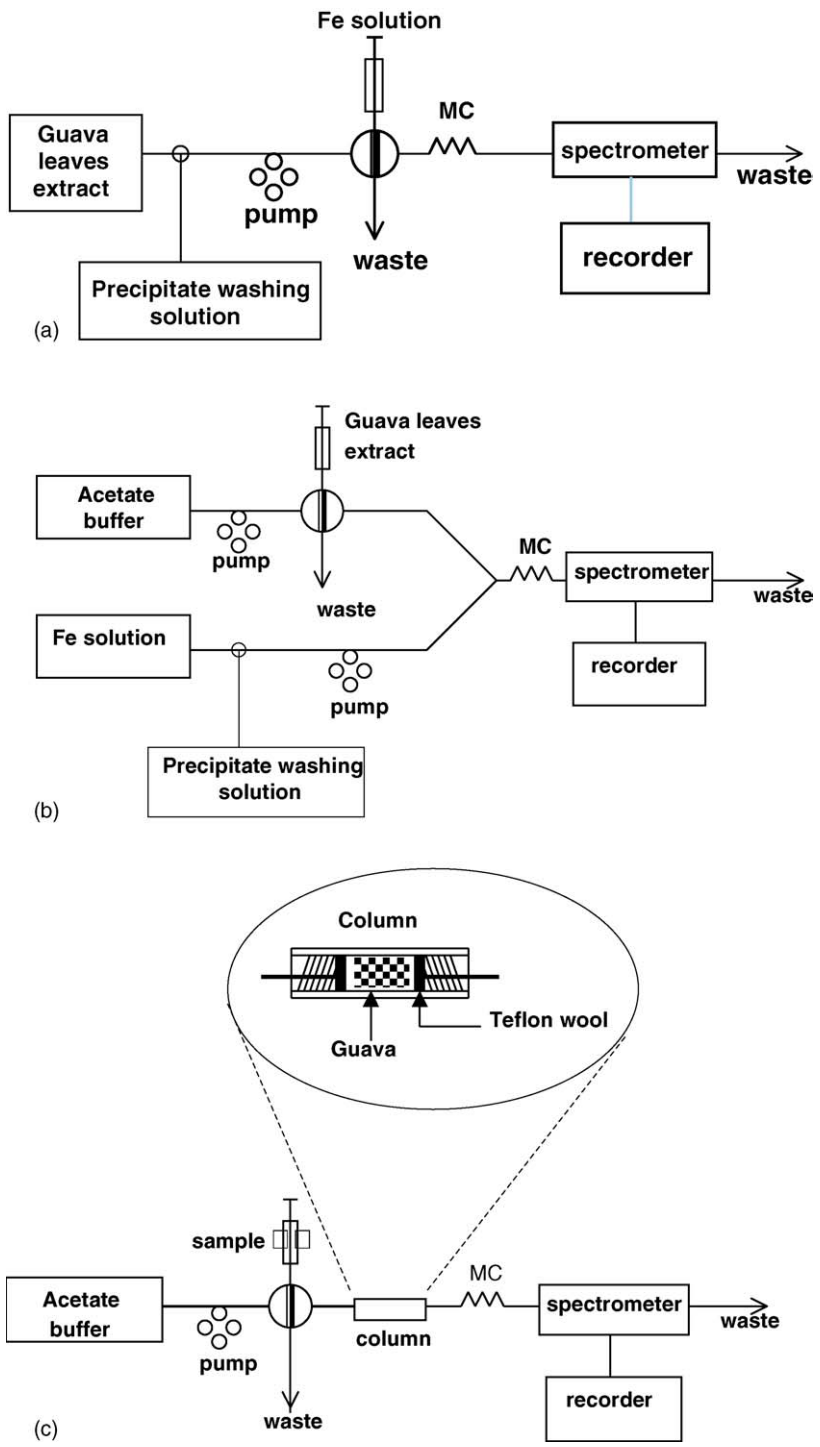

Fig. 1. (a) A normal FI manifold, (b) a reverse FI manifold and (c) a FI manifold with packed column.

of acidic pHs that can be prepared with easily available chemicals that would not interfere with the iron determination are rare. A suitable solution was sodium acetate buffer pH 4.8. Ammonium acetate was avoided because ammonium may form a complex with the metal ion of interest. To ensure the necessity of buffer solution in this study, water pH 7.0 and HCl diluted to pH 4.8 were also used as media for extraction to compare the results.

Fig. 2(a) and (b) represents the absorption spectra of guava leaf extract-iron complexes in water and buffer solution, respectively. In water (Fig. 2 (a)), no significant iron complex absorption is shown in the region of 450–700 nm, and except for high Fe (III) concentration, it is suppressed. This is likely because water cannot extract active species from the guava leaf. It could also be possible that there was no formation of iron complex with the active species or the

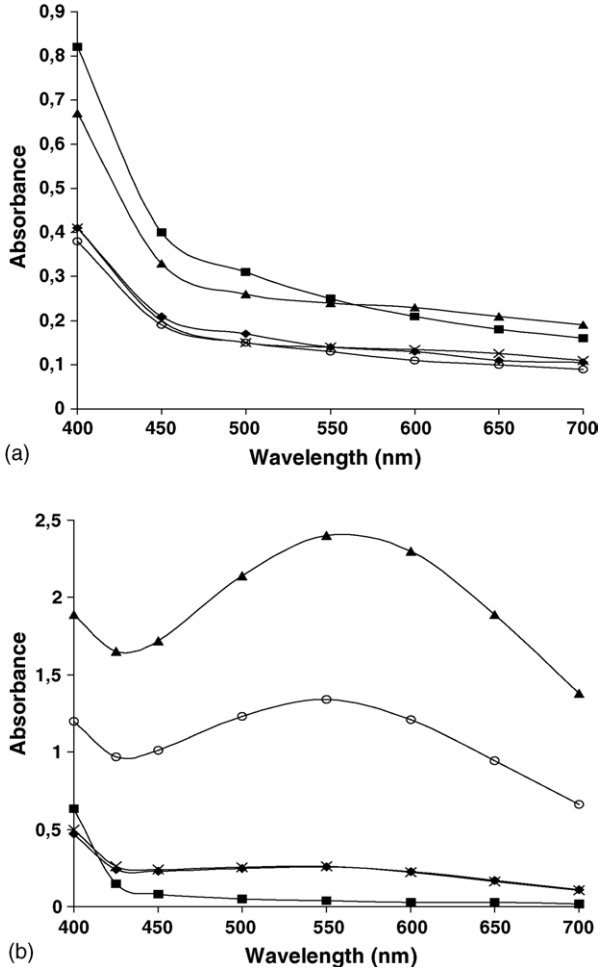

Fig. 2. (a) The absorbance spectra of guava leaf extract–iron complex in water, measured against water. (b) The absorbance spectra of guava leaf extract–iron complex in acetate buffer solution, measured against acetate buffer: (■) guava leaf extract; (▲) guava leaf extract with Fe (III) 100 ppm; (♦) guava leaf extract with Fe (III) 10 ppm; (◯) guava leaf extract with Fe (II) 100 ppm and (×) guava leaf extract with Fe (II) 10 ppm.

complex may not be stable in water. The exact cause of the results still needs further investigation, and the reason for the decreased absorbance is unknown. In acetate buffer solution pH 4.8 (Fig. 2 (b), both Fe (II) and (III) can form complexes with a chemical or chemicals in the guava leaf extract. Both Fe (II) and (III) complexes show maximum absorption at 570 ± 20 nm. The blank solution, guava leaf extract, did not show any significant absorption in the visible region. In a medium of diluted HCl pH 4.8, maximum absorption at 570 ± 20 nm was observed (not shown) only at high Fe (III) concentration but at much lower value as compared to the one in acetate buffer of the same pH. This indicates that guava leaf extract–iron complexes can only be formed at a suitable pH and that the use of buffer solution to control pH is necessary.

It has been observed that at low concentration of iron (10 ppm), the absorbances of Fe (II) and Fe (III) complexes were about the same while at high concentration of iron (100 ppm), the absorbance of the Fe (III) complex was higher

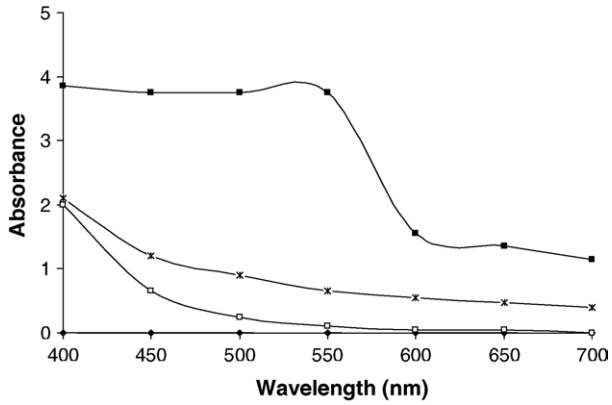

Fig. 3. The absorbance spectra studies for the reducing effect of ascorbic acid in. guava leaf extract: (♦) acetate buffer; (×) guava leaf extract and phenanthroline mixture; (□) Fe (III) 100 ppm with 0.3% (w/v) phenanthroline and (■) mixture of Fe (III) 100 ppm with 0.3% w/v phenanthroline and guava leaf extract.

than that of the Fe (II) complex. This might be explained as follows; at low concentration of iron, some chemicals (e.g., tannin) naturally present in guava leaves may reduce Fe (III) to Fe (II) and form guava leaf extract–Fe (II) complex. At high concentration of iron, there may be insufficient amount of reducing agents naturally present in the guava leaf extract to reduce all Fe (III). Therefore complexes formed were both Fe (II) and Fe (III) complexes. To test this hypothesis that guava leaf has a natural reducing effect, an additional experiment was carried out. Phenanthroline does not form a reddish complex with Fe (III) but does so with Fe (II). [10] Therefore, no significant absorbance should be observed at about 500 ± 20 nm when Fe (III) solution was mixed with phenanthroline. However, if some natural occurring substances in the guava leaf can reduce Fe (III) to Fe (II), the Fe (II)–phenanthroline complex should be formed when adding guava leaf extract into the mixture of Fe (III)–phenanthroline and the reddish colored complex with the absorption maxima at 500 ± 20 nm should appear. The absorbance at 570 ± 20 nm from the guava leaf extract–Fe (III) complex from excess Fe (III) that has not been reduced should also be observed.

In this experiment 3% (w/v) 1,10-phenanthroline, 100 ppm Fe (III) solution and guava leaf extract were mixed 1:1:1 by volume. The results shown in Fig. 3, comparing absorption spectra of 100 ppm Fe (III)–phenanthroline solution and 100 ppm Fe (III)–phenanthroline–guava leaf extract solution, confirm that guava leaf has a natural reducing effect. The mixture of Fe (III)–phenanthroline shows only slight absorbance at 500 nm and lower wavelengths. Guava leaf extract–phenanthroline mixture in the absence of Fe (III) was slightly turbid with white precipitate and might block the light, showing the result as if the solution had absorbed the light at 500 nm and lower wavelengths. The increase of absorbance at that region, when adding guava leaf extract into the Fe (III)–phenanthroline solution, was due to the reducing effect of guava leaf extract that changed Fe (III) to Fe

(II) which formed a reddish complex with phenanthroline that was observed with bare eyes. The comparable graph for 100 ppm Fe (III) with guava extract in acetate buffer is shown in Fig. 2(b) with the absorbance being about one half that when phenanthroline is added.

The results from both experiments in this study also indicate that the guava leaf extract–Fe (III) complex has higher absorbance (higher molar absorptivity) than that of the Fe (II) complex at 570 nm.

3.2. Stability of the guava leaf extract indicator

Guava leaf extract in acetate buffer was set-aside for 1, 2, 3, and 4 h before mixing with Fe (II) and Fe (III) solutions. Absorbances at 570 nm were recorded every hour. It was found that there were no significant changes in absorbance with time. This indicates that guava leaf extract is stable for at least 4 h, which is sufficient for a continuous experiment using FI.

3.3. Comparison of different manifold designs

In order to reduce the consumption of guava leaf extract, a reverse two line FI manifold was developed. Guava leaf extract was injected into the acetate buffer stream before merging with Fe solution.

In both the normal and reverse FI systems, purple precipitate adhering to the tubing and the detection cell was observed. Such a precipitate can cause unreliable and faulty results. Therefore, the procedure was modified by having a precipitate washing step with ascorbic acid. Fig. 4 shows the characteristics of the signals obtained from the reverse FI system which are similar to those obtained from the normal FI system. Signals from the Spectronic 21 are recorded as transmittance. Peaks between each run, which are constant throughout the experiment, are due to the Schlieren effect that occurs from injection of ascorbic acid for line washing.

Apart from minimizing the consumption of guava leaf extract, the reverse FI also showed better sensitivity than the normal FI system. This is possibly due to higher amount and less dilution of the Fe solution introduced by pumping as compared to injection. In terms of precision, the results obtained from the reverse FI system showed a slightly higher relative standard deviation (3–10%) as compared to the normal FI system (1–7%), for the Fe (III) concentration range of 1–10 ppm, which could be due to the high diffusion of reagent into the Fe solution stream thereby causing inconsistency of the complex formation.

Another attempt was to reduce the process of guava leaf extraction by packing the ground guava leaves in a column and placing it in the FI manifold. It was found that the guava leaves packed column-FI system could not be used effectively. The chemical from the leaves only came out at the edge of each piece, yielding a very small amount of color reagent in the flow stream. In addition, the active chemical was used up quickly, resulting in the need to change the column for every analysis, which is not practical.

3.4. Standard calibration graph and figures of merit

Calibration graphs for Fe (III) were prepared using both normal and reverse FI systems. Only calibration graphs of Fe (III) were constructed because species found in real samples such as water samples should be in the form of Fe (III).

Calibration graphs were obtained from standard Fe (III) solutions of 1, 2, 4, 6, 8 and 10 ppm. Both the normal FI and the reverse FI systems offer linear calibration graphs, represented as $Y = 11.09X + 4.06$, $R^2 = 0.9982$ and $Y = 24.93X - 20.05$, $R^2 = 0.9997$, respectively (where Y is peak height in mV and X is concentration of iron in ppm). The normal FI system showed higher blank signal due to the color of the guava leaf extract in the carrier line. In the reverse FI system, guava leaf extract was injected into the stream of colorless Fe solution and, therefore, the blank signal was low.

Even though the relative standard deviation is higher (3–10% R.S.D. in the Fe (III) working range of 1–10 ppm), the reverse FI system offers much better sensitivity than the normal FI system does. The practical lowest detectable concentration of Fe (III) obtained from the reverse FI system (estimated based on 3σ of blank signals [11]) was 1 ppm. Sample throughput was 20 injections/h. A sample with simple matrices, tap water, was spiked with Fe (III) standard solution at 2, 5, 7 and 30 ppm. Percent recoveries were found to be 95–97%.

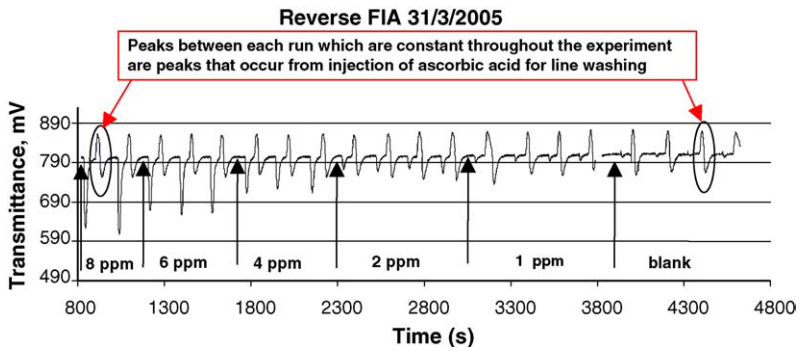

Fig. 4. Characteristics of the signals obtained from the reverse FIA system.

3.5. Interferences

It was found that the proposed system for iron (III) determination could tolerate Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+} and Cr^{3+} at least up to 1:1 concentration ratio (10 ppm Fe:10 ppm interference ion). Additional studies are required for general applicability.

4. Conclusion and future works

The results obtained from these preliminary studies indicate the potential use of guava leaf extract as an alternative reagent for quantification of iron in samples with simple matrices. The detection limit may possibly be improved by increasing the concentration of the guava leaf extract. No sample treatment is needed for iron concentrations at or below 30 ppm since Fe (III) and Fe (II) absorbances are similar, probably due to complete reduction of the Fe (III) by some chemicals naturally present in the guava leaves. Higher concentrations might be measured by adding a reducing agent such as hydroxylamine to convert the Fe (III) to Fe (II). Further studies are needed. Prior to applying the guava leaf extract in studies of samples containing complicated matrices, further studies should be made. Extensive interference studies, especially from other cations, should be evaluated. Even if the extract proves non-selective, it would serve for post-column detection of iron in separation or speciation studies. Although impure reagents can be used in a FI system, it would be interesting to find out what the exact chemical in guava leaf is that forms complexes with iron so that leaves of other plant species with similar chemical contents can be explored. Comparison of extracts from young and old leaves together with an additional separation technique such as HPLC or GC and NMR may help to identify the active chemical species. The use of dry leaves rather than fresh ones and the method of stabilizing the extract to be kept for future use should also be investigated. This will extend the use of the extract when guava leaf is not in season.

Acknowledgements

The Junior Student Talent Project, Thailand Research Fund (TRF) and the Post Graduate Education and Research in Chemistry (PERCH) are acknowledged for the support.

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A Simple Flow Injection Assay of Ca(II) in Mineral Supplement Using Mg(II)-EDTA

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Abstract

A new concept for a simple FI assay of Ca(II) employing a replacement reaction of Mg(II)-EDTA by Ca(II) in the present of Eriochrome Black T (EBT) is proposed. The released magnesium reacts with EBT. FI peak due to absorbance change of Mg(II)-EBT is proportional to Ca(II) concentration. Even with such a very simple FI set up, a through-put of 200 injections/h can be achieved. The procedure was applied to assay Ca(II) in mineral supplement samples. The results obtained agreed well with that of the British Pharmacopoeia titration method.

Key words Calcium, Magnesium, EDTA, Eriochrome Black T, mineral supplement

1. Introduction

Calcium is an important mineral for human body especially women. It reduces the risk of osteoporosis, the increased porosity or softening of bone, which usually occur in mature and postmenopausal women. However, too much of calcium consumption can cause problems. The effects of calcium overdose include renal damage and deposition of calcium in other areas of the body besides the bones. It can contribute to stone formation for people who are at risk of developing kidney stones.

Various analytical procedures have recently been developed for the determination of Ca(II) such as spectrophotometry [1], atomic spectroscopic techniques[2], electrochemical techniques [3] and complexometric titration [4-6]. The last ones are also employed as a standard method [4-6]. Flow based procedures [3, 7, 8] have also been reported for Ca(II) determination.

In this work, a new concept for a simple FI set-up with procedure employing a replacement reaction of Mg(II)-EDTA by Ca(II) in a present of Eriochrome Black T (EBT) is proposed. The released Mg (II) reacts with EBT in ammonia – ammonium chloride buffer of pH 9.5. Absorbance change due to Mg-EBT (at 530 nm) is followed. Peak height is proportional to the concentration of Ca(II). The procedure is demonstrated to be an alternative high sample through-put assay for Ca(II) in mineral supplement.

2. Experimental

2.1 Flow Manifold

FI manifold is schematically depicted in Fig. 1. It consists of simple components including a filter colorimeter.

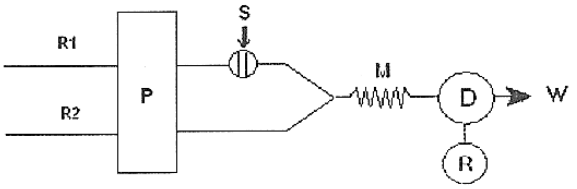

Fig. 1 Flow injection system for the determination of Ca(II) : R1= Mg(II)-EDTA reagent, R2 = EBT , P = peristaltic pump (Ismatec), S = injection valve (Rheodyne 6- Port valve), M = mixing coil, D = colorimeter (Cole Parmer 5564-15 with a green filter), R = recorder (Philips PM 8251) and W = waste.

2.2 Chemicals and Reagents

Stock standard Ca(II) solution (0.10 M) was prepared by dissolving 1.0010 g $CaCO_3$ (Fluka) in 1 ml of 1 M HCl. The solution was boiled to remove carbon dioxide before making the volume up to 100 ml with deionized water. Standard Ca(II) solutions of appropriate concentrations were obtained by further dilutions.

A solution containing Mg(II)-EDTA was prepared by dissolving 20.3300 g Na_2EDTA ($Na_2C_{10}H_{14}N_2O_8.H_2O$, Merck) and 37.2240 g $MgCl_2.6H_20$ (Fluka) in ammonia-ammonium chloride buffer (pH 9.5, 0.1 M) in a total volume of 1000 ml. The solution should have a Mg(II) : EDTA mole ratio of 1:1.5. A portion (0.01 g) of Eriochrome Black T (Aldrich) was dissolved in 100 ml of the ammonia-ammonium chloride buffer to become 0.01 % (w/v) solution.

2.3 Sample preparation

To prepare sample solutions from calcium carbonate tablets, the standard procedure was followed [6]. Not less than 20 tablets were weighed and finely powdered. A portion of the powder was weighed to obtain calcium equivalent to 100 mg and dissolved with 10 ml of 1 M HCl. The solution was boiled until clear solution was observed. After that it was diluted to 250 ml with deionized water. The treated sample was analyzed by the

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proposed method and the British Pharmacopoeia titration method [6].

For the capsule sample, 20 capsules were weighed. The accurate sample powder weight was obtained by the difference of the full and empty capsule.

3. Results and discussion

A set of conditions as summarized in Table 1 was selected. Fig. 2 illustrates the FI-gram.

Table 1 The conditions of the FI procedure for the determination of Ca(II).

R1	$[Mg^{2+}]$ in 0.1 M NH_3/NH_4Cl buffer	0.008 M
	[EDTA] in 0.1 M NH_3/NH_4Cl buffer	0.012 M
	Flow rate	5.1 ml/min
R2	Eriochrome Black T (EBT) in 0.1 M NH_3/NH_4Cl buffer	0.006 % w/v
	Flow rate	2.7 ml/min
S	Injection volume	50 µl
M	Mixing coil	75 cm
D	Detection wavelength	green filter (530 nm)

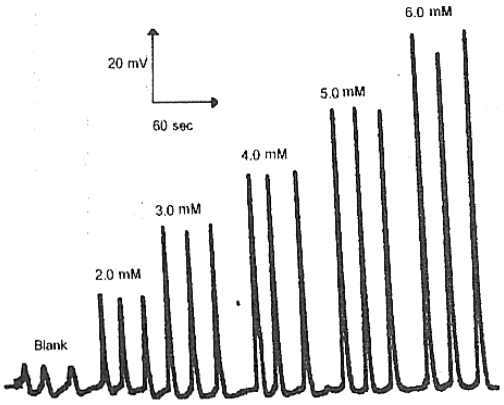

Fig. 2 FI-gram of Ca(II) 2.0-6.0 mM.

A linear calibration graph was obtained for 2.0-6.0 mM Ca(II) : $y = 22.094x - 14$, $r^2 = 0.9972$ with a detection limit (3σ) [9] of 0.6 mM, and 2% RSD (5 mM, n=11). A through-put of 200 injections per hour was achieved.

Effect of pH of solution injected was studied. According to the preliminary study, the standard solutions were chosen to be prepared in 0.01 M HCl (pH 2). Solutions adjusted to various pH values (2, 3 and 5) containing 5 mM Ca(II), were prepared. The solutions were injected into the FI system and evaluated for their analysis results by using a calibration graph prepared by employing standard solutions of pH 2. The analysis results were found to be the same as those of the standard Ca(II) prepared in pH 2 solution. This indicates that pH of the injected solution could be in the range of 2-5.

Although the procedure is not specific for Ca(II) but with its instrument simplicity and a high sample through-put, it should be useful to some certain types of samples which contain no interferents.

The results of the assay for Ca(II) in mineral supplement samples by the proposed procedure are summarized in Table 2 and agree well with the values obtained by the British Pharmacopoeia titration method [6]. This also indicates that no interference found in such assay of the samples.

Table 2 The determination results of Ca(II) mineral supplement samples by the proposed FI procedure and the British Pharmacopoeia titration method (average of triplicate results).

Sample[a]	Label	This work		BP Titration	
	mg/unit	mg/unit	%Label	mg/unit	%Label
No.1	600	573	96	610	102
No.2	250	243	97	246	99

[a] carbonate form, No.1 : tablet, No.2 : capsule

4. Conclusion

A simple FI determination of Ca(II) is proposed. It is based on the replacement of Mg(II)-EDTA by Ca(II). The released Mg(II) forms colored Mg(II)-Eriochrome Black T complex. The FI peak due to absorbance (at 530 nm) of the complex is proportional to Ca(II) concentration. The proposed procedure with very simple detection and high sample through-put is an alternative cost-effective assay of Ca(II) in mineral supplement.

5. Acknowledgements

We thank the Thailand Research Fund (TRF), the Postgraduate Education and Research Program in Chemistry (PERCH) and the Commission in higher Education including the University Development Committee (UDC) for supports and Dr. Yuthsak Vaneesorn for useful discussion.

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(Received March 18, 2005)
(Accepted March 30, 2005)

WATER RESEARCH 40 (2006) 2044–2054

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Interaction of trace elements in acid mine drainage solution with humic acid

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ARTICLE INFO

Article history:
Received 5 July 2005
Received in revised form
24 February 2006
Accepted 7 March 2006

Keywords:
Acid mine drainage
Trace metal ions
Humic acid
Flow field-flow fractionation

ABSTRACT

The release of metal ions from a coal mining tailing area, Lamphun, Northern Thailand, is studied by leaching tests. Considerable amounts of Mn, Fe, Al, Ni and Co are dissolved in both simulated rain water (pH 4) and 10 mg L^{-1} humic acid (HA) solution (Aldrich humic acid, pH 7). Due to the presence of oxidizing pyrite and sulfide minerals, the pH in both leachates decreases down to ~3 combined with high sulfate concentrations typical to acid mine drainage (AMD) water composition. Interaction of the acidic leachates upon mixing with ground- and surface water containing natural organic matter is simulated by subsequent dilution (1:100; 1:200; 1:300; 1:500) with a 10 mg L^{-1} HA solution (ionic strength: 10^{-3} mol L^{-1}). Combining asymmetric flow field-flow fractionation (AsFlFFF) with UV/Vis and ICP-MS detection allows for the investigation of metal ion interaction with HA colloid and colloid size evolution. Formation of colloid aggregates is observed by filtration and AsFlFFF depending on the degree of the dilution. While the average HA size is initially found to be $\leqslant 2$ *nm*, metal–HA complexes are always found to be larger. Such observation is attributed to a metal induced HA agglomeration, which is found even at low coverage of HA functional groups with metal ions. Increasing the metal ion to HA ratio, the HA bound metal ions and the HA entities are growing in size from <3 to >450 nm. At high metal ion to HA ratios, precipitation of FeOOH phases and HA agglomeration due to colloid charge neutralization by complete saturation of HA complexing sites are responsible for the fact that most of Fe and Al precipitate and are found in a size fraction >450 *nm*. In the more diluted solutions, HA is more relevant as a carrier for metal ion mobilization.

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1. Introduction

Approximately 20% of the total electricity in Thailand is generated by coal burning (Arbhabhirama et al., 1987). Most of the coal mines are the open-pit mines, where the tailings are placed around the mining area exposed to weathering and leaching by rain. It is well known that the tailings in mining areas are composed of various kinds of minerals containing high amounts of heavy metals (Kucukonder et al., 2003; Matlock et al., 2002; Lee et al., 2002; Dang et al., 2002). Residual waste crushed rock from mining activities represents a major source for metal rich acid mine drainage (AMD) water. The tailing minerals can release metal ions over long time scales due to oxidation of sulfide minerals leading to the production

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doi:10.1016/j.watres.2006.03.009

of sulfuric acid (e.g. Bunce et al., 2001) according to the overall equations:

$$FeS_2(s) + \tfrac{7}{2}O_2(g) + H_2O \rightarrow Fe^{2+}(aq) + 2H^+(aq) + 2SO_4^{2-}(aq),$$
$$2Fe^{2+}(aq) + \tfrac{1}{2}O_2(g) + 5H_2O(l) \rightarrow 2Fe(OH)_3(s) + 4H^+(aq).$$

Metals in the waste rock can be released by the low pH AMD (Kucukonder et al., 2003; Matlock et al., 2002; Lee et al., 2002; Dang et al., 2002; Bunce et al., 2001; Vigneault et al., 2001) and possibly be transported by rainwater and surface water away from the source. Quite a number of studies in many countries have been performed to study the release of metals from coal mine spoils, but only a few in Thailand. Those reports concerned the weathering process of the tailings using leaching experiment and sequential extraction (Yukselen and Alpaslan, 2001; Sun et al., 2001; Sahuquillo et al., 2002; Badulis, 1998). They focused on only how the metals are leached out. However, the mechanisms leading to the possible migration of heavy metals further away downstream from the mining site is still not clear. The geochemical behavior of metals released from the acidic tailing minerals depends on a number of different parameters. As soon as the AMD is diluted and pH increases, a number of precipitating phases have been observed: jarosite, schwertmannite, ferrihydrite (Hochella et al., 1999; Dinelli et al., 2001) and mixed amorphous gel like phases with more or less variable composition (Featherstone and O'Grady, 1997; Munka et al., 2002). Precipitation of those phases going along with metal adsorption and co-precipitation leads to the retention of released metal ions. The mobility of metal ions then is often determined by potentially forming particulate and colloidal species being stabilized and dispersed in the water. Particulate and colloidal species of metals such as Fe, As, Cd, Cu, Mn, Pb and Zn derived from tailing minerals have indeed been observed to migrate over long distances (Featherstone and O'Grady, 1997; Kimball et al., 1995; Zänker et al., 2002; Schemel et al., 2000). Purely inorganic colloidal species composed of high AlOOH and FeOOH amounts have their isoelectric point close to the pH of natural water and thus exhibit only low colloidal stability. Surface sorption or coating with silicate or natural organic matter as humic or fulvic acid is known to shift the isoelectrical point of colloidal particles to lower pH (e.g. Kretzschmar et al. 1998), and to increase inter-particle charge repulsion and thus colloidal stability. Humic and fulvic acids being present in natural water in concentrations ranging from 0.5 to 100 mg/L (Frimmel, 1998) can on the other hand form metal-complexes in certain pH ranges (Gundersen and Steinnes, 2003) and by this way enhance the metal mobility.

The aim of the present work is to study the colloid formation upon AMD dilution in presence of humic acid to simulate the interaction of acidic leachates upon mixing with ground- and surface water containing natural organic matter. Humic and fulvic acids are known to be major constituents of organic matter in most soils and groundwater (see e.g. Stevensen, 1994). Humic acid is thus taken to simulate the influence of natural organic matter on metal ion colloid formation. AMD is prepared by leaching of a tailing sample taken from a disposal of the Lamphun area with simulated rain water (RW) or humic acid containing solution. The solution compositions of the two leachates are compared with water samples taken from a pond fed by draining water from the mine. The leachate obtained with the humic acid solution (10 mg L^{-1}, pH 7) is then subsequently diluted with 10 mg L^{-1} humic acid solution to final pH values ranging from 4.6 to 7.6 and the colloidal species formed are characterized by using the asymmetric flow field-flow fractionation (AsFlFFF) combined with UV/VIS absorbance and ICP-mass spectrometric detection. Humic acid concentration and pH values lie in the range of natural conditions.

2. Materials and methods

2.1. *Analysis of the tailings and water samples*

AMD water samples from a coal mine disposal area in northern Thailand (Lamphun area) were collected from two sites of a pond during summer season (September: BP-S; LN-S) and rainy season (October: BP-O; LN-O). Tailing samples were taken from the same site. Grain particles with a size < 1 mm were separated by sieving and used for characterization by X-ray diffraction (XRD-3000, Seifert, Germany), scanning electron microscopy (SEM-EDX, CamScan CS44 FE, England), X-ray fluorescence (XRF, Siemens, SRS 3000, Germany), Organic carbon analysis (TOC-5000, Shimadzu, Japan) and trace element analysis by ICP-mass spectrometry (ELAN6000, Perkin-Elmer, USA) after total digestion (HNO_3/HF) in a microwave oven.

2.2. *Leaching of the tailing sample*

Batch leaching tests with two leachants were performed to follow the leaching behavior of some metals in the tailings: (1) simulated RW (2×10^{-5} mol L^{-1} HNO_3 (Merck, Germany), 3×10^{-5} mol L^{-1} H_2SO_4 (Merck, Germany), 1×10^{-5} mol L^{-1} HCl (Merck, Germany) and 2×10^{-5} mol L^{-1} NH_3 (Merck, Germany) (recipe: Sigg and Stumm, 1989) and (2) 10 mg L^{-1} Aldrich humic acid (pH 7, in 10^{-3} mol L^{-1} $NaClO_4$). Fifty mL aliquots of each leachant were added to 2.5 g of ground tailings material. Each mixture was rotated vertically at a rate of 1 rpm for 10 days. After the leaching period, the mixture was centrifuged and the leachate was separated and collected into a container. Metal ions and anions in the leachates were analyzed by ICP-MS and IC, respectively. Fe(II)/Fe(III) speciation was done by application of the classical spectrophotometric procedure described in American Water Works Association and Water Pollution Control Federation (1989).

2.3. *Sequential dilution of the leachate*

In order to simulate the interaction of metal ions released from the tailings to the surface- or groundwater, a sequential dilution of the humic acid solution leachates was performed. In experiment 1, aliquots of the leachate were diluted with a 10 mg L^{-1} humic acid solution of an initial pH 6.7 and containing 10^{-3} mol L^{-1} $NaClO_4$. In parallel, an analogous test was made with a 10 mg L^{-1} humic acid solution of higher initial pH (pH 9; ionic strength: 10^{-3} mol L^{-1} $NaClO_4$) (experiment 2). The dilution ratios studied in the present work were

1:100, 1:200, 1:300, and 1:500. The diluted leachate solutions were stored up to a week until the final pH established. The colloid particles < 450 nm in the filtered solutions (syringe filter with pore size: 450 nm) were characterized by the AsFlFFF-ICP-MS system. The solutions before and after filtration were analyzed by ICP-MS in order to determine the particulate metal ion fraction. The particulate fraction in this case is defined as particles $>450\,nm$.

2.4. *Analysis of colloidal species*

Characterization of the colloidal species in the leachates was performed by AsFlFFF (HRFFF 10.000 Series, Postnova Analytics, Germany). The carrier was degassed by a 1100 series vacuum degasser model G1322A (Hewlett–Packard, Waldbronn, Germany). Buffer solutions were used as carriers for the AsFlFFF experiments: MES (pH 5, $10^{-3}\,mol\,L^{-1}$) (Sigma, USA) and TRIS (pH 7, $10^{-3}\,mol\,L^{-1}$). Samples generated in experiment 1 (pH ranging from 4.6 to 6.1) were fractionated in the MES buffer (pH 5), experiment 2 samples (pH ranging from 6.0 to 7.6) in the TRIS buffer (pH 7). The pH of sample solutions and the respective carrier solution, therefore, did not vary by more than 1 unit. We do not expect a major influence of these pH differences in sample and carrier solutions on the HA colloids. In-line filters (teflon membrane; pore size: 0.1 μm) were placed in the carrier tubings before entering the channel in order to retain particulate impurities coming from the internal constituents of the FFF-equipment e.g. abraded material from pumps. Thickness of the channel was 350 μm. The ultrafiltration membrane used as accumulation wall consists of regenerated cellulose with 5 kDa cutoff (Postnova Analytics, Germany). Previous experiments proved that more than 85% humic acid passed the channel under the fractionation conditions used here even though the average molecular weight was clearly lower than 5 kDa (Ngo Manh et al., 2001). Major reasons for this finding are the molecular structure of humic acid being more extended than that of globular proteins for which the membrane cutoffs are usually defined and the negative charge of the humic colloids leading to repulsion from the membrane surface (Guo et al., 2000). For each experiment, sample aliquots of 500 μL were injected into the channel via a 6-port Rheodyne injection valve. A focusing time of 180 s was selected. Elution was carried out by applying an axial channel flow rate of $1\,mL\,min^{-1}$ and a cross flow rate of $0.7\,mL\,min^{-1}$.

From the channel the carrier solution was directed through an UV–Vis detector (LambdaCmax 481, Waters, USA) recording the signal at $\lambda = 225\,nm$. The ICP-MS (ELAN6000, Perkin Elmer, USA) was connected to the AsFlFFF -UV–Vis flow-through cell via a T-junction (UpChurch, USA) to analyze metal ions continuously in a simultaneous mode. The stream eluted from the AsFlFFF system was in-line mixed with a 6% HNO_3 solution containing Rh as an internal standard ($100\,\mu g\,L^{-1}$). The size calibration using latex standards (20, 50 and 100 nm in diameter, Duke Scientific Corporation, California) is made by plotting $\log V_R$ against $\log d_h$ ($\log V_R = 0.34 + 0.5\log d_h$, $R = 0.9968$), where V_R is the retention volume and d_h is the hydrodynamic diameter and R is the correlation coefficient.

3. Results and discussion

3.1. *Characterization of solids and solutions*

The elemental composition of the tailing sample used for leaching experiments is given in Table 1. Main mineral components as derived by XRD inspection are quartz (SiO_2), jarosite ($KFe_3(SO_4)_2(OH)_6$) (an oxidation product of pyrite (FeS_2)), gypsum ($CaSO_4 \cdot 2H_2O$), and additionally muscovite ($KAl_3Si_3O_{10}(OH)_2$) and kaolinite ($Al_2Si_2O_5(OH)_4$). Analysis of minor components by SEM-EDX showed the presence of iron sulfides and gypsum. The composition of the solutions resulting from the leaching experiments with $10\,mg\,L^{-1}$ HA solution and simulated RW is given in Table 2. As expected, the solutions become acidic (pH 2.6–2.7) and rich in sulfate in both leachates. These values remained more or less independent of the solid/liquid ratio of the experiment. The same pH values and sulfate concentrations were observed in column leaching experiments (not described here) with the identical solid material and solutions. Beside high Ca, Fe, Mn, Mg, Al and Na concentrations, transition metal ions as Co, Cu, Ni and Zn were present up to $3\,mg\,L^{-1}$. The final composition of the leachates is primarily determined by dissolution of acidic components from the tailings. The initial solution composition and notably the presence or absence of humic acid does not play a significant role. Weak complexing characteristics of the fully protonated HA functional groups and the potential sorption to the tailings material under the acidic conditions may explain why the HA solution does not mobilize more metal ions than the simulated RW. The composition of the leachates obtained in the laboratory experiments is quite similar to that found in some water samples collected at the mining areas (see also Table 2). Those samples were taken from a pond at the bottom of the mining site which is fed by drainage water from the mine in September (summer time in Thailand, sample BP-S and LN-S) and in October (raining season, BP-O and LN-O). The pH-values of the BP-S and LN-S samples are considerably lower than those of the BP-O and LN-O solutions likely due to the dilution by RW. In general, the

Table 1 – Composition of the tailings sample as analyzed by XRF, XRD, SEM-EDX, TOC and ICP-MS (grain fraction: < 1 mm)

Element	Content	Method
Si	40.5% (w/w)	XRF
Al	20.5% (w/w)	XRF
Fe	12.7% (w/w)	XRF
As	$137.5\,\mu g\,g^{-1}$	ICP-MS
TOC	6.2% (w/w)	TOC-analyzer
Minerals		
Quartz, Jarosite, Gypsum, Muscovite, Kaolinite	N/Q	XRD
Gypsum, FeS_2	N/Q	SEM-EDX

N/Q: Not quantified.

Table 2 – Composition of leachate solution (10 mg L^{-1} HA, 1×10^{-3} mol L^{-1} $NaClO_4$) and water samples collected from a pond on site. BP-S/LN-S are taken in September (summer season), BP-O/LN-O in October (rainy season). (Analytical uncertainties are < 10%.)

	Tailings leachate		BP-S	LN-S	BP-O	LN-O
	HA-solution	sim. rain-water solution				
pH	2.6	2.7	2.43	2.41	5.88	5.22
DOC (mg L^{-1})	N/A	N/A	29.7	28.8	3.8	4.6
Trace elements (mg L^{-1})						
Fe	84.7	119.4	331 (80% Fe(II))	339 (75% Fe(II))	1.4 (86% Fe(II))	2.2 (59% Fe(II))
Al	15.9	21.5	28.4	31.1	0.7	2.2
Mn	23.0	26.9	23.8	24.2	3.0	3.6
Ca	133	164	99.6	104.6	30.4	33.0
Mg	17.3	20.4	12.4	13.3	7.2	7.3
Na	14.9	0.1	5.5	5.9	36.8	35.2
Trace elements (μg L^{-1})						
Co	500	517	349	373	27	28
Ni	1200	1467	1900	900	100	100
Cu	900	1069	182	172	23	16
Zn	2900	3377	1600	1700	N/A	N/A
Cd	11	14	N/A	N/A	N/A	N/A
Pb	0.74	0.5	37	34	1.3	1.3
Ce	267	320	137	148	0.5	1.3
Eu	8.5	8.6	N/A	N/A	N/A	N/A
La	101	107	N/A	N/A	N/A	N/A
Th	41	42	10	11	0.5	0.1
U	23	25	18	19	0.6	0.9
Anions (mg L^{-1})						
SO_4^{2-}	1182	1152	1487	1524	166	173
Cl^-	3.8	2.7	47	57	40	37
NO_3^-	< 0.1	3.6	< 0.1	< 0.1	4.3	1.3
PO_4^{3-}	< 0.1	<0.1	< 0.1	< 0.1	< 0.1	< 0.1

N/A: not analysed.

concentrations of inorganic and organic components in the water samples collected during the rainy season are lower (1) due to the dilution effect and (2) due to oxidation of Fe(II) with subsequent formation of Fe(III)OOH precipitates scavenging the trace elements. The compositional similarity of natural AMD taken from the pond at the site and the leachate obtained from the tailing material in the laboratory demonstrates that the leachate composition is typical for AMD in the investigated system. The nature of the relatively high organic carbon content in the pond water samples is not yet clear and no attempt was made within the present study to elucidate the nature of those compounds.

3.2. *Metal ion speciation in AMD leachates*

We performed simulation experiments in order to estimate the formation of colloidal and precipitating metal species upon dilution of the AMD in presence of humic acid. The acidic solution extracted from the tailing soil was subsequently mixed with humic acid containing solutions with initial pH values of 6.7 (experiment 1) and 9 (experiment 2), respectively. The leachate was diluted by factors of 100, 200, 300 and 500. Resulting Fe and Al concentrations and the pH values of the diluted solutions are given in Tables 3 and 4 and respective data are plotted in Figs. 1 and 2 for both dilution experiments. The pH values established in the final solutions cover a range relevant to natural conditions. Diluted solutions were aged for 1 week in order to reach steady-state conditions and to avoid reaction kinetics overlaying the results. Aim of the experiments was to provide an estimate of the conditions where metal ions in AMD could be mobilized due to colloid formation or could be rather retained by precipitation or co-precipitation.

In a first step we consulted existing thermodynamic data bases in order to estimate the speciation of metal ions in our diluted leachate solutions under oxidizing conditions. We used the speciation code ECOSAT (Keizer and van Riemsdijk, 1994) for all calculations. If not specified explicitly in the text, we took the thermodynamic data summarized by Lindsay (1979), which are implemented in the ECOSAT data base. Solubility of Fe(III) and Al(III) is calculated as a function of pH taking the solution composition of the 1:100 diluted leachate (Table 3) in absence of HA as a basis. The final pH values, Fe and Al concentrations of the diluted leachates obtained in experiments 1 and 2 are compared with calculated solubility curves in Fig. 1. As a result, over-saturation for Fe(III) with

Table 3 – Fe concentration in the leachate solutions diluted with humic acid solution (10 mg L^{-1}) with an initial pH 6.7 (exp. 1) and pH 9 (exp. 2)

Dilution factor for the leachate solution	Fe-conc. after dilution (mol L^{-1})	HA-COO$^-$ concentration (mol L^{-1})	Molar ratio Fe(III)/HA-COO$^-$	Exp. 1		Exp. 2	
				Final pH (initial HA solution pH 6.7)	Fe fraction >450 nm(%)	Final pH (initial HA solution pH 9)	Fe fraction >450 nm (%)
1 : 500	3.94×10^{-6}	5.3×10^{-5}	1 : 13.4	6.1	17.7	7.6	0.2
1 : 300	6.57×10^{-6}	5.3×10^{-5}	1 : 8.1	5.6	17.9	7.2	7.2
1 : 200	9.85×10^{-6}	5.3×10^{-5}	1 : 5.4	5.2	18.7	6.9	17.9
1 : 100	1.97×10^{-5}	5.3×10^{-5}	1 : 2.7	4.6	40.0	6.0	53.4

Initial concentration of Fe in the leachate is 110 mg L^{-1} (1.97×10^{-3} mol L^{-1}); the concentration of carboxylate groups in the HA is taken as 5.3×10^{-3} mol g^{-1} (Rabung et al., 1996).

regard to ferrihydrite (log (K_{sol}): -38.46, Lindsay, 1979) in the pH range of interest ($pH \geqslant 4$) is expected for both dilution experiments. In case of Al(III), the solubility limit with regard to amorphous $Al(OH)_3$ (log (K_{sol}): -32.34, Lindsay, 1979) is expected to be exceeded slightly in a pH range 6.5–7.5. However, oversaturation occurs at $pH \geqslant 5$ when assuming gibbsite as solubility determining solid phase (log (K_{sol}): -33.96, Lindsay, 1979). The presence of humic acid is anticipated to prevent precipitation due to formation of humate complexes. Using the generic parameters for humic acid and its complex formation constants with Al(III) and Fe(III) as published by Milne et al. (2003) and applying the Nica Donnan model (Kinniburgh et al., 1999), results in a speciation plot as given in Fig. 2. According to this calculation, a concentration of 10 mg L^{-1} HA appears to be insufficient to suppress ferrihydrite precipitation completely. According to the calculations, precipitation of ferrihydrite is prevented by humic acid in the higher diluted leachates. The same is true for Al(III) if we consider gibbsite precipitating. If we, however, assume that rather amorphous than crystalline phases are generated under given conditions, Al(III) species should exist as dissolved species in presence of humic acid for all dilutions. The solution speciation of Fe(III)/Al(III) is clearly dominated by humic acid complexes. The calculations predict that humic acid is able to keep considerable amounts of both metal ions via complexation in solution and thus to enhance their mobility.

Experimental data reveal that significant fractions of Fe and Al can be separated from all diluted solutions by filtration through a 450 nm membrane filter (see Tables 3 and 4). This is somewhat in contradiction to the calculations saying that even $Fe(OH)_3$ phases should not precipitate in the higher diluted samples. The presence of relatively high particulate Al fractions (>450 *nm*)-similar to what is found for Fe—is at first glance also not compatible with the speciation calculations. The Al behavior might be explained by formation of amorphous Fe/Al co-precipitates in the concentrated solutions (1:100 diluted leachate). Another more plausible explanation is considerable flocculation of humic acid by interaction with Fe/Al ions. A number of spectroscopic studies verified that hard Lewis acids like Al(III) as well as Tb(III) and Th(IV) mainly interact with humic acid carboxylate groups (Lee and Ryan, 2004; Monsallier et al., 2003; Denecke et al., 1999). Assuming three carboxylate ligand groups interacting with one trivalent cation and taking the potentiometrically determined carboxylate concentration of the Aldrich HA (5.3×10^{-3} mol g^{-1}; Rabung et al., 1998) leads to the molar metal ion/HA ratios given in Tables 3 and 4. Note that the experimentally determined concentration of carboxylate groups (taken from Rabung et al., 1998) is quite consistent with the generic value for functional HA-groups assumed by Milne et al. (2003) (5.7×10^{-3} mol g^{-1}). The concentration of those metal ions which are strongly interacting with humic acid is dominated by Fe(III) in the leachate. The carboxylate ligand to Fe(III) ion ratio is close to 3:1 in the 1:100 diluted solution, so that HA functional groups are completely occupied with Fe(III), if we consider three carboxylate groups interacting with one trivalent Fe(III) ion. Deprotonated carboxylate groups are mainly responsible for the overall negative net charge of the humic acid macromolecule

Table 4 – Al concentration in the leachate solutions diluted with humic acid solution (10 mg L^{-1}) with an initial pH 6.7 (exp. 1) and pH 9 (exp. 2)

Dilution factor for the leachate solution	Al-conc. after dilution (mol L^{-1})	HA-COO^- concentration (mol L^{-1})	Molar ratio Al(III)/HA-COO^-	Exp. 1		Exp. 2	
				Final pH (initial HA solution pH 6.7)	Al-fraction >450 nm (%)	Final pH (initial HA solution pH 9)	Al-fraction >450 nm (%)
1 : 500	1.48×10^{-6}	5.3×10^{-5}	1: 35.8	6.1	27.1	7.6	9.1
1 : 300	2.47×10^{-6}	5.3×10^{-5}	1: 21.6	5.6	34.6	7.2	31.9
1 : 200	3.71×10^{-6}	5.3×10^{-5}	1: 14.2	5.2	33.8	6.9	31.7
1 : 100	7.41×10^{-6}	5.3×10^{-5}	1: 7.2	4.6	43.3	6.0	53.8

Initial concentration of Al in the leachate is 20 mg L^{-1} (7.4×10^{-4} mol L^{-1}); the concentration of carboxylate groups in the HA is taken as 5.3×10^{-3} mol g^{-1} (Rabung et al., 1996).

preventing agglomeration and precipitation. Charge neutralization of the HA by the interacting cations will certainly lead to extensive HA flocculation. Flocculation in addition to ferrihydrite precipitation explains the presence not only of Fe(III) but also of Al(III) in the >450 *nm* fraction of the 1:100 diluted solution. The existence of Fe and Al in the size fraction >450 *nm* in the higher diluted sample solutions, where ferrihydrite precipitation becomes unlikely, must then mainly be due to HA flocculation. The Fe or Al fraction larger than 450 nm decreases at higher dilutions and this particulate fraction is obviously lower for Fe in experiment 2 where the pH is higher. This pH effect may be the consequence of the higher dissociation degree of the humic carboxylic acid groups impeding aggregation. We should also mention that the assumption of three carboxylate groups interacting with one trivalent cation must not necessarily be true. Ternary complex formation has been found for the trivalent actinide ion Cm(III) where e.g. significant fractions of hydroxy humate complexes may easily exist at near neutral pH (Panak et al., 1996). More metal ions can interact with the humic acid until full charge neutralization will be achieved in case of ternary humate carbonate or hydroxy complex formation.

Ca(II) is also known to induce HA flocculation (Schimpf and Petteys, 1997; Benincasa et al., 2002; Bryan et al., 2001). Wall and Choppin (2003) found in their experiments critical coagulation concentrations for Ca(II) of around 10^{-3} mol L^{-1} before significant HA flocculation ([HA] ~ 100 mg L^{-1}, pH 8.3) is observed. Ca(II) concentrations of up to 3.3×10^{-5} mol L^{-1} (in the 1:100 diluted leachate) are two orders of magnitude lower than this threshold. According to our calculations, considerable Ca(II) complexation with HA is only expected at pH>6.2 so that due to the higher charge and the stronger interaction with HA, the influence of the Fe(III)/Al(III) cations has to be assessed as more relevant for charge neutralization and the experimentally observed HA flocculation.

3.3. *Colloid characterization*

We studied the colloidal fraction (size<450 nm) in the sequentially diluted leachates by AsFlFFF combined with UV/Vis and ICP-MS detection. The photometric detection allows monitoring of the HA in the FFF effluent while ICP-MS detects colloid-bound metal ions.

The fractograms (Figs. 3 and 4) clearly visualize the existence of an agglomeration process depending on metal ion concentration in the leachate. A clear increase of the average HA colloid size is observed by UV/Vis absorbance with increasing Fe or Al to HA ratio. The peak maximum of the HA size distribution under given conditions lies at ⩽2 *nm* related to the calibration with polystyrene particles. The average HA size increases considerably by increasing the Fe(III) to HA(carboxylate functional group) ratio. In the fractograms of the solution prepared in experiment.1 containing the highest metal ion concentration (dilution: 1:100), we could hardly find any peak for HA. Only at very large retention times (corresponding to a size of ~200 nm) a small peak could be observed at the same position as for Al and Fe. The major part of the Fe/Al/HA species, however, is located in the >450 nm fraction as has been mentioned already earlier (see Tables 3 and 4). In experiment 2, i.e. at higher pH, the observation is

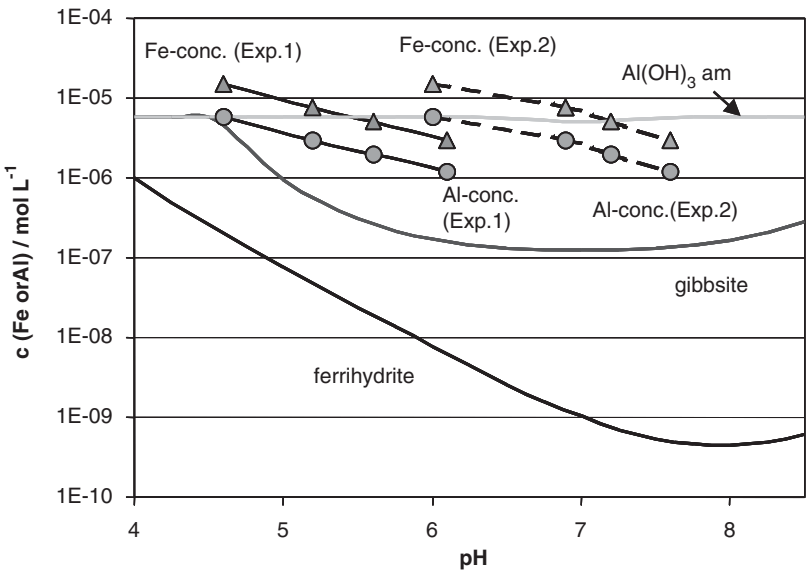

Fig. 1 – Solubility of Fe(III) and Al(III) in absence of humic acid as a function of pH in the diluted (1:100) leachate from coal mining tailings.

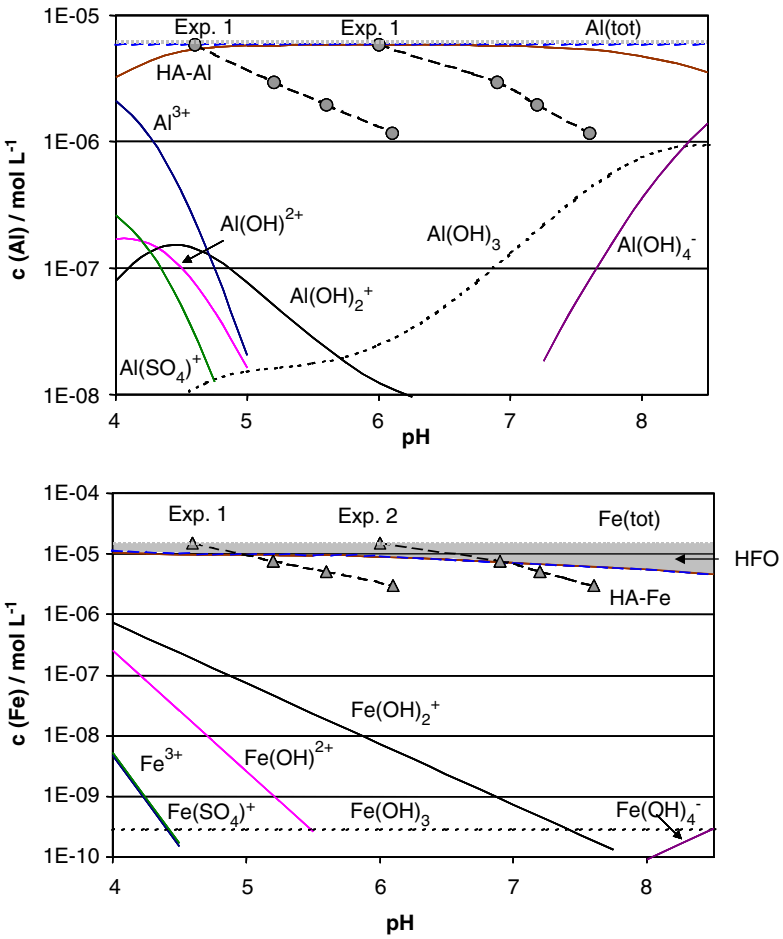

Fig. 2 – Speciation of Fe(III) and Al(III) in presence of humic acid as a function of pH in the diluted (1:100) leachate from coal mining tailings (HFO: hydrous ferric oxide).

somewhat different. The colloid fraction passing the 450 nm filter is not such strongly agglomerated as observed at the lower pH values of experiment 1. The average colloid size at a Fe(III) to HA (carboxylate functional group) ratio of ~1:3 is ~7 nm, where the size distribution ranges from 3 to 20 nm. It appears that there are still functional groups not coordinated

to metal ions which at the higher pH induce a sufficiently high negative surface charge, thus leading to a stronger stabilization of a relatively small colloid fraction in solution. One explanation for this observation could be the formation of hydroxy or carbonate humate ternary complexes at the higher pH with less coordination of HA carboxylate groups to the metal ion.

The combination of FFF with UV/Vis and ICP-MS detection allows not only to observe the evolution of the HA size but also of the HA-bound metal size distribution. Fractograms as given in Figs. 3,4,5 reveal a marked feature: at low metal loading, HA and the metal humate complexes exhibit different size distributions with significantly different peak maxima. Those differences are perceptible in both dilution experiments. It is also striking that the size of colloid borne divalent metal ions such as Cu(II) is always closer to that of HA than that of the trivalent cations (Fe(III) and Al(III)). Colloidal metal ion species are larger than the average HA entities, whereby the higher metal charge seems to increase the observed colloid size. Such behavior was already observed in earlier studies (Hassellöv et al., 1999; Geckeis et al., 2002, 2003; Bouby et al., 2002; Lyven et al., 2003). One possible explanation is the occurrence of metal induced agglomeration of HA macromolecules. Our studies show that this effect appears at even low metal loading. This finding points to the interaction of polyvalent metal ions with more than one HA entity thus acting as bridging unit. A second possibility is that complexed metal ions effectively screen the HA surface charge and thus favor agglomeration of those units. Such processes are in line with the observation that for lower charged metal ions (e.g. Cu(II)) the size increasing effect is smaller than for metals with higher charge (e.g. Fe(III)/Al(III)). This effect is illustrated exemplary in Fig. 5 for U, Ce, Th. measured by ICP-MS additionally after injection of the 1:500 diluted leachate solution (experiment 2) where those metal ions are present at trace concentrations $<1\,\mu g\,L^{-1}$ in the investigated HA solution. The shifts in the observed size distribution pattern are significant and reproducible. Under given conditions uranium exists in the hexavalent state as humate complex of the divalent UO_2^{2+} ion where cerium as a representative of the lanthanides is trivalent and thorium tetravalent. The observations that (1) the average size of the metal bearing colloids is larger than the average size of the HA entities and (2) the dependence of average metal-humate size on the metal ion charge both suggest the existence of metal induced agglomeration processes influenced by the individual metal ion charge.

With increasing metal loading, HA and metal–HA entities progressively grow in size and finally the fractograms for both species approach to almost coincidence. The difference between the peaks of HA (detected by UV–VIS) and the metal–HA fractograms (detected by ICP-MS) obviously can be

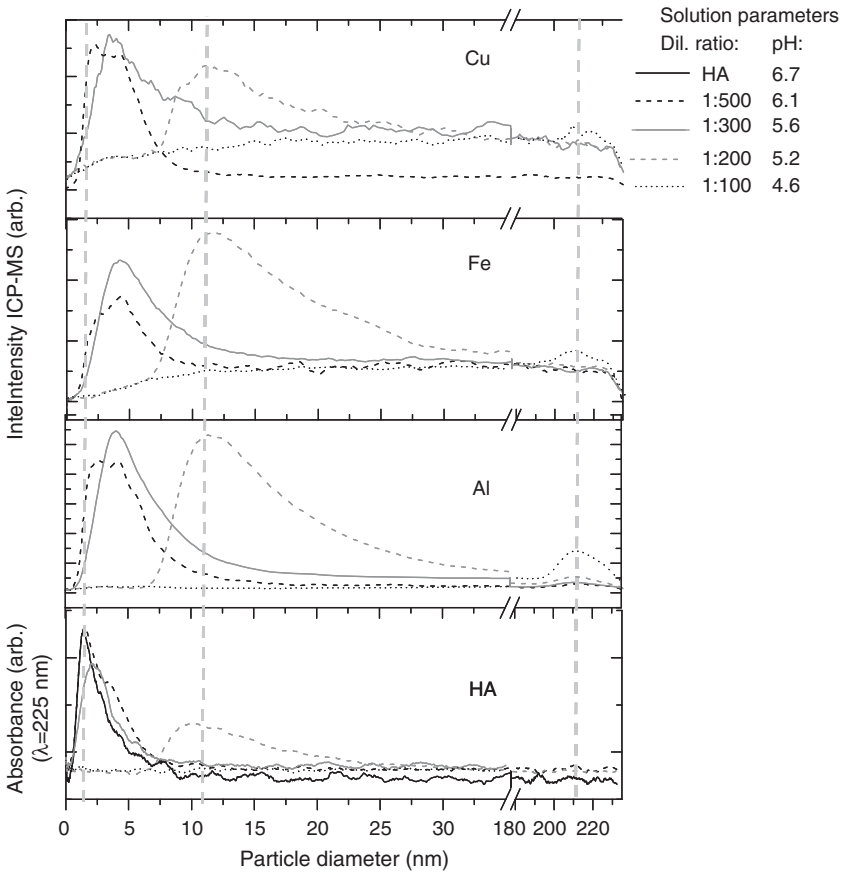

Fig. 3 – AsFlFFF-ICP-MS fractograms for solutions of experiment 1; dilution of the leachate with HA ($10\,mg\,L^{-1}$ in $0.1\,mol\,L^{-1}$ $NaClO_4$; initial pH: 6.7). A fractogram of the HA solution are included for comparison.

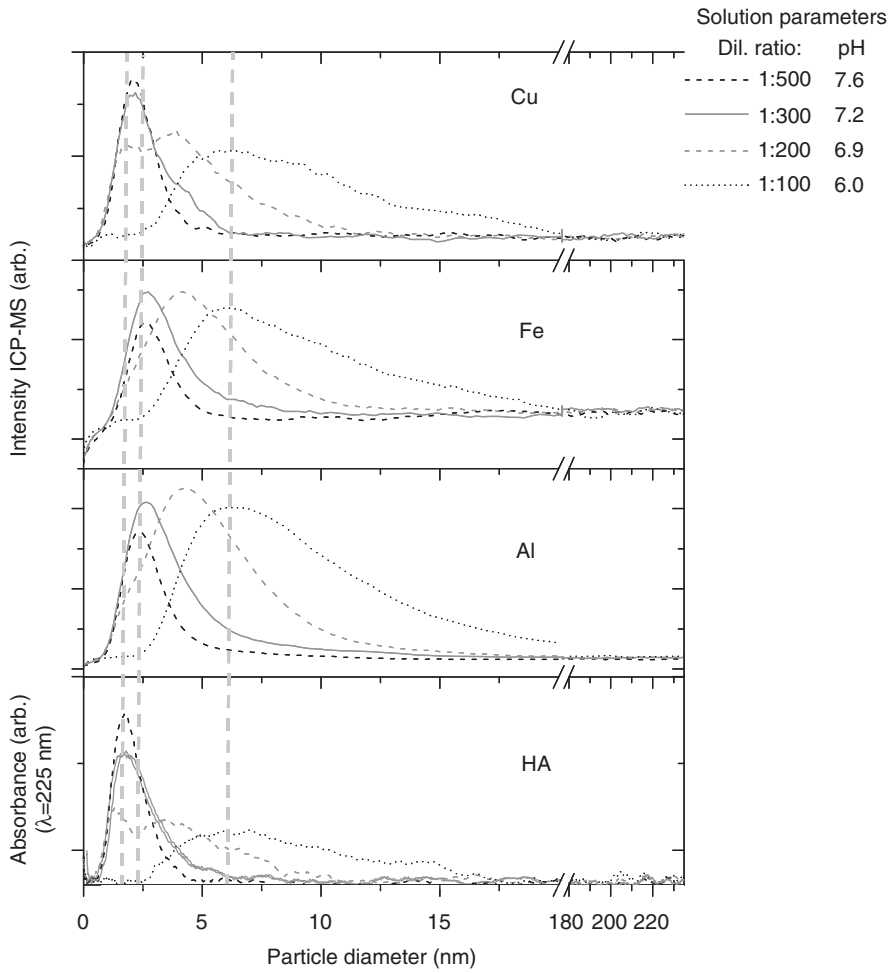

Fig. 4 – AsFlFFF-ICP-MS fractograms for solutions of experiment 2; dilution of the leachate with HA (10 mg L^{-1} in 0.1 mol L^{-1} $NaClO_4$; initial pH: 9).

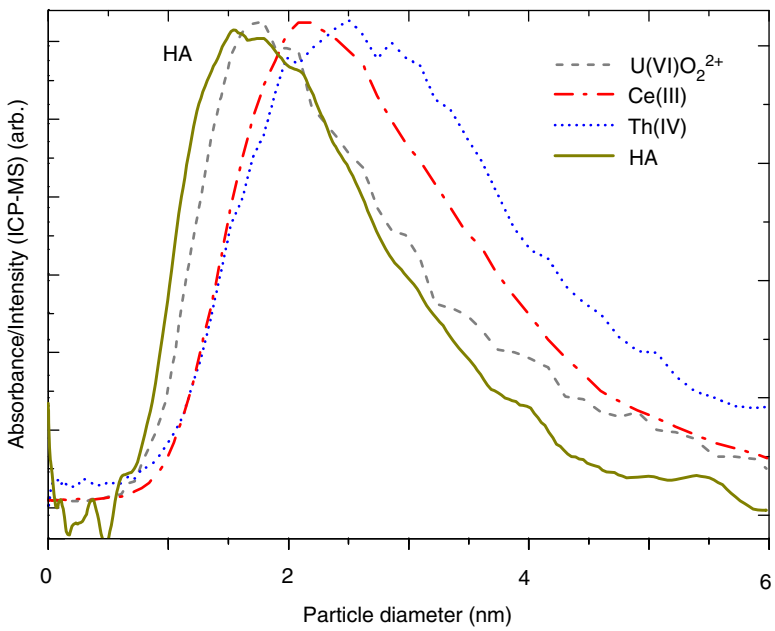

Fig. 5 – AsFlFFF-UV/Vis and ICP-MS fractograms for a diluted leachate (experiment 2; 1:300 dilution; pH 7.2).

taken as a measure for the degree of metal-loading: The larger the difference between the peak maxima, the lower the overall HA loading. In our experiments, the complete HA loading with Fe(III) seems to correspond closely to the complete occupation of three carboxylate groups with one trivalent Fe(III) ion at pH $\sim$ 6 (experiment. 2) while at pH $\sim$ 5 (experiment. 1) loading is already complete if only $\sim$60% of the functional HA groups are occupied (again assuming one Fe ion interacting with three HA carboxylate groups). Under those conditions about half of Fe and Al already form agglomerates with a size $>$450 nm.

Size evolution of HA upon interaction with another trivalent metal ion (Eu(III)) has been also studied earlier at very low HA concentration (0.26 mg L^{-1}) by using the laser-induced breakdown detection (LIBD) at pH 6.0 (Bouby et al., 2002). A clear HA agglomeration could be established in this experiment only at metal loading $\geqslant$60% (assumption: Eu(III) interaction with three HA carboxylate groups) quite consistent with our present results. Various other investigations by using e.g. using atomic force microscopy (AFM) (Plaschke et al., 2000) or in the frame of spectroscopic studies on actinide complexation with HA (e.g. Czerwinski et al., 1996) show qualitatively the same HA behavior. Precipitation of significant HA fractions has been already stated at M(III)/HA ratios as low as 0.05 – 0.1 if pH decreases below 4 at an ionic strength of $I = 0.1$ mol L^{-1} (M(III): trivalent metal ion, Eu(III) or Am(III)) (Czerwinski et al., 1996). The Fe(III) induced coagulation of natural organic matter was investigated in a recent study by using transmission electron spectroscopy (TEM) and electron energy loss spectroscopy (EELS) (Jung et al., 2005). Fe ions were mainly found to be associated by carboxylate groups in coagulates and, therefore, a charge neutralization/complexation mechanism was suggested to be responsible for the aggregate formation.

4. Conclusions

The experiments indicate that metal ions dissolved in AMD may experience either retention or mobilization when released from the source. When diluted in near neutral natural water, metal ions can be kept to a certain degree in solution by humic substances through complexation. Agglomeration to larger HA aggregates and thus precipitation, sedimentation or attachment to surfaces can predominate under conditions where polyvalent metal ions as Fe(III) and Al(III) saturate the complexing functional HA groups. At very high metal ion concentrations, co-precipitation of Fe-oxyhydroxide/humic acid may also become relevant. Such behavior is in agreement with the outcome of earlier investigations: Transformation of marine dissolved organic matter into agglomerated polymer gels is known to have a shorter oceanic residence time due to enhanced sedimentation (Chin et al., 1998). Flocculation through interaction with polyvalent metal ions as Ca(II), Al(III) (Römkens and Dolfing, 1998) and Fe(III) (Jung et al., 2005) is reported to control the DOC concentration in soil pore water and during drinking water treatment processes. The complexation reaction is responsible for charge neutralization of HA entities thus reducing HA colloid stability (see also Jung et al., 2005). Our studies show that even at lower loading of HA with polyvalent metal ions a size increase is found for the metal–HA complexes as compared with the average HA colloid size. We interpret this result as an indication for polyvalent metal induced humic agglomeration taking place at even low metal ion concentration. Those species nevertheless remain suspended in solution, thus being mobile, and may act as carriers for metal ions.

Acknowledgements

The authors acknowledge the German Academic Exchange Service-Royal Golden Jubilee (DAAD-RGJ) Ph.D. Scholarship to S. S.. The Postgraduate Education and Research in Chemistry (PERCH) scholarship and the Thailand Research Fund (TRF) are grateful for partial financial support. S. S. would like to thank all the co-workers at Forschungszentrum Karlsruhe, Institut für Nukleare Entsorgung, for their help during performing the research in Germany notably assistance with analytical methods. The sample from the mine tailing was kindly provided by Dr. Ponlayuth Sooksamiti, the Office of Mineral Resources (Region III), Chiang Mai, Thailand. We thank Dr. Ron Beckett, Water Studies Centre, Monash University, Melbourne, Australia, for valuable discussions on the FFF results.

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Electroanalysis of sulfonamides by flow injection system/high-performance liquid chromatography coupled with amperometric detection using boron-doped diamond electrode☆

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Received 23 May 2005; received in revised form 15 August 2005; accepted 15 August 2005
Available online 23 September 2005

Abstract

Sulfonamides (SAs) were electrochemically investigated using cyclic voltammetry at a boron-doped diamond (BDD) electrode. Comparison experiments were carried out using a glassy carbon electrode. The BDD electrode provided well-resolved oxidation, irreversible cyclic voltammograms and higher current signals when compared to the glassy carbon electrode. Results obtained from using the BDD electrode in a flow injection system coupled with amperometric detection were illustrated. The optimum potential from a hydrodynamic voltammogram was found to be 1100 mV versus Ag/AgCl, which was chosen for the HPLC-amperometric system. Excellent results of linear range and detection limit were obtained. This method was also used for determination of sulfonamides in egg samples. The standard solutions of 5, 10, and 15 ppm were spiked in a real sample, and percentage of recoveries was found to be between 90.0 and 107.7.
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Keywords: Sulfonamides; Boron-doped diamond thin film electrode; Cyclic voltammetry; Flow injection system; HPLC; Amperometric detection

1. Introduction

Sulfonamides (SAs) have been used as antibacterial agents for over 60 years. They are often used for prevention or treatment of poultry leucocytozoonosis and coccidiosis, and are generally co-administered in feed. The European Union (EU) has set a maximum residue limit (MRL, 100 ng g^{-1}) for SAs in foods of animal origin such as meat, milk and eggs [1]. Therefore, the determination of such residues in meat and other animal by-products (milk and egg) used for human consumption has become an important task.

Owing to concern over sulfonamide residues in food products of animal origin, a number of techniques have been proposed for their detection, including, immunoassay [2,3], thin layer chromatography (TLC), gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) [4], capillary electrophoresis [5], and high performance liquid chromatography (HPLC) [6–17] and high performance liquid chromatography–mass spectrometry (HPLC-MS) [18–20]. HPLC with UV and a fluorometry detector were common methods for determining these drugs [6–9]. The alternative for determination of these SAs was HPLC with an electrochemical detector (HPLC-EC) using the amperometric technique [14]. HPLC-EC has been proved to be quite sensitive and inexpensive.

The diamond is one of nature's best insulators, but when doped with boron, the material can possess either semiconducting or semimetallic electronic properties depending on the doping level [21]. Therefore, the boron-doped diamond thin film electrode has shown unique characteristics that make it partic-

☆ Presented at the 13th International Conference on Flow Injection Analysis, April 24–29, 2005, Las Vegas, Nevada, and erroneously omitted from Talanta 68(2) 2005.

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doi:10.1016/j.talanta.2005.08.040

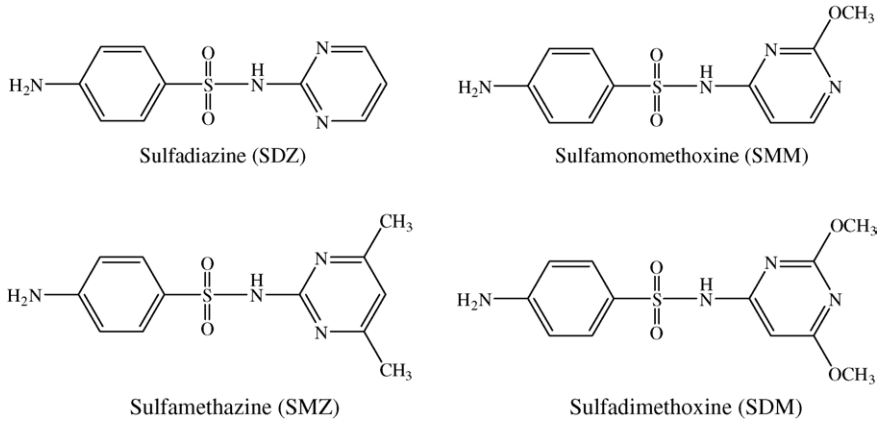

Fig. 1. Chemical structures of four SAs analyzed in this study.

ularly attractive for electrochemical applications, such as: (i) very low and stable voltammetric background current, which results in the improvement of signal-to-background [22,23]; (ii) long-term response stability as well as excellent activity towards any redox species without any pre-treatment [24–27]; (iii) low sensitivity to dissolved oxygen [28]; (iv) a wide working potential window in aqueous solution [29,30]; (v) slight adsorption of polar organic molecules [25]; and (vi) high resistance to deactivation. These material properties are the impetus for our interest in studying and developing diamond electrodes for electrochemical applications. With respect to the outstanding properties of BDD film, quite a number of applications, based on the use of this new material for electrochemical quantitation, have been reported [14,27,31–47]. This synthetic type of diamond film has already been applied as an amperometric detector in flow-injection (FI) analysis [27,31–37,40,42–46] and liquid chromatography [14,37–41,47]. Furthermore, the BDD electrode has been used for the treatment of wastewater [48,49].

This paper reported the use of the BDD electrode to detect four SAs: sulfadiazine, SDZ; sulfamethazine, SMZ; sulfamonomethoxine, SMM; and sulfadimethoxine, SDM. The structures of these four compounds are shown in Fig. 1. Cyclic voltammetry, flow injection analysis and HPLC with an amperometric detector were used in this study. The applicability of the method to analyze hen egg laying samples was demonstrated.

2. Experiment

2.1. Apparatus

The FIA and HPLC system used in this study consisted of a Water Model 510 solvent delivery system (Water Associates Inc, Milford, MA, USA), an injector system (Rheodyne no. 7125) with a 20-μL loop, Inertsil C4 column (GL Science, 150 mm × 4.6 mm i.d.; particle size, 5 μm), a thin layer flow-cell (GL Science Inc.), and an amperometric detector (Autolab Potentiostat 30; Metrohm, Switzerland). The following apparatus used in the sample preparation comprised a mini centrifuge (Cole Parmer, USA); an ultrasonic-homogenizer (Ney Dental, USA); Mixer (National, Matsushita Electric industrial Co. Ltd., Japan); and a micro-centrifugal ultrafilter unit (Ultrafree-MC/PL, regenerated cellulose ultra-filtration membrane, nominal molecular mass limit = 5000, capacity ≤0.5 mL, Millipore, Bedford, MA, USA).

2.2. Reagents

HPLC grade acetonitrile and ortho-phosphoric acid were purchased from Merck (Darmstad, Germany). Deionized water was from a Milli-Q-gradient system (Millipore, $R \geq 18.2$ MΩ cm). Sodium dihydrogen orthophosphate 1-hydrate and disodium hydrogen phosphate were purchased from BDH (VWR international Ltd., England). SMM, SDM, SMZ and SDZ standards were obtained from Sigma (St. Louis, MO, USA). Stock standard solution (500 ppm) of each SA was prepared in acetonitrile:deionized water (50:50, v/v). The stock standards were stored at 4 °C. Working mixed standard solutions of these four SAs were prepared by diluting the stock solutions with 0.1 M phosphate solution.

2.3. Electrode

The BDD electrode grown on conductive Si (1 0 0) substrate using the microwave plasma-assisted chemical vapor deposition (MPCVD) system was obtained from Associate Professor Yasuaki Einaga's laboratory [23]. A mixture of acetone and methanol at a ratio of 9:1 (v/v) was used as the carbon source. B_2O_3, used as the boron source, was dissolved in the acetone–methanol solution at a B/C atomic ratio of 1:100. The BDD electrode was rinsed with isopropanol and then deionized water prior to use.

The glassy carbon (GC) electrode was purchased from Bioanalytical System Inc. (area 0.07 cm^2). It was pre-treated by sequential polishing with 1 and 0.3 μm of alumina/water slurries on felt pads, followed by rinsing with deionized water prior to use.

2.4. Electrochemical measurements

2.4.1. Cyclic voltammetry

Electrochemical measurements were carried out in a single compartment three-electrode glass cell, with a volume of

50 mL. The BDD electrode was pressed against a smooth ground joint at the bottom of the cell, and isolated by an O-ring (area 0.07 cm^2). Ohmic contact was made by placing the backside of the Si substrate onto a brass plate. The GC electrode was also used as a working electrode in a comparison study with the BDD electrode. A platinum wire and Ag/AgCl with a salt bridge were used as the counter and reference electrodes, respectively. Cyclic voltammetry was performed with an Autolab Potentiostat 30. The electrochemical equipment was housed in a faradaic cage to reduce electronic noise.

2.4.2. Flow injection and HPLC analysis with amperometric detection

The measurements (FIA) using the BDD electrode as an amperometric sensor were carried out in a 0.1 M phosphate solution (pH 3.0) at an applied potential of 1100 mV versus Ag/AgCl. The FIA and HPLC system used in this study consisted of a Water Model 510 solvent delivery system, with a flow rate of 1.0 mL min^{-1}; the length of the tubing connecting the injector and the detector in the flow injection system was 20 cm, an injector system, with a 20-μL loop; a thin layer flow-cell; and an amperometric detector. The potential of the electrochemical detector was set using a computer-controlled potentiostat. The thin layer flow-cell consisted of the Ag/AgCl reference electrode and a stainless steel counter electrode. A 1-mm thick silicon rubber gasket was used as a spacer in the cell. The geometric area of the electrode in the cell was estimated at 0.6 cm^2. During the measurements, the flow cell was maintained at room temperature (25 ± 1 °C). An Intertsil C4 column was used for the separation of the SAs. The 0.1 M sodium dihydrogen phosphate (pH 3.0), acetonitrile (80:20; v/v), was used as the mobile phase for FIA experiments, and eluent in the liquid chromatographic experiments.

2.5. The preparation of egg samples

This method used less organic solvent [16]. An accurate 0.2 g of the sample was taken into a 1.5 mL micro-centrifuge tube and homogenized in 0.4 mL of 10% (v/v) perchloric acid solution (in water) with an ultrasonic-homogenizer for 1 min. Next, this micro-centrifuge tube was centrifuged at 6000 rpm for 3 min. A 0.3 mL portion of supernatant liquid was put into an Ultrafree-MC/PL and centrifuged at 6000 rpm for 5 min. The 20 μL of solution in ultra-filtrate was injected into the HPLC system.

2.6. Recovery test

The recoveries of SAs were determined from three egg blank samples, accurately weighed at 1.0 g, and each spiked with mix standards for a concentration of 5, 10, and 15 ppm, respectively. Then an accurate 0.2 g homogenate was transferred into a 1.5 mL micro-centrifuge tube and homogenized in 0.4 mL of 10% (v/v) perchloric acid solution (in water) with an ultrasonic-homogenizer for 1 min. Preparation steps for the egg samples followed.

3. Results and discussion

3.1. pH dependence study

In initial experiments, the electrochemical behavior of four SAs was investigated at the BDD electrode in 0.1 M phosphate solution from pH 2.0 to 7.0. Cyclic voltammograms of SAs at various pH phosphate solutions were obtained. It was found that changing pH phosphate solution effected the oxidation peak current. The best-resolved and highest anodic signals of SAs were obtained at pH 3. Therefore, pH 3 was chosen as the optimal pH.

3.2. Cyclic voltammetry

Fig. 2A and B show the cyclic voltammograms for 50 μM SDM together with the corresponding background voltammogram in 0.1 M phosphate solution (pH 3.0) at the BDD and GC electrodes. The background current for the GC electrode was ∼10 times higher than that obtained from the BDD electrode. The BDD exhibited a well-defined irreversible oxidation peak at ∼1100 mV versus Ag/AgCl, whereas the GC electrode provided an ill-defined irreversible oxidation peak. No cathodic peak was

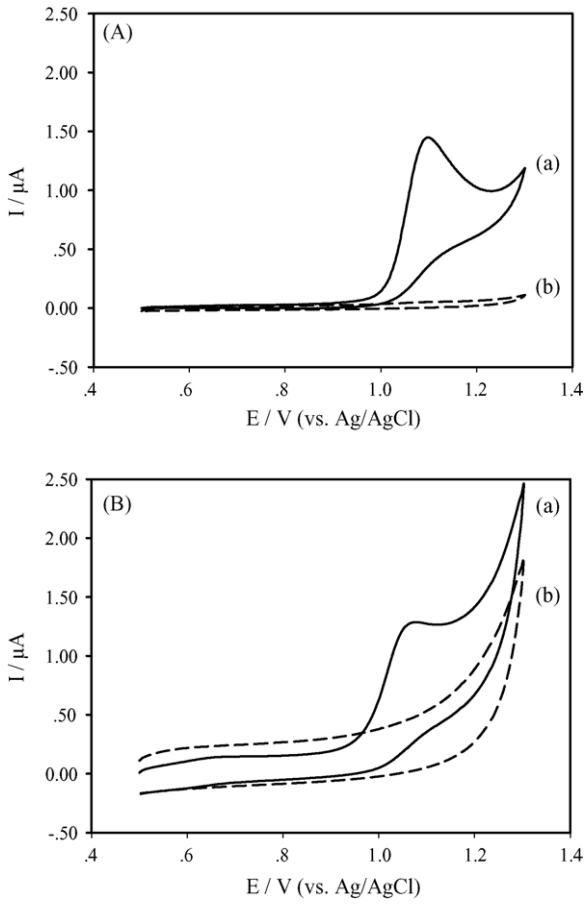

Fig. 2. Cyclic voltammograms for (A) BDD and (B) GC electrodes vs. Ag/AgCl in 50 μM SDM in 0.1 M phosphate solution pH 3.0 (a) and 0.1 M phosphate solution pH 3.0 (b). The sweep rate was 50 mV s^{-1}. The area of electrodes was 0.07 cm^2.

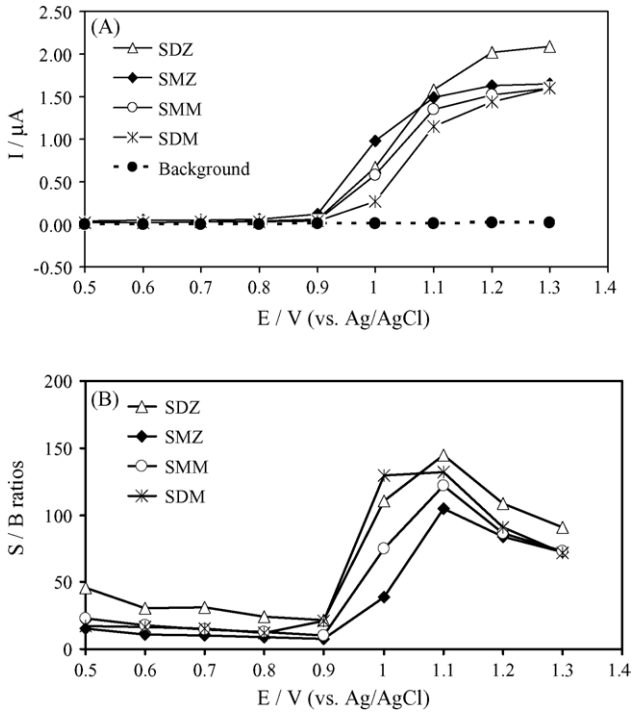

Fig. 3. Hydrodynamic voltammetric results for 10 ppm of each SA. The average peak current obtained from injections ($n = 4$) of (A) background (0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v), SDZ, SMZ, SMM and SDM in 0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v). Phosphate solution was used as a carrier stream. The flow rate was 1 mL min^{-1}. (B) Hydrodynamic voltammogram of signal-to-background ratios.

observed at either electrode on the reverse scan within the investigated potential range (+500 to +1300 mV).

3.3. Flow injection analysis with amperometric detection

To obtain the optimal potential for amperometric detection in flow injection analysis, the hydrodynamic behavior of SAs was studied. Fig. 3 shows a hydrodynamic voltammetric *I–E* curve obtained at the BDD electrode for 20 μL injections of 10 ppm to each SA in 0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v) as the carrier solution. Each datum represents the average of four injections. The absolute magnitude of the background current at each potential is also shown for comparison. The S/B ratios were calculated from Fig. 3A at each potential to obtain the maximum potential point. The hydrodynamic voltammetric S/B ratios versus potential curve are shown in Fig. 3B, with the maximum S/B ratio at 1100 V. Hence, this potential was set for quantitative amperometric potential detection in HPLC analysis experiments.

3.4. HPLC analysis with amperometric detection

In previous papers [7–10] on reversed-phase HPLC analysis of SAs, the C18 or C8, non-polar sorbent columns were used the most frequently. The C18 and C8 sorbents required a large volume of strong elution solvents as the mobile phase. The separation in this experiment was performed using a C4 column, which remarkably reduced the volume of elution solvents required and provided a high signal and clear separation, as described previously [15–17].

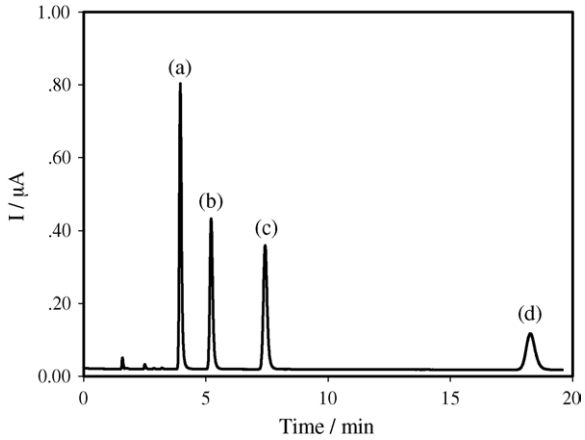

Fig. 4. HPLC-EC chromatogram of a standard mixture containing 10 ppm concentration of (a) SDZ; (b) SMZ; (c) SMM; and (d) SDM at the BDD electrode. The mobile phase was 0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v). The injection volume was 20 μL, and the flow rate 1 mL min^{-1}.

The results were analyzed by a chromatographic technique (HPLC), coupled with amperometric detection on the BDD electrode. The pH of the mobile phase was selected at 3.0 in order to reduce the above information. The chromatogram of standard solution of the four SAs in 0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v) solution, as the mobile phase, is presented in Fig. 4. The retention times of the four SAs; SDZ, SMZ, SMM, and SDM, at a concentration of 10 μg mL^{-1}, were 4.0, 5.2, 7.5, and 18.0 min, respectively. Twenty minutes were required to complete separation of the four SAs.

3.5. Method characteristics

The calibration characteristics of the SAs at the BDD electrode are shown in Table 1. The detection limit (DL) and quantitative limit (QL) for the four SAs under these experimental conditions were obtained from $DL = 3S_B/b$ and $QL = 10S_B/b$, when S_B was the standard deviation of the mean value for 10 signals of the blank and b was the slope of the straight line in the analytical curve [50].

From the standard deviation (S_B), the straight line slope of the analytical curve (b), the calculated value of DL and the QL is shown in Table 1. The current responses of SAs var-

Table 1
Linear range (LR), detection limit (DL), slope (b), and quantitative limit (QL) of SDZ, SMZ, SMM, and SDM

SAs	LR (ppm)	Equation $y = bx + a$	R^2	DL (ppm)	QL (ppm)
SDZ	0.050–100	$y = 0.2922x + 0.6232$	0.9905	0.011	0.037
SMZ	0.050–100	$y = 0.2915x + 0.1610$	0.9974	0.012	0.040
SMM	0.050–100	$y = 0.2939x + 0.3084$	0.9959	0.011	0.037
SDM	0.100–300	$y = 0.2898x + 0.6433$	0.9910	0.032	0.107

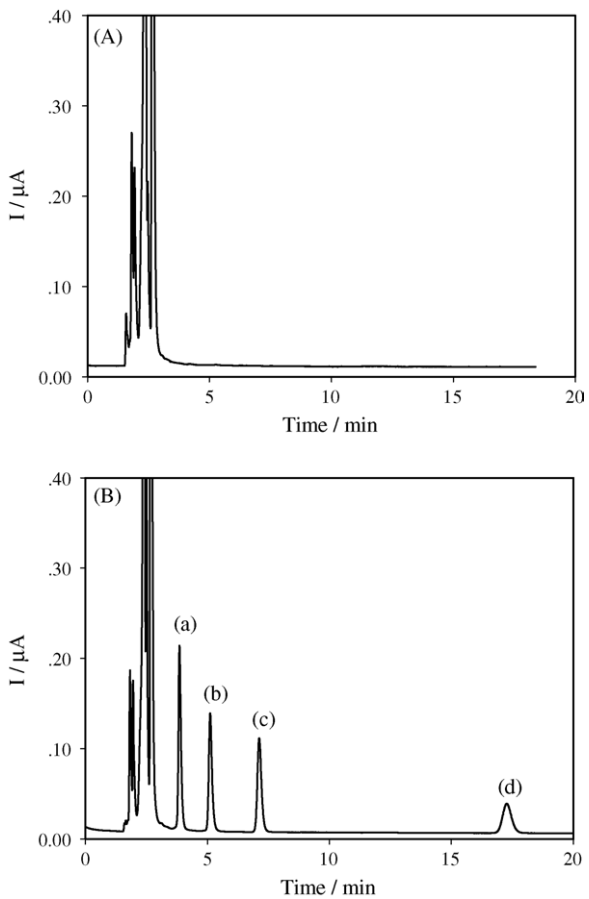

Fig. 5. HPLC-EC chromatograms obtained from egg samples: (A) blank egg sample and (B) egg sample spiked to a 10 ppm concentration of (a) SDZ; (b) SMZ; (c) SMM; and (d) SDM at the BDD electrode. The mobile phase was 0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v). The injection volume was 20 μL, and the flow rate 1 mL min^{-1}.

ied linearly with the standard concentrations, covering a range of 0.05–100 ppm for SDZ, SMZ and SMM, and 0.1–300 ppm for SDM. The results indicated that this proposed method can be applied for the determination of four SAs in egg samples.

3.6. Application

Egg samples were analysis by the HPLC/EC system. Fig. 5 shows clearly the chromatograms, with clear/sharp peaks and short retention times by using a C4 column and an isocratic mobile phase of 0.1 M sodium dihydrogen phosphate (pH 3.0):ACN (80:20; v/v). The experiment was repeated three times in order to assess the reproducibility in real sample. The recoveries of four SAs were determined by injecting a blank egg sample, spiked together with the standard samples. The recoveries and %R.S.D. of SDZ, SMZ, SMM, and SDM at three different spiked levels are summarized in Table 2. This procedure allowed rapid and efficient purification of SAs and resulted in high recovery (Mean of percentage of recoveries was found to be between 90.0 and 107.7) and reproducibility (%R.S.D. < 4.9% and S.D. < ± 4.7).

Table 2
Analysis four SAs in egg samples ($n = 3$)

SAs	Mean of percentage recovery ($x \pm$ S.D.)			%R.S.D.		
	5 ppm	10 ppm	15 ppm	5 ppm	10 ppm	15 ppm
SDZ	103.0 ± 4.7	96.9 ± 1.9	93.3 ± 1.6	4.5	2.0	1.7
SMZ	107.7 ± 3.8	98.8 ± 2.3	95.3 ± 2.1	3.5	2.3	2.2
SMM	103.2 ± 2.9	94.9 ± 2.3	91.3 ± 1.9	2.8	2.4	2.1
SDM	94.7 ± 1.2	90.0 ± 2.5	95.0 ± 4.7	1.3	2.8	4.9

4. Conclusions

BDD electrodes exhibit excellent performance for the electrochemical detection of SAs (SDZ, SMZ, SMM, and SDM) in egg samples. The optimum potential from the hydrodynamic voltammogram was found to be 1100 mV versus Ag/AgCl, which was chosen for the HPLC-amperometric system. An excellent linearity, and detection and quantitation limit were obtained. This proposed method was used for determination of SAs in egg samples. Percentage recoveries were found to be acceptable.

Acknowledgements

We would like to acknowledge support from the Thailand Research Fund (TRF) for Research Senior Scholars, The Enhancement on the Country's Performance and Competitiveness Program, and the Ratchadaphisek Somphot Grant, Chulalongkorn University.

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Flow-injection in-line complexation for ion-pair reversed phase high performance liquid chromatography of some metal-4-(2-pyridylazo) resorcinol chelates

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Received 22 December 2004; accepted 9 March 2005
Available online 21 September 2005

Abstract

Flow injection (FI) was coupled to ion-pair reversed phase high performance liquid chromatography (IP-RPHPLC) for the simultaneous analysis of some metal-4-(2-pyridylazo) resorcinol (PAR) chelates. A simple reverse flow injection (rFI) set-up was used for in-line complexation of metal-PAR chelates prior to their separation by IP-RPHPLC. The rFI conditions were: injection volume of PAR 85 μL, flow rate of metal stream 4.5 mL min^{-1}, concentration of PAR 1.8×10^{-4} mol L^{-1} and the mixing coil length of 150 cm. IP-RPHPLC was carried out using a C_{18} μBondapak column with the mobile phase containing 37% acetonitrile, 3.0 mmol L^{-1} acetate buffer pH 6.0 and 6.2 mmol L^{-1} tetrabutylammonium bromide (TBABr) at a flow rate of 1.0 mL min^{-1} and visible detection at 530 and 440 nm. The analysis cycle including in-line complexation and separation by IP-RPHPLC was 16 min, which able to separate Cr(VI) and the PAR chelates of Co(II), Ni(II) and Cu(II).
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Keywords: Flow injection; In-line complexation; Ion pair reversed phase high performance liquid chromatography; Metal-PAR chelates

1. Introduction

Liquid chromatography has been widely recognized as one of the methods for multi-element and sensitive analysis of metal ions. Various modes of liquid chromatography have been used, including normal phase [1–3], reversed phase and ion exchange chromatography (IEC) [4–10]. Since the introduction of ion-pair reversed phase high performance liquid chromatography (IP-RPHPLC) [11,12] for the separation of charged solutes, IP-RPHPLC has gained wide acceptance as an alternative method to IEC for charged analytes, including metal ions. IP-RPHPLC offers multi-element detection capacity, selectivity and sensitivity of analysis. Moreover, the reversed-phase stationary phase has the benefit of lower cost compared to the IEC stationary phase.

Most of the reports on IP-RPHPLC for metal analysis [13–16] are based on the separation as their chelates. Pre-complexation of metal ions with appropriate ligands has many advantages such as increasing selectivity between metal ions, the ability to determine speciation and increasing sensitivity for chelates with high absorptivity. Among the many ligands successfully used for IP-RPHPLC separation of metal ions, 4-(2-pyridylazo) resorcinol (PAR) is one of the most widely used ligands. PAR is an azo dye has been used for the spectrometric determination of over 40 different metals [17]. PAR forms ionic complexes with large absorptivity ($\sim 10^4$ L cm^{-1} mol^{-1}) [18] at about 500 nm. It has been shown to be an effective reagent for the determination of metals using HPLC with either pre-column [19] or post column complexation techniques [20,21].

Typically, complexation of metal ions is performed by batch or external to the chromatographic system before injection. External complexation is time consuming and the large amounts of chemicals used mean more waste to discharge. It is prone to contamination, especially for trace level determinations. Nowadays, the main consideration includes automation of the method, low operating costs, less waste as well as high sample throughput.

Flow injection (FI) has been known with features of a simple operational basis, using inexpensive hardware, straightforward thus leading to convenient operation, high sample throughput, cost effective performance and versatility. FI has been widely

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doi:10.1016/j.talanta.2005.03.043

used as an analytical tool and also combined with the other analytical techniques [22].

Flow injection coupled with HPLC systems is usually intended to improve general features of the analytical process such as sensitivity, precision, rapidity, cost, etc. [23]. FI coupled with HPLC is used in two different modes, i.e., pre- or post-column arrangements. For the pre-column arrangement, as in the present study, the FI port is placed before the HPLC. The specific objectives of pre-column coupling are automation of sample clean-up and/or preconcentration steps, automatic implementation of derivatization reactions and saving reagents. Two methods have been used to couple FI as precolumn of HPLC. The first method, the sample plug is injected through the FI valve and then passed through HPLC loop. In the second method, the sample from the FI system is retained in a precolumn placed in HPLC loop.

Previous work in this laboratory has involved metal analysis by IP-RPHPLC via batch complexation with PAR [24]. In the work described here, a simple FI system was developed as the in-line precolumn for complexation of some metal-PAR chelates before being analysed by IP-RPHPLC. The FI part (rFI) is operated by injecting a PAR reagent solution into a metal ion solution flowing stream. A portion of the PAR-metal mixture zone is then sampled with the HPLC injection valve for subsequent separation and further detection.

2. Experimental

2.1. Chemicals and reagents

All the reagents used were of analytical reagent (AR) grade, 4-(2-pyridylazo) resorcinol and tetrabutylammonium bromide (TBABr) were purchased from Fluka (Switzerland). 2-Diethylaminoethanol was obtained from Merck (Germany). Methanol and acetonitrile were of HPLC grade from Lab-Scan (Thailand). The atomic absorption standard solutions (1000 mg L^{-1}) of Cu(II), Cd(II), Co(II), Hg(II), Zn(II), Fe(III) and Pb(II) were obtained from Ajax Finechem (Australia) whereas Ni(II) was purchased from BDH (England). Cr(VI) oxide was obtained from Merck (Germany). Aqueous solutions were prepared with deionized water obtained from RiOs^{TM} type I simplicity 185 (Millipore Waters, USA) throughout the experiment. Standard solutions of metal ions were prepared daily by stepwise dilution of 1000 mg L^{-1} stock solution with water. Stock PAR solution (0.001 mol L^{-1}) was prepared by dissolving an accurately weighed amount of 4-(2-pyridylazo)resorcinol in water and stored in a dark bottle. Working solution was prepared daily with water and appropriate volume of 2-diethylaminoethanol was added to make the concentration of 2.5×10^{-4} mol L^{-1} in such a PAR solution.

2.2. Instruments

A schematic representation of the rFI coupled with the HPLC system is shown in Fig. 1.

The rFI system used a 505s 505LA peristaltic pump (Watson Marlow, England). PFA Teflon tubes (1.5 mm i.d.) were employed for the reaction coils and were connected to a six-port low-pressure injection valve, four way switching valve (Upchurch, USA) was used to allow the metal-chelates flow to HPLC system. A manual operation using a stopwatch was for time control.

The chromatographic set-up consisted of a Waters 6000A Dual Pump, a Rheodyne injector with 20 μL sample loop and a Waters 484 Tunable Absorbance Detector (Waters, USA) equipped with Waters 740 Data Module Integrator (Waters, USA), and the Millinium 32 Software data acquisition system was used. A 996 photodiode array (Waters, USA) was also used for the study of interferences. A C_{18}-μBondapak (3.9 mm i.d. × 300 mm) coupled to a guard column (Waters, USA) was used as the stationary phase.

The spectra of the metal chelates in batchwise experiments were obtained with a Agilent 8453 (USA) UV–vis spectrophotometer equipped with a 1 cm quartz cell.

2.3. Procedure

Once the baseline of the HPLC was steady, a complete cycle (4 steps) of the rFI-HPLC manifold was started. The 4 steps include prefill, complexation, separation and washing. In the first (prefill) step, the aqueous solution containing metal ions was pumped through the rFI manifold for 30 s, this period was long enough to fill the transmission line with metal solution. The HPLC was in the load mode throughout this step to maintain a steady baseline of mobile phase.

During the prefill step, an aliquot of PAR was filled into the loop connected to V2 (at LOAD position).

Step 2, the complexation step, was started by switching V2 to INJECT position. The metal ions merged with PAR and complexation occurred during their passage through the reaction coil (RC). To avoid a dilution edges of the zone and to allow only the middle zone of the PAR chelates to pass into the HPLC-loop, the valve V3 was switched after 8 s of injection of PAR. The subsequent time period of 2 s, was enough to rinse and fill the HPLC-loop (20 μL).

Then, step 3 (separation step), was initiated via HPLC-valve (V4). The PAR-chelates were introduced and then separated in the HPLC system.

Finally, step 4 (washing step), was to wash the rFI and HPLC-loop for the next analysis, while separation was taking place on the HPLC column.

3. Results and discussion

3.1. Coupling of rFI to IP-RPHPLC

The rFI was chosen instead of normal FI because of its low background noise for HPLC baseline as well as lower PAR consumption. The rFI was coupled to the HPLC by switching valve (V3) shown in the diagram (Fig. 1).

Factorial design was used to investigate the influence of parameters of the rFI system on the peak height (absorbance). The four variables studied were: flow rate of metal ions stream, injection volume of PAR, length of the mixing coils and concen-

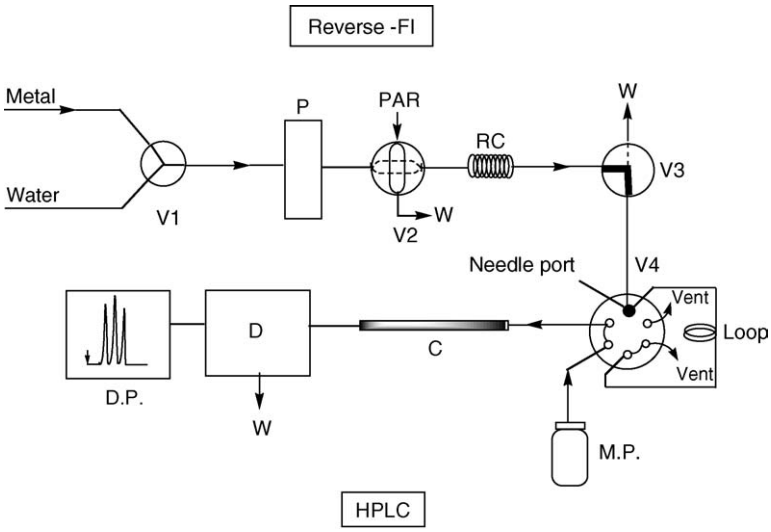

Fig. 1. Diagram of FI-HPLC in-line derivatization system: P, peristaltic pump; RC, reaction coil; V1, three way valve; V2, low pressure injection valve; V3, switching valve; V4, high pressure injection valve; C, analytical column; M.P., mobile phase; D, UV–vis detector/photodiode array detector; D.P., data processor; W, waste.

tration of PAR. A factorial design for four variables at two levels (2^4 resolution, 16 experiments) was performed. According to the results obtained from the factorial design, the chosen parameters to be optimized were the concentration of PAR, injection volume of PAR solution and flow rate of metal stream. The variable size simplex was then employed for optimization. The mixing coil length of 150 cm was used throughout the experiment.

In-line complexation of metal-PAR chelates was performed using the rFI which the optimum conditions were: mixing coil length of 150 cm, injection volume of PAR 85 μL, flow rate of metal stream 4.5 mL min^{-1} and concentration of PAR 1.8×10^{-4} mol L^{-1}. The PAR chelates were then separated via IP-RPHPLC.

Synchronization of the FI manifold and the HPLC is very important to achieve good performance of the coupling system. The time intervals and valve positions of the rFI-HPLC were investigated using the results obtained from the study of the optimization of the rFI. Manual operation of the rFI-HPLC system was found to provide satisfied precision.

The complete cycle of rFI coupling to IP-RPHPLC was 120 s, where as the analysis time of the HPLC was 14 min. Operating periods and valve position for rFI-IPRPHPLC are summarized in Table 1 and Fig. 2.

3.2. IP-RPHPLC of metal-PAR chelates

In IP-RPHPLC, the metal chelates, which are successfully separated, have to be stable and kinetically inert [25]. It is known that the retention behavior of chelates in IP-RPHPLC depends strongly on complex composition (metal:ligand), which is governed by the nature of the central metal ion. The mobile phase composition is also govern the separation. The principal parameters of interest in the mobile phase are pH, buffer (type and concentration), organic modifier and ion pairing agent (long-chain alkyl ions with a charge opposite that of analytes). There are several mechanisms [26–28] explaining the retention behavior of IP-RPHPLC, such as the ion-exchange mechanism, the solvophobic theory and dynamic equilibrium.

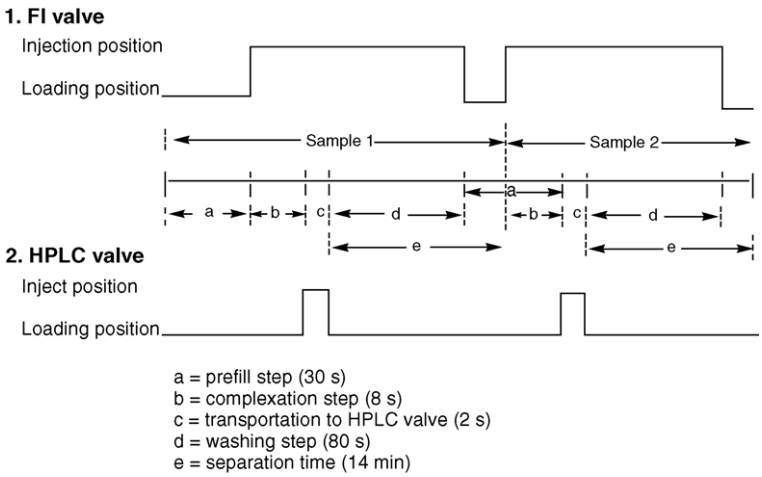

Fig. 2. Diagram of timing control for operation of valves in the FI-HPLC system.

Table 1
Valves positions and operating times for rFI-HPLC

Step	Time (s)	Valve position				In-line complexation operation	
		V1	V2	V3	V4	Medium pump	Stage of operation
Sample 1							
1	30	To V2	Load	To waste	Load	Metals	Prefill
2	38	To V2	Inject	To V4	Load	Metals	Complexation
3	40	To V2	Inject	To V4	Inject	Metals	Separation
4	120	To V2	Load	To V4	Load	Water	Washing
Sample 2							

Typically, PAR forms anionic chelates with metals at the metal to ligand ratio of 1:2 [29]. Thus, cationic ion pairing agent, tetrabutyl ammonium bromide was used. The optimum mobile phase was obtained by slightly adjusting the one obtained in our previous work [24]. The mobile phase composition was 37% acetonitrile, 6.2 mmol L^{-1} TBABr and 3.0 mmol L^{-1} acetate buffer pH 6.0.

Baseline separation of three metal-PAR chelates was achieved within 14 min, with the elution order of Co(II)-PAR, Ni(II)-PAR and Cu(II)-PAR. The excess PAR was detected at the retention time of 9.6 min. The chromatogram is shown in Fig. 3.

Using the optimum mobile phase and the detection at 440 nm, Cr(VI) was retained shortly (4.9 min) after unretained peak (at 3.3 min), as shown in Fig. 4. The spectrochromatogram corresponding to Fig. 4 is shown in Fig. 5. For the condition used, Cr(VI) might present as its oxyanion ($HCrO_4^-$) [30]. Thus, it could interact with ion pairing agent in the same manner to the anionic chelates. The peak at 4.9 min which was identified as Cr(VI) gives the absorption spectrum (as shown in Fig. 6) identical to the spectrum of Cr(VI) detected by UV–vis spectrometer. The resolutions between pairs were as follow: 1.4 for Cr(VI) and Co(II)-PAR, 1.0 for Co(II)-PAR and Ni(II)-PAR, 2.4 for Ni(II)-PAR and excess PAR, and 1.3 for excess PAR and Cu(II)-PAR.

3.3. Performance of rFI-IP-RPHPLC

Quantitative features including linearity and reproducibility for retention time and peak area were studied using the optimum condition. Calibration graphs were prepared by plotting the concentration of each metal ion (μg mL^{-1}) against the peak area. The limit of detection (LOD) was deduced based on three times of baseline signal. The calibration equation, coefficient of correlation (r^2), recovery, reproducibility and LOD are summarized in Table 2.

3.4. Interferences

The effect of interferences on the chromatography of metal-PAR chelates was investigated. The chosen ions are the ions able to form chelate with PAR including Cd(II), Cr(III), Hg(II), Mn(II), Fe(III), Pb(II) and Zn(II). These metal ions were individually injected into the rFI-HPLC. All of the studied ions could not form chelates with PAR under the condition used. Only peak, which was identified as PAR (9.6 min) was observed.

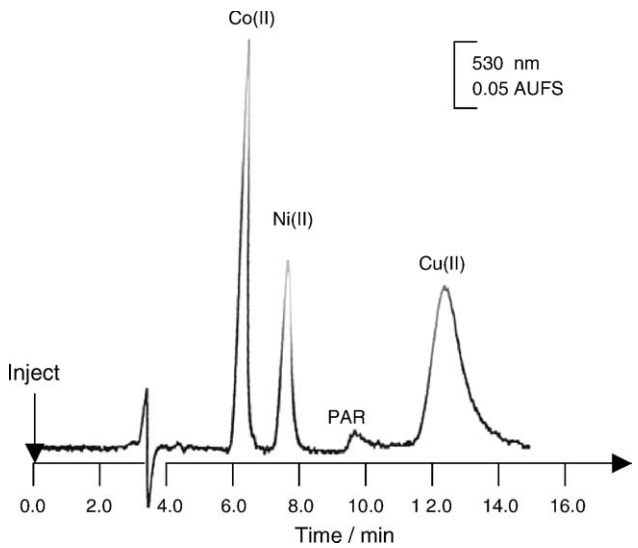

Fig. 3. Chromatogram of metal-PAR chelates; condition: C_{18} column, mobile phase 37% acetonitrile, 6.2 mmol L^{-1} TBABr and 3.0 mmol L^{-1} acetate buffer pH 6.0, flow rate of mobile phase 1.0 mL min^{-1} visible detection at 530 nm; peak: 0.10 μg mL^{-1} Co(II), 0.20 μg mL^{-1} Ni(II), excess PAR and 0.80 μg mL^{-1} Cu(II).

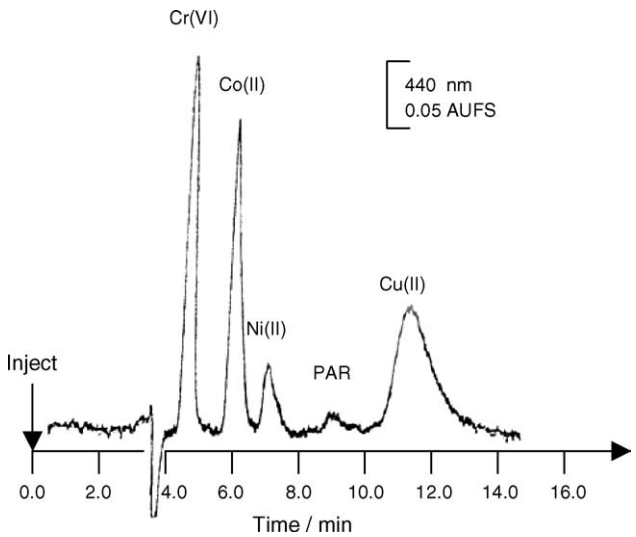

Fig. 4. Chromatogram for Cr(VI) and metal-PAR chelates; peaks: 5.0 μg mL^{-1} Cr(VI), 0.10 μg mL^{-1} Co(II), 0.10 μg mL^{-1} Ni(II), excess PAR and 0.40 μg mL^{-1} Cu(II) (condition as described in Fig. 3, except visible detection at 440 nm).

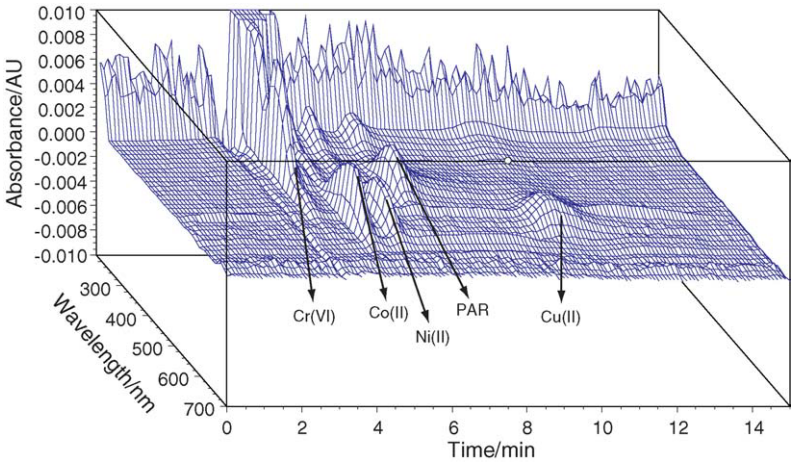

Fig. 5. 3D plot of Cr(VI) and metal-PAR chelates of chromatogram in Fig. 4.

Table 2
The quantitative features of rFIA-HPLC

Chelates	Concentration of metal ion ($\mu g\,mL^{-1}$)	Linear equations $Y = AX + C$	Correlation coefficient (r^2)	R.S.D.[a] (%) ($n = 11$)		LOD (3σ) ($\mu g\,mL^{-1}$)	Percent recovery
				t_R	Area		
Cr(VI)	1.00–6.00	$473.51X - 0.17$	0.9961	2.0	0.94	1.00	113
Ni(II)-PAR	0.01–0.20	$341.66X - 4.73$	0.9983	2.1	0.91	0.02	84
Co(II)-PAR	0.01–0.40	$547.21X - 0.61$	0.9952	0.6	0.97	0.03	96
Cu(II)-PAR	0.05–0.60	$293.80X + 5.49$	0.9986	2.4	0.71	0.15	102

[a] Concentration of each metal was described in Fig. 4.

The study on tolerance level of the metal ions which could not form chelates with PAR was studied by individually spiking the metal ions at difference amounts (ranging from 0.5 to 10.0 $\mu g\,mL^{-1}$) into the mixture of 0.10 $\mu g\,mL^{-1}$ Co(II), 0.20 $\mu g\,mL^{-1}$ Ni(II), 0.40 $\mu g\,mL^{-1}$ Cu(II) and 5.0 $\mu g\,mL^{-1}$ Cr(VI). It was found that the presence of the foreign ions did not affect the retention time of the PAR chelates of Co(II), Ni(II) and Cu(II). However, the quantitative signals (both peak height and peak area) were affected by the addition of the foreign ions. Cu(II)-PAR was strongly influenced when the concentrations of the foreign ions increase to 2.5 times resulted in decreased of peak height and peak area. The effect on Ni(II)-PAR was observed when the foreign ions increase to five times greater than Ni(II), resulted in the decreasing of peak height and peak area. This effect was also observed for Co(II) when the concentration of the foreign ion was 10 times to Co(II). The peak area of Cr(VI) was not affected by the addition of the foreign ion. However, the obtained spectra and the 3D plots (results not shown) revealed that neither PAR chelates of the foreign ions nor the ternary complexes were formed. According to the obtained results indicating that in such a condition, quantity of PAR was enough for all of metal ions. Furthermore, to ensure the excess amount of PAR, 10 times higher concentration of PAR, i.e., 1.0×10^{-3} mol L^{-1} was used. Similar results were obtained and large peak of excess PAR overlapped the analyte peaks. The effect of interference on the present method was obviously seen when compared to the previous work [24] on precomplexation of metal-PAR chelates by batch method prior to the analysis by IP-RPHPLC. This may attribute to the nature of the flow system, which a short time that stream of reagents are reacted. Neither physical equilibrium nor chemical equilibrium (i.e., the completeness of reaction) has been attained by the time it was detected.

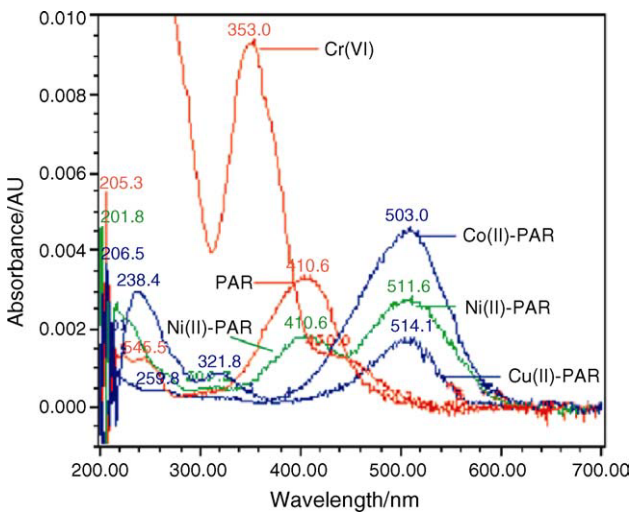

Fig. 6. The absorption spectra of chromatogram in Fig. 4.

3.5. Analysis of real sample

According to the study, it is possible to analyse Cr(VI) simultaneously with Ni(II). The present method was applied to the analysis of chrome plating waste water. The samples were collected from chrome plating plant in Khon Kaen and were

Table 3
Results of analysis of chrome plating waste water

Metal	Concentration ($\mu g\,mL^{-1}$)			
	Sample 1		Sample 2	
	FI-HPLC	AAS	FI-HPLC	AAS
Cr(VI)	984.5 ± 6.4[a]	980.8 ± 7.9[a]	69.3 ± 10.7[a]	72.6 ± 9.3[a]
Co(II)	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
Ni(II)	1574.6 ± 10.2[a]	1580.9 ± 12.0[a]	121.4 ± 7.4[a]	126.8 ± 8.2[a]
Cu(II)	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]

[a] S.D. ($n = 3$).
[b] Not detected.

analysed after dilution, pH adjustment and filtration through 0.45 μm membrane. The results obtained are listed in Table 3, which were in good agreement with that of AAS.

4. Conclusion

In the present study, a simple combination of rFI and HPLC resulted in a powerful technique for simultaneous analysis of metal ion as their PAR chelates. The rFI was coupled to HPLC with simple operation. Using the developed rFI for in-line complexation gives benefit of less PAR consumption, less analysis time and less waste disposed comparison to the batch derivatization. The analysis cycle consists of in-line complexation (ca. 2 min) and separation by IP-RPHPLC (14 min). The method was successfully applied for the separation of Co(II), Ni(II) and Cu(II) as their PAR chelates.

Acknowledgements

The authors are grateful for the financial support from the Thailand Research Fund (TRF) and the Postgraduate Education and Research Program in Chemistry (PERCH).

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<Review> J. Flow Injection Anal., Vol. 23, No. 2 (2006) 94–101

Down scaling: From operation on lab bench space to manipulation at a valve

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Abstract

Down scaling for chemical analysis with flow-based techniques with the emphasis on sequential injection systems is reviewed. Sequential Injection (SI) systems with Lab-on-Valve (LOV) offer various advantages. SI with Lab-at-Valve (LAV) approach serves as an alternative cost effective means for automation and miniaturization in chemical analysis. Development of the techniques and their features are discussed.

Keywords miniaturization, flow based techniques, sequential injection analysis, lab-on-valve (LOV), lab-at-valve (LAV), cost effective analysis

1. Introduction

Research and development have favored the direction of "zero emission" and "greener" chemistry. This has also led to various approaches for cost effective chemical analysis. Flow-based techniques offer not only advantages gained in term of automation but also to down scaling in chemical analysis such as reduction in chemicals/ volumes used and waste, and in sample consumption; much less analysis time (leading to high sample through-puts), and less activities involving glassware cleaning. All concern cost effectiveness [1].

Flow injection analysis (FIA) may be known as an analytical technique based on microfluidic manipulation of samples and reagents. Usually, a sample is injected into a carrier/reagent solution which transports the sample zone into a detector to monitor changes due to the desired (bio)chemical reactions which have been taken place. Chemical information will be obtained from the detector response (absorbance, conductance, current, fluorescence, radioactivity, mass spectra, etc.), such as concentration from a calibration graph (signal value vs. concentration) [2, 3, 4].

Microfluidics have been employed for flow analytical techniques either in continuous flow modes, such as FIA and chromatography with constant unidirection (forward) flow of carrier to transport a sample from an injector to a detector, or programmable flow modes such as sequential injection analysis (SIA) with flow reversal to mix sample with reagent(s) or to stop flow to accommodate reaction time [2, 3, 5].

FIA was introduced by Ruzicka and Hansen as an approach for downscaling from batch analysis to flow analysis-from a beaker to microfluidics [3, 6, 7]. As the second generation, SIA was developed for more degrees in automation by having a bi-directional pump for programmable flow.

2. Lab-on-Valve (LOV) Approaches

Ruzicka [8] proposed a SI with "Lab-on-Valve" (LOV) system, as the third generation, to perform micro- or nanoanalysis by integrating all the necessary laboratory facilities for a variety of analytical schemes, including sampling, chemical reaction, and detection in a conduit at a multiposition valve. The conduit is a very precisely positioned engraved Plexiglas using computer-aided-design (CAD). This is mounted to replace the headpiece of a selection valve (see Figure 1 [3]). As SI-LOV is a system/device for analytical processes taking place, it could serve then as a type of micro-total analysis system (μ-TAS). SI-LOV has proven to have more tolerance than that of chip based approaches for real applications to dirty samples or samples with complicate matrices.

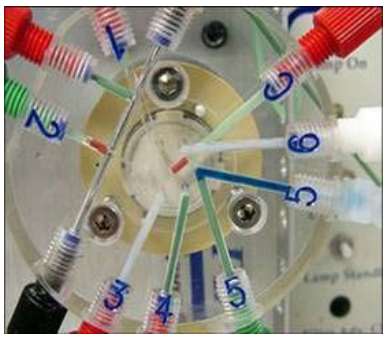

Fig. 1 SIA -Lab-On-Valve®
(more detail in www.flowinjection.com)

SI-LOV has become a platform for microSI manipulation using microliter volumes of sample and reagents per analysis. SI-LOV has been cooperated well with various techniques apart from UV-VIS such as bead injection, sequential injection affinity chromatography, mass spectrometry, capillary electrophoresis, atomic spectroscopy (AAS, ICP-AES and ICP-MS) and potentiometry.

Ruzicka has demonstrated the usefulness of SI-LOV systems to various applications [7, 9, 10, 11], including environmental analysis, pharmaceutical and bioanalytical assays.

Hansen, Wang and Miró have extensively reviewed the applications of the microSI-LOV systems with various sample

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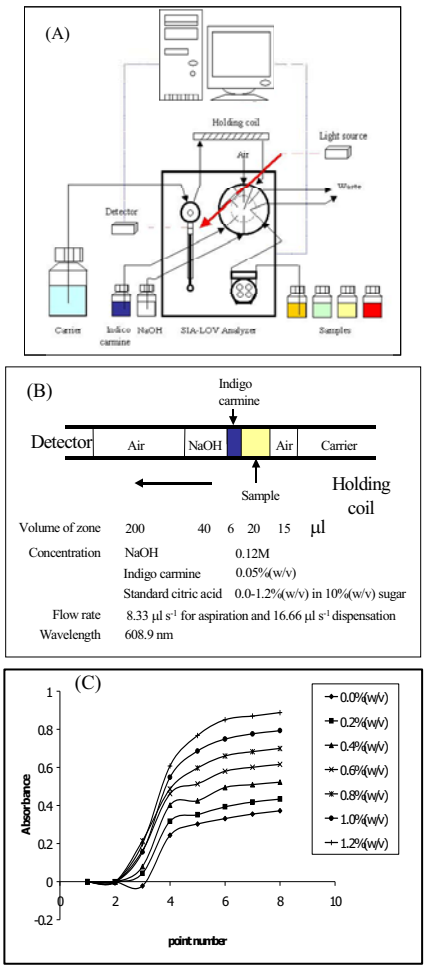

Fig. 2 Mono-segmented flow using SI-LOV for microtitration (adapted from ref 27): (A) the SI-LOV system, (B) the conditions and (C) the signal profiles.

pretreatment methods for determination of trace levels of elements using ETAAS and ICPMS. These sample pretreatments include sample dilution, dialysis, derivatization, hydride generation, liquid-liquid and solid phase extraction, chromatographic separation and preconcentration with beads and packed column [12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23].

Coupling of the SI-LOV system with other detection systems such as AAS, FT-IR, electrochemistry has also been reviewed [24, 25].

Some previous reviews may be sources for information (Table 1). Table 2 summarizes some SIA-LOV developments.

Employing SI-LOV, micro-segmented microflow analysis [27] can be performed. Air segments are introduced to sandwich the sample-reagent plug to eliminate dispersion between the sample-reagent zone and the carrier stream. This enables promotion of mixing of analyte and reagent(s), with longer residence time and better sensitivity with low reagent consumption being possible. This was demonstrated by microtitration. By flow reversal, three solutions can be mixed together well in a mono-segmented zone. The air bubbles are to be discarded prior to absorbance measurement to obtain a smooth response. It should be noted that the signal profiles are not in the shape of peaks, which are different from normal SI-grams. (see Fig. 2). The concentration of a sample analyte can be obtained via a calibration graph. By the mono-segmented SI-LOV approach, the determination of copper using 2-carboxy-2'-hydroxy-5'-sulfoformazyl benzene (Zincon) has been reported [32].

3. Lab-at-Valve (LAV) Approaches

As an alternative cost effective micro-total analysis system, the Lab-at-Valve (LAV) approach has been investigated. A LAV unit can be fabricated using an ordinary and less precise machine tool by designing for suitable function for the chemistry of interest, and for easy attachment at a port of a conventional selection valve in a simple usual way. There is no need to take out any part of a usually available selection valve [1, 47].

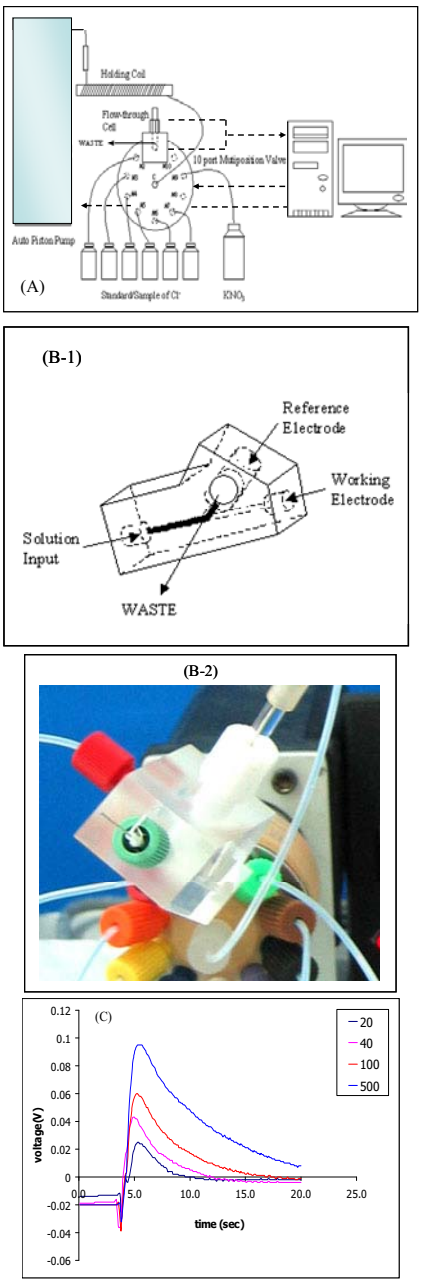

Fig. 3 SI-LAV for the potentiometric determination of chloride (adapted from ref 1, 47): (A) the SI-LAV system (B) the LAV flow cell and (C) the signal profiles

Table 1: LOV reviews and related articles

Analyte/sample/application field(s)	**Features (detection)**	**ref**
affinity separation, fermentation monitoring and functional cellular assay	µSI-LOV with various possible detectors for bionalytical assays	10
trace level metals	implementation of FI/SI-sample separation/preconcentration, mentioned LOV *(ETASS/ ICPMS)*	22
pharmaceutical samples	SI-LOV for chromatography and BI	9
trace level metals	progress in implementing miniaturized FI/SI systems for online separation and preconcentration, mention LOV *(ICPMS)*	19
ultra-trace level heavy metals	SI-BI-LOV for online solid phase extraction and preconc. *(ETASS/ ICPMS)*	20
trace level metals	SI-LOV with BI microcolumn for preconcentration *(ETASS/ ICPMS)*	21
	SI-LOV with other cost effective flow-based technique development	1
	impact of FI in modern chemistry including BI-LOV	17
	3 generations of FIA (FI, SI, BI-LOV)	18
	SIA for complicated online sampling manipulation (dilution, dialysis, gas diffusion, enzymatic and immunoassay) mentioned LOV *(ETAAS and ICPMS)*	25
trace level metals in complex matrices	FI/SI LOV for liquid-liquid extraction, precipitation, hydride generation, ion exchange/chelating packed column	14
	LOV mesofluidic system	15
	progress in implementing miniaturized FI/SI systems for online separation and preconcentration, mentioned LOV *(ETAAS)*	16
	programmable ufluidics to replace batch wet chem. *(various)*	7
nutrient (phosphorus) in fresh water and environmental solids	µSI-LOV microcolumn for dynamic fractionation *(spectrophotometry/ fiber optic)*	12
environmental field	SI with solid reactors, packed column for chem. derivatization, chrom. sep. and preconcentration including SI-BI with jet ring or LOV to accommodate dynamic fractionation of trace elements *(many)*	13
pharmaceutical samples	SIA (mentioned LOV) *(spectro, fluorescence, chemiluminescence, electrochem.)*	24
trace level metals	impact of FIA and SIA, LOV	23

Table 2: LOV with various detection techniques and applications

Detection	Analyte/sample	Features	ref
spectrophotometry	phosphate	molybdenum blue chemistry	8
	protease	enzymatic activity assay	
	immunoglobulin G	bioligand interaction assay protein G immobilized on Sepharose beads	
	ammonia, glucose, glycerol, free iron	μSI-LOV for offline and online monitoring of small scale fermentation	25
	nitrate, nitrite and orthophosphate	μSI-LOV with Cd foil filled microcolumn utilizing stopped flow	26
	acidity in fruit juice	mono-segmented flow micro-titration with SI-LOV	27
	DNA assay	crystal violet solution was de-colored inside the flow cell of the LOV at the presence of 5 l - DNA/HindIII within a certain pH range	28
	ketoprofen, naproxen, bezafibrate, diclofenac, ibuprofen and salicylic acid in surface water, urban wastewater, and urine	lab-on-valve (LOV) with bead injection (BI) for on-line solid-phase extraction (SPE) as a front end to highperformance liquid chromatography (HPLC)	29
	enzyme AChE and ACE	SI-LOV micro-reactor for enzyme kinetic and inhibition studies	30
	Cu (II) and Fe (II)	SI-LOV using reaction of 5-Br-PSAA) with Cu (II) and/or Fe (II)	31
	Cu (II)	SI-LOV with air segment using reaction of Cu(II) and Zincon	32
spectrofluorometry	nucleic acid sequences	SIA-LOV for sandwich hybridization of specific DNA probes to the target sequence	33
chemiluminescence	Tetracycline	LOV with bismuthate immobilized microcolumnfor in situ oxidation of KBr and generation of bromine as oxidant for the bromine-hydrogen peroxide-tetracycline (TC) chemiluminescent reaction	34
ICPMS	Ni (II) and Bi (II) in reference materials and spiked urine	SI-LOV with renewable ion exchange micro-column	35
UV-Vis and electrospray ionization MS	biotin containing conjugates and lysosomal-b-galactosidase in human cell homogenates	LOV-BI system for biotin conjuates using immobilized streptavidin	36
mass spectrometry	peptide mixture	affinity chromatographic-LOV system using multiple ligand affinities to proteins immobilized on beads.	37

Table 2 (con)

Detection	Analyte/sample	Features	ref
ETAAS	Pb (II)	SI-BI-LOV using Sephadex G-25 impregnated by dithizone	38
	Cd (II)	PTFE material for use as a means for separation and preconcentration of trace levels of metal ions with SI and SI-LOV	39
	Cd (II)	Octadecyl Immobilized Surface for Precipitate Collection with a Renewable Microcolumn in LOV	40
	Cr (VI) and Cr (III)	bead injection (BI) with renewable reversed-phase surfaces in a sequential injection-lab-on-valve (SI-LOV) mode - C18 sorption of Cr(VI) - DPC	41
	Cd (II), Pb (II) and Ni (II)	SI-LOV system using chelating Sepharose beads as sorbent material	42
	Ni (II) in saline matrices	Lab-On-Valve (μSI-LOV) for bead injection separation/pre-concentration of Ni-dimethylglyoxime (DMG) chelate	43
CVAAS	Hg (II)	SI-LOV for hydride generation	44
potentiometry	Ca (II)	SIA-LOV with solid contact ion selective electrode and pH electrode based on polyaniline	45
capillary electrophoresis	10 anions including chloride and sulfate	μSI-CE interfaced with LOV and demonstrated various injections such as electrokinetic, hydrodynamic and head column field amplification sample stacking injections	46

Table 3 Comparison of SI-LAV features to those of ion-selective electrode (ISE), ion chromatography (IC) and conventional titration with silver nitrate solution.

	LAV	ISE	IC	Titration with Ag^+solution	
Sample throughput	++++	+(+)	++	++	
Consumption:					
• sample	+	+++	+	+++	
• reagent	+	+	+++	+++	
Glassware	-	++	++	+++	
Cleaning activities	-	++	++	+++	
Automation	+++	++	+++	+	(conventional)
				+++	(auto-titrator)
Cost					
• system/ apparatus	++	++	+++	+	(conventional)
				+++	(auto-titrator)
• per sample	+	+	++	+++	

"+" refers to more scale

SI-LAV for chloride determination was proposed (Fig. 3) [47]. For potentiometry, a simple LAV flow through electrode system can be assembled. Both are Ag/AgCl electrodes, one a reference electrode (silver chloride activated surface-silver wire soaked in a constant chloride concentration in a small tube covered with a membrane), another as a working electrode situated in a flow channel. The potential difference due to the concentration cell effect is recorded as a peak. The SI-LAV provides a very simple, fast, precise, accurate, automatic and economical procedure for chloride determination and was applied to some water samples. Comparisons of features of SI-LAV to that of the conventional titration using silver nitrate solution, ISE, and ion-chromatography may be considered as illustrated in Table 3.

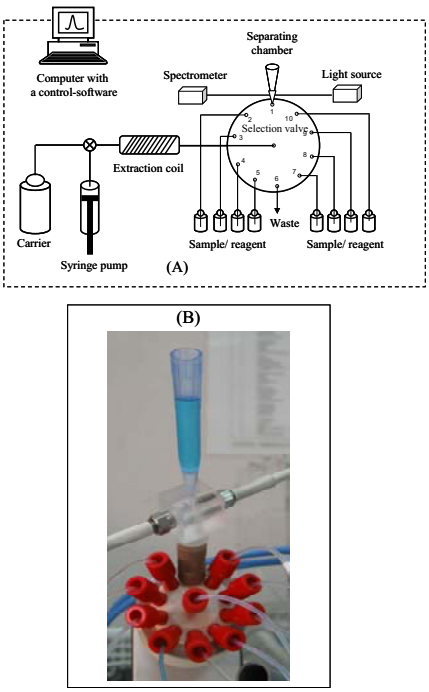

Fig. 4 SIA-LAV on-line micro solvent extraction (adapted from ref 48): (A) the SI-LAV system and (B) the LAV unit

The SI-LAV approach has been proposed for a novel alternative for simple on-line automated liquid-liquid microextraction [48, 49]. Sample, reagent and organic solvent are sequentially aspirated into an extraction coil connected to a central port of a conventional selection valve. Flow reversal enables good efficient extraction.

The aqueous and organic phases can be separated in a conical separating chamber, situated with a fiber-optic spectrophotometer to monitor the absorbance change in either the organic or aqueous layer. The unit (Fig. 4) is attached to one port of the valve. Applications have been made to the assays of anionic surfactant in water samples and diphenhydramine hydrochloride in pharmaceutical preparations by forming ion-association compounds with methylene blue and bromocresol green, respectively. Table 4 demonstrates some features of solvent extraction in comparison with conventional batch, FIA and SI-LAV formats.

Table 4 Features of solvent extraction performed in conventional batch, FIA and SI-LAV formats

	Conventional batch	FIA	SI-LAV
Consumption:			
reagents	+++	++	+
sample	+++	++	+
solvent	+++	++	+
Sample through-put	+	+++	++
Less hazardous (safety)	+	+++	+++
Cost			
• apparatus/ instrument	+	++	++
• per sample	+++	++	+

"+" refers to more scale

The SI-LAV setup for the previously described on-line solvent extraction may also be used for some applications employing stopped flow [51] (see Fig. 5(A)), such as the kinetic study of the persulfate-iodide reaction:

$$S_2O_8^{2-} + 2I^- \longrightarrow 2SO_4^{2-} + I_2$$

Employing a constant concentration of persulfate but varying the iodide concentration, signal profiles for a stopped flow mode can be obtained, as shown in Fig. 5(B). Rates for each reaction condition can be obtained from the slope of the peak profile. The order of reaction in a rate law can then be deducted from the graph (log [iodide] vs. log [rate], as illustrated in Fig. 5(C)).

Exploiting SI-LAV with air-segmented flow for automated on-line bead-based immunoassay has been made. The system provides precise delivery of micro-volumes of reagent and precise time of incubation and washing steps via manipulation of the syringe pump. Based on competitive enzyme linked immuno sorbent assay (ELISA), hyaluronan (HA), a biomarker, can be assayed by the competition of HA immobilized on beads and HA in the solution, to bind with a fixed amount of biotinylated HA-binding proteins (b-HABPs). After separation, anti-biotin conjugated with enzyme and a suitable substrate are introduced to follow the binding reaction of the immobilized HA and b-HABPs, whose degree of binding is indirectly proportional to the

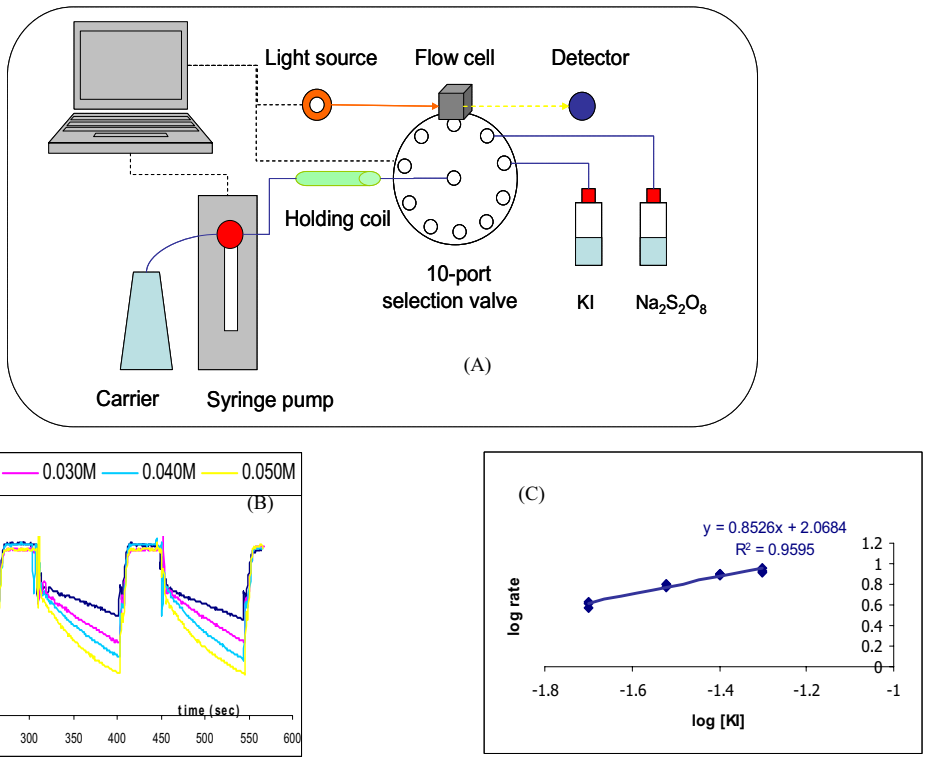

Fig. 5 SI-LAV for kinetic study of perfulfate-iodide reaction: (A) the SI-LAV system, (B) the signal profiles and (C) relation of log [Iodide] vs. log [Rate]

amount of HA in solution. Total analysis time for this automation is 30 min, comparing to 5-8 h for the conventional batch well ELISA. The system has been applied to human serum [52].

Investigation in progress is another SI-LAV system with bead injection for application to clinical analysis for the immunoassay for chondroitin 6-sulfate (C6S) as a biomarker for cartilage disease [53].

4. Conclusion

Flow based techniques provide advantages in automation and miniaturization, favoring "zero emission" and "greener" chemistry approaches. Sequential Injection (SI) with Lab-on-Valve (LOV) features give benefit in down scaling for chemical analysis. SI with Lab-at-Valve (LAV) approach may serve as a cost effective alternative. This enables down scaling chemical analyses, from operation on lab bench space to the manipulation at a valve.

Acknowledgements

The authors thank The Thailand Research Fund (TRF) (for support), Postgraduate Education and Research Program in Chemistry (PERCH) (for partial support), Professor Shoji Motomizu (for collaborative work), Professor Tadao Sakai (for collaborative work under the Frontier Research Project: Materials for 21st Century- Materials Development for Environment, Energy and Information) and Professor Gary Christian (for valuable comments).

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(Received October 29, 2006)

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Analytica Chimica Acta 575 (2006) 151–158
ANALYTICA CHIMICA ACTA
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An absorbance-based micro-fluidic sensor for diffusion coefficient and molar mass determinations

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Received 4 April 2006; received in revised form 19 May 2006; accepted 24 May 2006
Available online 3 June 2006

Abstract

The H-Sensor reported herein is a micro-fluidic device compatible with flow injection analysis (FIA) and high performance liquid chromatography (HPLC). The device detects analytes at two separate off-chip absorbance flow cells, providing two simultaneous absorbance measurements. The ratio of these two absorbance signals contains analyte diffusion coefficient information. A theoretical model for the sensing mechanism is presented. The model relates the signal Ratio to analyte diffusion coefficient. The model is qualitatively evaluated by comparing theoretical and experimental signal Ratio values. Experimental signal Ratios were collected via FIA for a variety of analytes, including sodium azide, benzoic acid, amino acids, peptides, and proteins. Measuring absorbance at multiple wavelengths provides higher order data allowing the analyte signals from mixtures to be deconvolved via classical least squares (CLS). As a result of the H-Sensor providing two simultaneous signals as a function of time for each sample injection, two simulated second-order HPLC chromatograms were generated using experimental H-Sensor data. The chemometric deconvolution method referred to as the generalized rank annihilation method (GRAM) was used to demonstrate chromatographic and spectroscopic deconvolution. GRAM also provides the signal Ratio value, therefore simultaneously obtaining the analyte diffusion coefficient information during deconvolution. The two chromatograms successfully serve as the standard and unknown for the GRAM deconvolution. GRAM was evaluated on chromatograms at various chromatographic resolutions. GRAM was found to function to a chromatographic resolution at and above 0.25 with a percent quantitative error of less then 10%.
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Keywords: Absorbance; Chemometrics; Flow injection; Liquid chromatography; Sensor; Molar mass; Diffusion coefficient

1. Introduction

The power of hyphenated chromatographic techniques and their subsequent higher order data sets has been known for some time [1,2]. Hyphenation within separation science falls within two categories. The first category generally refers to coupled-column separation techniques such as two-dimensional separations (i.e., LC × GC, GC × GC). The second category concerns a separation combined with a multi-channel detector (i.e., LC–MS, GC-FTIR) and is referred to as a hyphenated system [3]. This report concerns the development and initial proof-of-principle evaluation of such a multi-channel detector, termed the H-Sensor.

Based upon the concept of the previously developed microfabricated T-Sensor [4–9], and H-Filter [10], the H-Sensor selectivity is diffusion coefficient dependent. The name of the detector arises from its characteristic H-shaped flow channel. The H-shape allows two flow streams to merge under laminar flow conditions. The two streams are termed the sample stream and the receiving stream. The only difference between the two streams is that the sample stream initially contains analyte(s) within a host solvent while the receiving stream initially contains only the host solvent. Both enter the microfabricated flow channel at the left hand branches of the H, as depicted in Fig. 1. After merging, the two streams travel down the central channel. While in the central channel, the analytes within the sample stream are allowed

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doi:10.1016/j.aca.2006.05.083

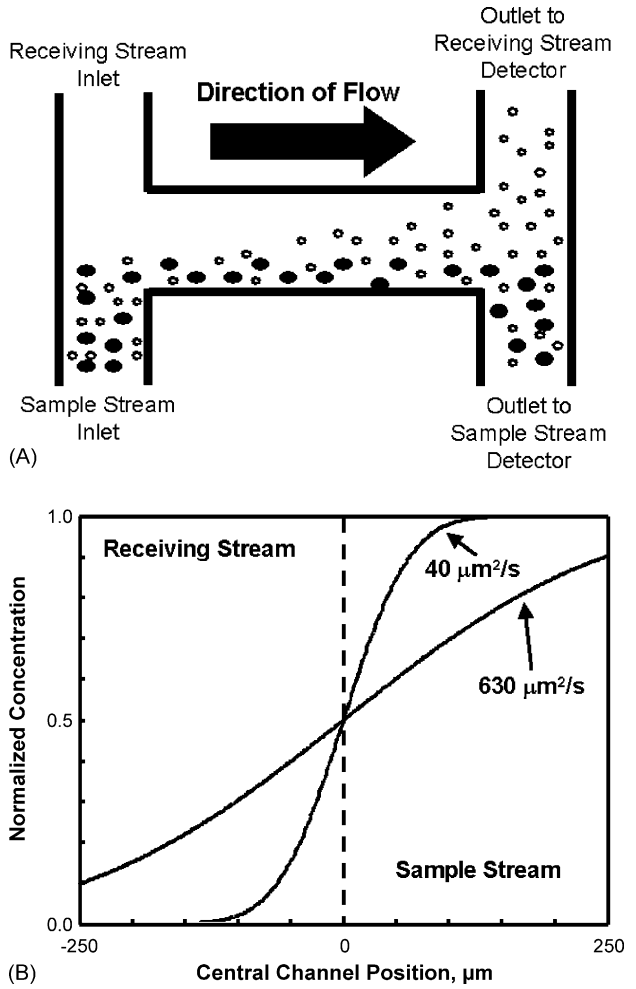

Fig. 1. (A) A schematic diagram of H-Sensor flow cell. The sample stream enters from the lower left hand side of flow channel. The sample stream merges with the receiving stream and flows from left to right down central channel. In the central channel, the smaller analytes (illustrated by small open symbols) within the sample stream diffuse into the receiving stream. The larger analytes (illustrated by larger black symbols) pre-dominantly remain in the sample stream. Upon reaching the end of the central channel, the two streams diverge and exit to their respective absorbance detector. (B) Theoretical diffusion profiles of two analytes having diffusion coefficients of 630 and 40 $\mu m^2/s$. Generated by Crank's solution (Eq. (2)) for the H-Sensor dimensions used in these studies: 500 μm wide × 200 μm deep × 2 cm long at a total flow rate of 4 μL/min (residence time of 30 s). The Ratio of the analyte having a diffusion coefficient of 630 $\mu m^2/s$ was predicted to be 0.38 while the Ratio of the analyte having a diffusion coefficient of 40 $\mu m^2/s$ was predicted to be 0.09.

to diffuse into the receiving stream. Smaller analytes, having larger diffusion coefficients, diffuse more rapidly from the sample stream into the receiving stream than larger analytes with smaller diffusion coefficients, as illustrated graphically in Fig. 1. Upon reaching the end of the central channel, the two streams diverge and exit the channel at the right-hand branches of the H. Each stream then flows into a separate off-chip absorbance flow cell. Both the sample and receiving stream fluidic paths and detectors should be identical. The detectors are capable of monitoring the absorbance at one wavelength or over a wavelength spectrum.

This report begins with the development of a quantitative theoretical description of the H-Sensor's detection principles. The theory relates an experimental signal Ratio to analyte diffusion coefficient. The signal Ratio is defined as the absorbance of the receiving stream over the absorbance of the sample stream. The experimental signal Ratios obtained via flow injection analysis (FIA) are reported and compared to theoretical predictions. The report also outlines the potential that the H-Sensor holds in sample mixture deconvolution. Absorbance recorded at multiple wavelengths creates a three-dimensional data set (time, absorbance, and wavelength). This multi-dimensionality allows for data deconvolution. We report successful application of classical least squares (CLS) deconvolution to H-Sensor data of a binary mixture. Lastly, using a simulated chromatographic time axis, we examine the future applicability of the H-Sensor for high performance liquid chromatography (HPLC) by performing the chemometric deconvolution and quantification method referred to as the generalized rank annihilation method (GRAM) [11,12] on co-eluting chromatographic peaks.

2. Theory

To provide a better understanding of the proof-of-principle experiments discussed in this report, a theoretical description of the H-Sensor is provided. This theory aims to derive a theoretical expression that relates the aforementioned signal Ratio value to analyte diffusion coefficient. The Ratio is defined as the experimentally measured absorbance of the receiving stream, A_R, divided by the experimentally measured absorbance of the sample stream, A_S, as presented in Eq. (1).

$$\text{Ratio} = \frac{A_R}{A_S} \tag{1}$$

A larger Ratio value indicates a larger diffusion coefficient. To theoretically determine the Ratio value one must first determine the theoretical absorbance values of each stream at the time of detection. As absorbance is proportional to concentration, it is apparent that the theoretical analyte concentration in each stream as the stream exits the H-Sensor chip needs to be determined. Towards that goal, a more thorough understanding of the on-chip fluid dynamics is needed.

The H-Sensor's dimensions are such that laminar flow dominates. Therefore, mixing between the sample and receiving streams occurs primarily via diffusion. Crank's solution for one-dimensional diffusion, from an initial step profile at x equal to zero, when the diffusion is not influenced by the analyte concentration, describes the concentration of an analyte at time t and position x as [13]:

$$C = \int \frac{C_0}{2}[1 + \text{erf}(\xi)] \tag{2}$$

where erf(ξ) is the error function of argument $\xi = x/(4Dt)^{1/2}$, and C_0 is the initial analyte concentration at the time when the streams merge ($t = 0$). Applying Crank's solution to the H-Sensor is straightforward. The zero time point, $t = 0$, is defined as the time at which the sample and receiving streams merge, while maintaining laminar flow [5]. At this instant, the sample concentration profile across the channel is assumed to be a step function. Crank's solution provides analyte concentration across

the width of the channel (the x dimension) at any time, t. Solving Eq. (2) at a given time for a variety of x-positions yields a concentration profile, such as those seen in Fig. 1B. In Fig. 1B, the x-axis is the width of the central flow channel. Fig. 1B presents theoretical concentration profiles for two analytes with different diffusion coefficients: 630 and 40 $\mu m^2/s$. The profiles were generated using a typical residence time of 30 s beyond the instant of stream merging. The analyte with the larger diffusion coefficient diffuses more readily across the channel resulting in a shallower concentration gradient than the analyte with the smaller diffusion coefficient.

This model assumes that the receiving and sample streams split at the center of the chip, $x = 0$, before exiting and heading to the two detectors. Therefore, the theoretical absorbance values of each stream are directly proportional to the total concentration of analyte in each side of the flow channel at a time equal to the residence time. The analyte concentrations can be calculated by taking the integral of the concentration profile (Eq. (2)) over the appropriate limits at time equal to the residence time. Allowing w to equal the channel width, the sample stream's limits are from $x = (w/2)$ to 0. The receiving stream's limits are from $x = 0$ to $-(w/2)$.

$$\text{Sample stream concentration} = \int_0^{w/2} \frac{C_0}{2}[1 + \text{erf}(\xi)] \quad (3)$$

$$\text{Receiving stream concentration} = \int_{-w/2}^{0} \frac{C_0}{2}[1 + \text{erf}(\xi)] \quad (4)$$

Dividing the receiving stream concentration by that of the sample stream yields the theoretical Ratio, R_t, for an analyte with known diffusion coefficient. This Ratio is independent of the initial analyte concentration, as shown in Eq. (5).

$$R_t = \frac{\text{Recieving stream concentration}}{\text{Sample stream concentration}} = \frac{\int_0^{w/2}[1 + \text{erf}(\xi)]}{\int_{-w/2}^{0}[1 + \text{erf}(\xi)]} \quad (5)$$

The initial concentration of the analyte entering the sample stream does not significantly influence the theoretical Ratio as long as the concentration is low enough as to not significantly influence the diffusion coefficient of the analyte.

For ease of visualization and discussion, we have taken the liberty of relating the theoretical and experimental Ratio values to molar mass rather than diffusion coefficient. The following generalized equation was used to find the diffusion coefficient of an analyte with a known molar mass.

$$D = 10^4 \times M^{-0.6} \quad (6)$$

In Eq. (6), M is molar mass and D is diffusion coefficient in $\mu m^2/s$. The values for the coefficient and exponent in Eq. (6) are representative of a large selection of specific values given in the second edition of the polymer handbook [14]. Eq. (6) serves as an adequate tool for determining the trend of the diffusion coefficient to molar mass relationship for a large group of analytes (i.e., random coil linear molecules in a good solvent). This theory is not designed for predicting accurate diffusion coefficients or molar masses for a particular analyte. However, the theory provides an adequate tool for evaluating the Ratio data given by the H-Sensor is terms of molar mass for a large sampling of diverse analytes.

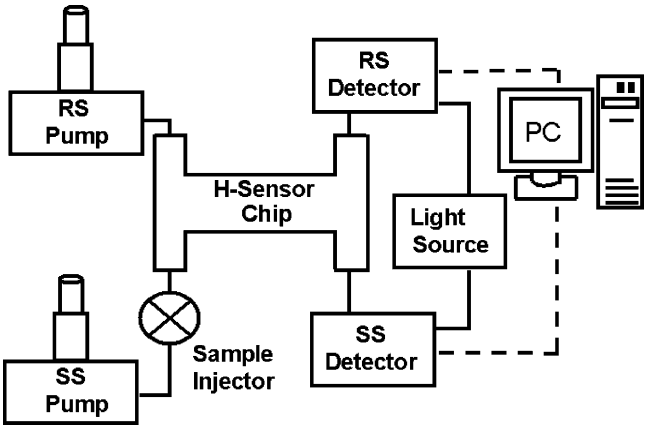

Fig. 2. A schematic diagram of the H-Sensor set up for flow injection analysis (FIA). RS and SS stand for receiving stream and sample stream, respectively.

Theoretical Ratios were calculated using Eq. (5) for the hypothetical analytes shown in Fig. 1B. The analyte with a diffusion coefficient of 630 $\mu m^2/s$ has a theoretical Ratio of 0.38. The second hypothetical analyte, with a diffusion coefficient of 40 $\mu m^2/s$, has a theoretical Ratio value of 0.09. Using Eq. (6) to convert the diffusion coefficients to molar mass, one sees that a $D = 40$ $\mu m^2/s$ is indicative of an analyte with an approximate molar mass of 10,000 g/mol. The much higher 630 $\mu m^2/s$ diffusion coefficient corresponds to a lower molar mass of about 100 g/mol.

3. Experimental

The H-Sensor system was assembled as shown in Fig. 2. The receiving stream and sample stream pumps were microgradient syringe pumps (Microgradient System, Brownlee Labs, Santa Clara, CA). The sample stream pump carried fluid through an injector fitted with a 1.5 μL sample loop (6 port, Rheodyne, Rohnert Park, CA). Detector flows cells, for the RS and SS detectors, respectively, were constructed from PEEK crosses (Upchurch Scientific, Oak Harbor, WA). Fluid entered and exited the flow cells in a straight line. It is essential that the fluid flow in both streams on the chip and off the chip be identical. Great care was taken in ensuring the lengths, and inner diameters of the tubing as well as all fluidic fittings were identical. Specifically, the flow cells should be positioned such that they create an equal backpressure (i.e., identical fluidic path length). Equalizing the backpressure helps maintain an equal on-chip split of the two streams. With great care any H-Sensor induced disturbance of the original analyte peak profile can be minimized. The light source for each flow cell entered perpendicular to the fluid stream and exited in the straight line. A bifurcated fiber optic cable (300UM Aluminum ZFQ-1437, Ocean Optics, Dunedin, FL), modified in house such that it has 1/16 in. outer diameter, was used to carry light to each flow cell from a continuous Xenon lamp (Hamamatsu, Bridgewater, NJ). The light for each flow cell was collected via a second fiber optic cable (FIA-P400-SR

Table 1
Analytes used for Ratio and deconvolution studies

Analyte	Molar mass (g/mol)	Concentration in PBS buffer (ppm)
Sodium azide[a]	65	2.15
2-Hydroxypyridine[a]	95.1	10.92
Sodium pyruvate[a]	110	2.96
Benzoic acid[b]	122.4	2.38
Phenylalanine[a]	165.2	2.54
Tryptophan[a]	204.2	2.92
Peptide 451, ggyr[a]	451.5	0.94
Peptide 848, WAGGDASGE[a]	848.8	0.83
Peptide 1009, APRLRFYS[a]	1009.2	1.03
Cytochrome *C*[a]	12327	1.1
Myoglobin[a]	16700	0.87
Carbonic anhydrase[a]	29000	0.64
Ovalbumin[a]	43000	0.93
Bovine serum albumin[a]	68000	1.13

[a] Sigma–Aldrich, St. Louis, MO.
[b] Eastman Kodak, Rochester, NY.

FMD30971, Ocean Optics, Dunedin, FL) that was then connected to a dual-channel UV–vis absorbance detector (SD-2000, Ocean Optics, Dunedin, FL) for the absorbance measurements.

The H-Sensor glass-chip was etched in glass using methods previously described [7]. The branches of the H-shaped flow channel are 1 cm long and 250 μm wide. The central H channel is 2 cm long and 500 μm wide. The entire chip was etched to a depth of 200 μm. Holes were bored at the sample and receiving stream inlets and outlets using 0.75 mm diamond tipped drill bits (C.R. Laurence Co., Los Angeles, CA). Nanoport assemblies (N-126S, Upchurch Scientific) were used to connect the H-Sensor micro-fluidic chip to PEEK tubing. For all experiments, both the receiving and sample stream pumps were filled with 10 mM pH 7.4 phosphate buffer solution. All samples were dissolved in the same buffer solution. Each stream had a flow of 2 μL/min, for a total flow through the central channel of the H-Sensor of 4 μL/min. The samples analyzed are shown in Table 1. All samples and buffer chemicals were purchased from either Sigma–Aldrich (St. Louis, MO) or Eastman Kodak (Rochester, NY); see Table 1 for details. Five solution mixtures of benzoic acid and peptide 848 (WAGGSASGE) were also prepared for deconvolution studies. Each sample contained a total injected concentration of 1000 ppm. Two pure samples as well as three mixtures were prepared. The three mixtures had 3:1, 1:1, and 1:3 mass/mass ratios of peptide 848:benzoic acid.

4. Results and discussion

We began by performing proof-of-principle experiments with the absorbance-based H-Sensor coupled to FIA. These early experiments were aimed at obtaining experimental Ratios (Eq. (1)) and comparing them to the theoretical Ratios obtained via Eq. (5). Fig. 3A shows the multi-wavelength sample stream data for benzoic acid. The receiving stream data is not shown for brevity. Initially, for benzoic acid, experimental Ratios were calculated using only the absorbance at 280 nm. Fig. 3B presents the 280 nm data for both the sample and receiving streams for the same benzoic acid analysis. The Ratio for benzoic acid (0.45) was calculated by dividing the area under the receiving stream signal (shown in Fig. 3B) by the area of the sample stream signal (also in Fig. 3B).

In this manner, Ratio values were experimentally determined for all of the analytes in Table 1. For many analytes, it is more informative to relate to molar mass than to diffusion coefficient, since often the diffusion coefficient information is not readily available (as was the case for many of the analytes in this report). Fig. 4 shows the Ratio values for all analytes tested versus each analyte's molar mass. The solid curve in Fig. 4 is the theoretical relationship between the Ratio and molar mass as estimated by Eqs. (5) and (6). The experimentally obtained Ratio values show an adequate agreement with theoretically calculated curve. It is important to note that the curve has been calculated from a general expression (Eq. (6)). The proximity of any one data point to the curve is not as important as the correlation between the experimental data and the theoretical curve.

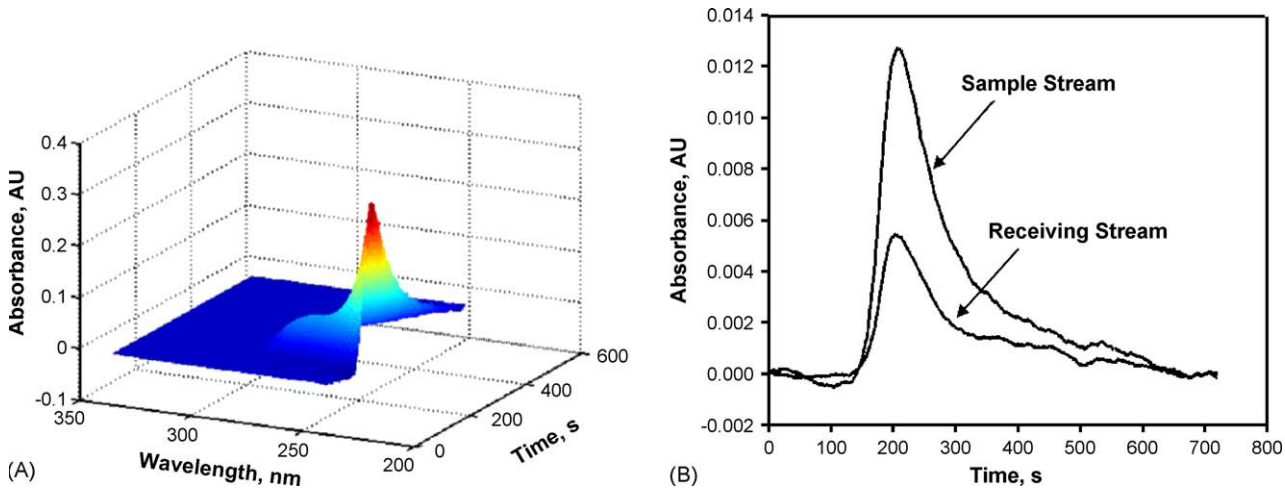

Fig. 3. (A) The sample stream data collected for a single injection of benzoic acid. Note that a similar, yet with a less intense signal, data file is collected for the receiving stream. Each injection results in two data files. (B) Data initially collected as in (A) for benzoic acid with only the absorbance at 280 nm plotted. Both the sample and receiving stream data are shown. The experimental Ratio for benzoic acid is 0.45.

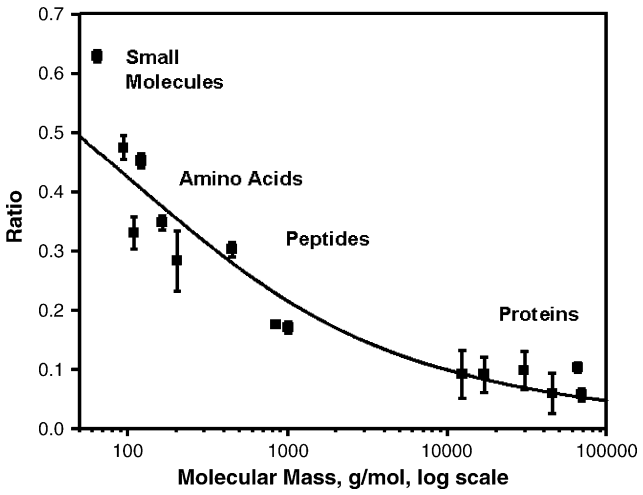

Fig. 4. Comparison between several experimental Ratios (Eq. (1)) plotted along with the theoretical Ratio curve (Eqs. (5) and (6)). The experimental Ratio values plotted as a function of molar mass. The theoretical conversion between diffusion coefficient and molar mass was performed using Eq. (6).

The correlation between the experimental and theoretical Ratio values was reassuring as we began investigating the analytical utility of the multi-wavelength features of the H-Sensor. Thus, our next step was to investigate the deconvolution of individual analyte signals from mixtures using the multi-wavelength information provided by this absorbance-based H-Sensor. A series of binary mixtures were created to test the applicability of a classic least squares (CLS) deconvolution of H-Sensor data. Five solutions of benzoic acid and peptide 848 (mixture details given in the Section 3) were prepared and injected into the H-Sensor. Fig. 5A presents the sample stream signal for a mixture of 250 ppm benzoic acid and 750 ppm peptide 848. Note that Fig. 5A is only one-half of the data acquired from a single analyte injection. There is also a unique signal detected for the receiving stream. For ease of analysis, the absorbance at each wavelength was summed along the time axis, thus removing the time dependence at this juncture, leaving two (both receiving and sample stream) two-dimensional data sets.

With the data reduced to absorbance versus wavelength, the classical least squares method was used to find the contribution of each analyte in the mixtures for both the sample and receiving streams, and thus, be able to determine the Ratio values of each deconvoluted analyte. The CLS method assumes that the mixture spectrum was due to a linear combination of the pure components of the mixture. Both the mixture and the pure component sample stream spectra are shown in Fig. 5B. Each pure spectrum is then multiplied by a factor and summed with the other pure spectrum. This process is repeated until the summed pure spectra match, as closely as possible, the mixture spectrum. The concentration of each analyte in the mixture is calculated by multiplying the concentration of the pure spectrum by the same factor that yielded the best-matched spectrum (minimizing the error of the CLS fit). Lastly, Fig. 5B also shows a plot of the difference (i.e., the minimized error of the fit) between the predicted mixture spectrum as compared to the experimentally measured mixture spectrum. The intensity of the difference plot is less than one order of magnitude of the original spectra. This suggests a reasonable CLS fit, albeit with some run-to-run variation from the individual standards and the mixture.

We now turn our attention to investigate the utility of the CLS deconvolution of the receiving and sample stream signals for several mixtures of benzoic acid and peptide 848, in order to provide consistent Ratio values. The theory above assumes that diffusion, and therefore the Ratio value, is independent of analyte concentration. CLS deconvolution provides a receiving and sample stream concentration for each analyte in the mixture. If the assumption is correct, the Ratio value for each analyte should be constant across all mixtures, suitably near infinite dilution conditions, which is often the case for many analyses. Recall

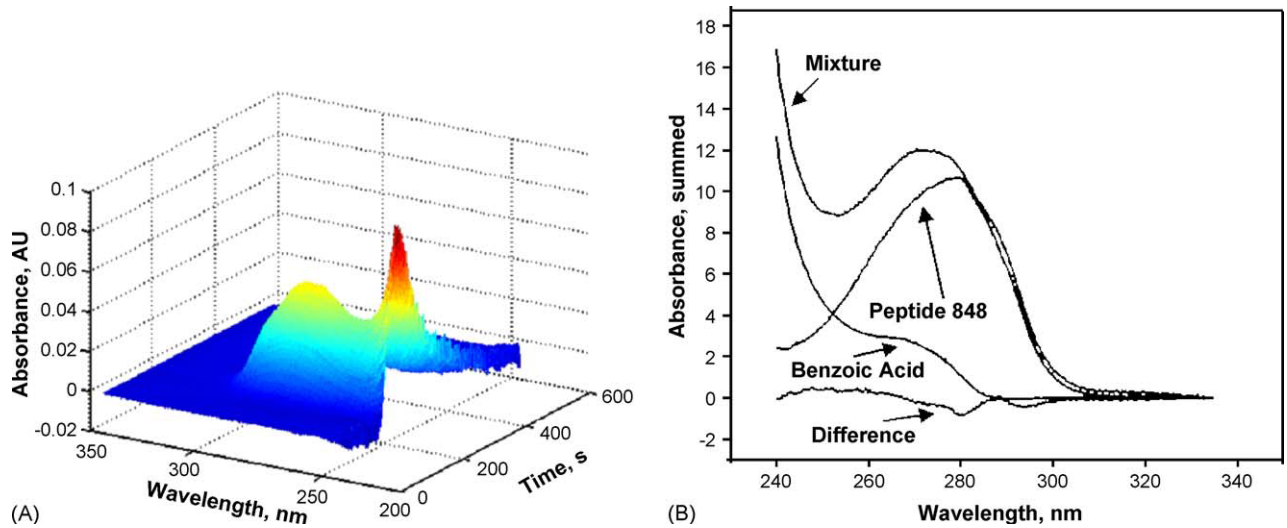

Fig. 5. (A) Sample stream signal for a mixture of 250 ppm benzoic acid and 750 ppm peptide 848. Five mixtures were created and analyzed via CLS. (B) Pure and mixture sample stream spectra of benzoic acid and peptide 848 used for CLS deconvolution. The absorbance values were summed across all time points to yield the two-dimensional representation. Also shown is the difference plot. The difference plot was generated by subtracting the summed deconvolved spectra from the mixture spectrum. This mixture data was from the 1:3 mass:mass mixture of benzoic acid and peptide 848.

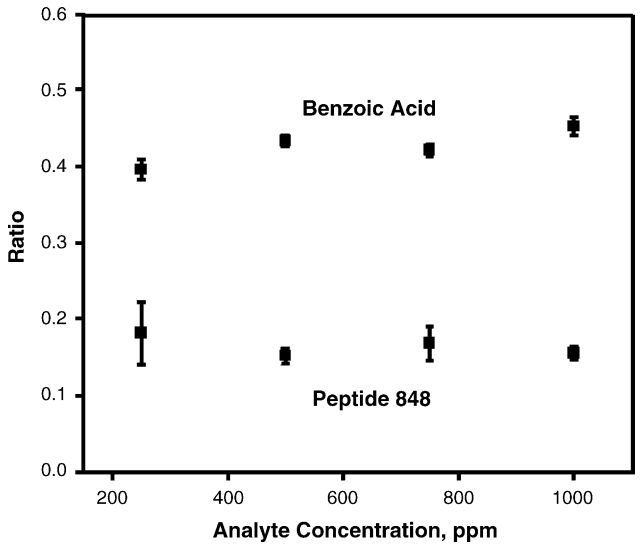

Fig. 6. The Ratio values calculated from the CLS fit. The Ratios for benzoic acid and peptide 848 remain relatively independent of concentration.

from the Section 3, two pure samples as well as three mixtures were prepared, at 3:1, 1:1, and 1:3 mass/mass ratios of peptide 848:benzoic acid. For example, the 1:3 mixture was 750 ppm benzoic acid to 250 ppm peptide 848. After CLS deconvolution, a Ratio value for each analyte in the mixture can be easily found by dividing the calculated receiving stream concentration by the calculated sample stream concentration. Fig. 6 shows the Ratio values for both benzoic acid and peptide 848 calculated in this manner. The Ratio values are relatively constant, regardless of the concentration of each analyte in the mixture. This fact supports the assumption made above that we are operating sufficiently within the infinite dilution regime, where the diffusion of an analyte is independent of analyte concentration. Working within the infinite dilution range also insures that the sample stream viscosity will not increase significantly due to the analyte presence. If this were to occur the merge point on-chip would shift, artificially increasing the receiving stream signal intensity. For more information concerning viscosity-induced flow changes, readers are referred to two previous publications [7,9]. In this regard, a more thorough quantitative study specifically for the H-Sensor is warranted after the success of this proof-of-principle study.

The final portion of the current study involved simulating chromatographic data using actual experimental H-Sensor data to determine the applicability of the generalized rank annihilation method deconvolution of H-Sensor data [11,12]. Overlapped HPLC peaks were simulated by offsetting the pure peptide 848 FIA signal by varying lengths of time from the pure benzoic acid FIA signal. The length of the offset was such that the chromatographic resolution between the peaks ranged from 0 to 1, equating to a retention time difference of 0–300 s. The time axis of the peptide 848 data was changed by adding the desired offset (in seconds) to each time point. The two data sets were then added together to create the simulated chromatographic data set. In this manner, several simulated chromatographic separations of benzoic acid and peptide 848 mixtures were generated, each with a different resolution. The simulated separations are chemometrically defined as second order for either the receiving or sample stream signals when considered separately (simulated HPLC/multi-wavelength absorbance). However, the separations are third order when the receiving and sample stream data are considered jointly. The three axes of interest are simulated separation time, spectral selectivity, and analyte diffusion coefficient selectivity.

To successfully deconvolute the individual analytes of a co-eluting mixture, GRAM requires knowledge of how many analytes are in the mixture, as well as two second-order data files (a ‘standard’ and an ‘unknown’) of the co-eluting mixture. Arbitrarily, the sample stream was used as the standard, and the receiving stream was used as the unknown. No alignment was performed on the two data sets, as great care was taken to experimentally ensure equal flow rates (i.e., residence times from the chip to the two detectors) in both streams to preserve the original analyte peak profile. In principle, one could also image the absorbance on-chip in order to obtain the diffusion coefficient information, thus circumventing the issue of requiring equal flow paths following the chip to both detectors. Application of GRAM for this data returns the individual pure analyte chromatographic concentration profiles in time, their absorbance spectra, and the Ratio of each analyte between the standard and unknown chromatograms (thus diffusion coefficient information as well). Generally, the success of applying GRAM to chromatographic data is typically determined by the amount of retention time shifting between the two separately injected chromatograms. The difficulty of averting or correcting the retention time shifting problems before performing GRAM has been the subject of previous study [15,16]. However, in principle, retention time reproducibility is averted with the H-Sensor, as a chromatographic detector, since all of the necessary data for a successful GRAM deconvolution is provided with one injection. This is a result of the H-Sensor producing both a sample stream and a receiving stream chromatogram upon each injection. The two chromatograms can serve as the ‘standard’ and ‘unknown’ chromatogram as long as the chromatographically overlapped components have sufficiently unique diffusion coefficients. Unique diffusion coefficients ensure that the receiving stream chromatogram’s composition is not co-linear to the sample stream chromatogram for the overlapped components. If the chromatographic separation were based on size or diffusion coefficient, co-eluting analytes would have very similar diffusion coefficients, thus, adversely effecting GRAM deconvolution.

Demonstration of this concept is shown in Fig. 7. The sample and receiving stream chromatograms with a resolution of 0.3 are presented in Fig. 7A and B, respectively. The chromatograms are shown as a contour plot to allow for easier visualization of the change in relative signal intensity between the two. If the two analytes’ diffusion coefficients were similar the receiving stream signal would look like a less intense sample stream signal, and GRAM would be less successful. GRAM was run on each of the simulated chromatograms, the pure component peaks were shown in time, the pure component spectra were provided, and a Ratio for each analyte in the simulated chromatographic peak

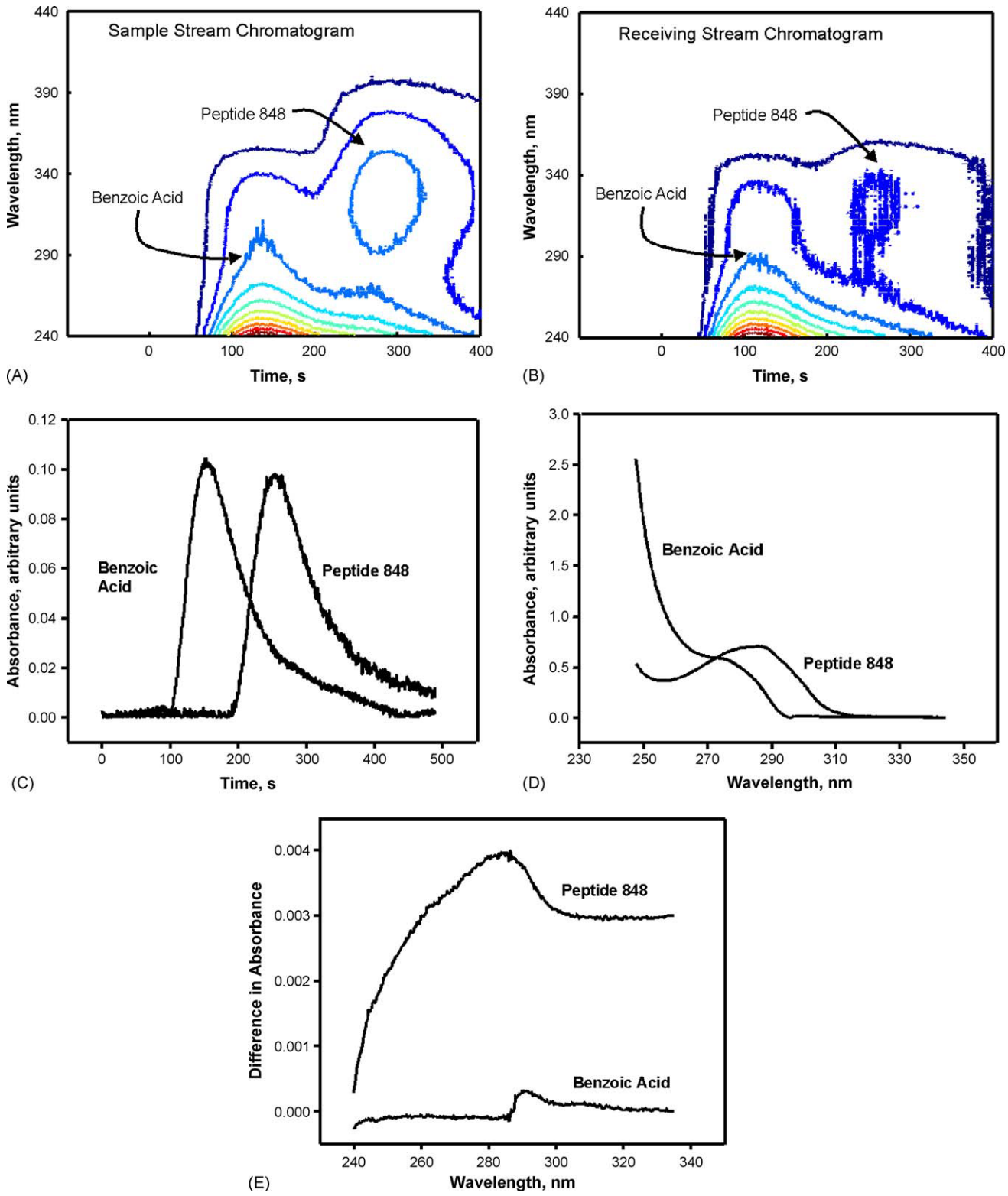

Fig. 7. (A) Contour plot of the simulated sample stream chromatogram used for GRAM. Experimental FIA sample stream peaks for 3000 ppm peptide 848 and 1000 ppm benzoic acid were offset 150 data points (90 s), giving a resolution of 0.3. The data files were then summed to yield simulated chromatograms. (B) The contour plot of the simulated receiving stream chromatogram used for GRAM. Note the different relative intensities of benzoic acid and peptide 848 in (A) and (B). (C) The GRAM provided chromatographic concentration traces of each deconvolved analyte in the mixture. (D) The spectroscopic deconvolution results of GRAM for the 0.3 resolution chromatogram for both 3000 ppm peptide 848 and 1000 ppm benzoic acid. (E) The difference between FIA obtained pure spectra for 1000 ppm benzoic acid and 3000 ppm peptide 848 spectra and the deconvolved spectra provided by GRAM. The differences are very small. Absorbance units in (E) are quantitatively relative to (D). The peptide 848 plot is offset by +0.003 for clarity.

was determined. Fig. 7C and D, shows the GRAM results for the simulated chromatogram with a resolution of 0.3. Fig. 7C shows the deconvolved concentration traces for each component in the mixture. Fig. 7D shows the deconvolved absorbance spectra for each component. Fig. 7E presents one method for evaluating the success of the GRAM deconvolution. Fig. 7E shows the two difference plots between each of the deconvolved component absorbance spectra (provided by GRAM) and the pure spectra. The peptide 848 difference plot has been offset +0.003 units for clarity. Note that the intensity of the difference plots is approximately two orders of magnitude smaller than that of the absorbance spectra provided by GRAM (Fig. 7D), indicating successful deconvolution. The results of GRAM were also evaluated by determining the percent error in the GRAM Ratio of benzoic acid and peptide 848 as resolution varies. The GRAM Ratio at a resolution of 1.0 was used as the known value. The percent error was therefore the GRAM Ratio at each resolution divided by the GRAM Ratio at a resolution of 1.0 multiplied by 100%. At all resolutions above approximately 0.25, the percent error for both analytes was less than 10%, which is consistent with previously published work [17,18]. In a worst-case scenario the flow rates between streams would not be the same and the resulting receiving and sample stream data would need to be aligned before application of GRAM. Fig. 7D and the low percent error in the GRAM Ratio provide evidence that the flow rates are very similar. However, that is not to say that flow rate fluctuations are completely unavoidable. Flow rate fluctuations could result from pump malfunction, unequal backpressure on the exit streams, or large changes in relative stream viscosity. Current and future work will entail new H-Sensor experimental designs to dampen the effect of flow rate fluctuations. However, the initial proof-of-principle GRAM results are very promising. Assuming the overlapped analytes have distinct diffusion coefficients the results show that the H-Sensor does provide all of the information for successful application of GRAM to HPLC/multi-wavelength absorbance data without the need for a second injection, or, in principle, retention time alignment.

5. Conclusions

The absorbance based H-Sensor has been introduced as a novel FIA detector. An H-Sensor model has been developed that relates an experimentally determined Ratio value to analyte diffusion coefficient and therefore molar mass. The theory adequately describes the experimental Ratio molar mass trend. The multi-dimensionality of H-Sensor data allows successful implementation of CLS methods of FIA data. CLS deconvolution of benzoic acid and peptide 848 mixtures are reported. When combined with a simulated chromatographic time axis, H-Sensor data can be analyzed with GRAM, thus providing the Ratio of each deconvoluted analyte. The H-Sensor produces two non-colinear chromatograms with each analysis. These chromatograms can serve as both the standard and the unknown in GRAM. The GRAM results show great promise in deconvolving overlapping analytes in future studies with actual chromatographic separations.

Acknowledgments

R.C. thanks the Commission on Higher Education in Thailand for financial support for her visit to the University of Washington.

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Enhancing chemical analysis with signal derivatization using simple available software packages

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Received 19 February 2007; received in revised form 19 March 2007; accepted 20 March 2007
Available online 31 March 2007

Abstract

Derivative techniques for analytical signal processing are useful for solving some noise and signal resolution problems in various fields of study such as titrimetry, spectrophotometry, chromatography and electrochemistry. The broad use of these techniques, however, is often limited by costly inflexible built-in software packages in commercial analytical instruments. We propose here the application of commercial simple software packages such as Microsoft® Excel and Microcal Origin for signal smoothing and fitting, and for obtaining derivative analytical signals in batch and flow-based analyses, including potentiometric titration, spectrophotometry, chromatography, voltammetry and sequential injection analysis (SIA). The worldwide (especially Excel) software packages are easy-to-use for less experienced users and have also capabilities for advanced users, and therefore employing such packages can result in expansion of useful derivative techniques. We demonstrate application of the available package-aided derivative capabilities for enhancing some chemical analyses, including potentiometric acid–base titration, Bradford assay of protein, chromatographic separation of ajmaline and reserpine and anodic stripping voltammetry of copper. The derivative signals from smoothed and fitted curves offer better accuracy and precision, even for non-resolving peaks and tailing peaks. In some cases, the optimization of experimental conditions is not further required, which can lead to fast method development.
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Keywords: Signal derivatization; Microsoft Excel; Microcal Origin; Enhancement of chemical analyses

1. Introduction

Derivative spectrophotometry was introduced in the early stage by Giese and French [1] to identify low intensity bands overlapped by bands of higher intensity. Afterwards, with the rapid development of data processing techniques, a great number of works on mathematical derivatization of analytical signals have been reported in many fields of study, *e.g.* spectrophotometry [2], atomic absorption spectrometry [3], voltammetry [4], and chromatography [5,6] to overcome the signal overlapping of analyte with matrices and to improve the S/N ratio. Some applications have been reported, such as, illicit drugs [7], biomedical [8], pharmaceutical, clinical and environmental analyses [9].

Derivative signals can be obtained by using hardware [5,6] and software [10–13]. Hardware derivatization has been carried out with laboratory-made electronic circuits or a derivatization unit which accompanies an analytical instrument, so that the derivative signal(s) are automatically obtained. However, the typical drawback is that the shape of the derivative signal strongly depends on the resistor–capacitor (RC) time constant of the circuit. Software derivatization may be accomplished by built-in software or by a derivatization algorithm written using the software package. Modern and costly instruments may

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0026-265X/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.microc.2007.03.005

possess a derivative function provided by built-in controlled-software for performing derivatization of signals. However, the function is usually inflexible and limited. Most general-purpose instruments or measuring devices, *e.g.* ultraviolet-visible spectrometers, fluorescent spectrometers, etc., usually do not possess a derivative function. In addition, to write software for calculating derivatives requires user's skill in programming and mathematics, which is difficult for many users.

Usually in chemometrics, one uses complicated software, but we are interested in using simple software packages as derivatization tools. They include Microsoft® Excel [14] and Microcal Origin [15]. Excel is very familiar to almost all computer users and does not require skill of programming. Hence, in many works, Excel (usually with Visual Basic) has been exploited for non-linear curve fitting [16–18], for calculating equilibrium gas speciation [19] and for estimation of pharmacokinetic parameters [20]. Origin is a very useful software package for scientific graphing and data analysis with C programming capabilities. Both software packages can be used, not only for computing derivatives of signals, but also for other data evaluation.

The aim of this work is to use such readily available software packages for derivatization of analytical signals to solve some analytical problems, so that cost-effective batch and/or flow-based chemical analyses [21] can be achieved. The application of signal derivatization for solving analytical problems such as titrimetric end point detection, separation of overlapping signals, and peak tailing is discussed.

2. Experimental

In order to perform derivatization of analytical signals with the software packages of Excel and Origin, it is necessary to obtain a digital file of the *X*–*Y* data set. The file extensions should be available for the two packages before data processing of derivatization.

Continuous flow acid–base potentiometric titration (titrant was continuously added to titrand in a vessel at a constant flow rate of 2 ml/min) was performed with a home-made flow system. The potential during the titration was recorded by a lab-made electronic unit and the data was saved as Excel files (xls).

The batchwise and SIA methods for determination of protein content by the Bradford assay [22,23] were performed using a fiber optic based spectrophotometer for detection (Ocean Optics, Inc.). The analytical signals were recorded via FIALab software [24] and were saved as DAT files (dat).

Chromatographic determination of ajmaline and reserpine was performed with an Agilent HPLC-UV model 100 at 280 nm. The absorbance–time data was recorded and saved as CSV file (csv).

To determine Cu by anodic stripping voltammetry, a 757 VA Computrace voltammetric system (Metrohm, Switzerland) was used. The voltammetric measurement was performed by using a glassy carbon-disc working electrode, a carbon auxiliary electrode and an Ag/AgCl reference electrode. The 757 PC

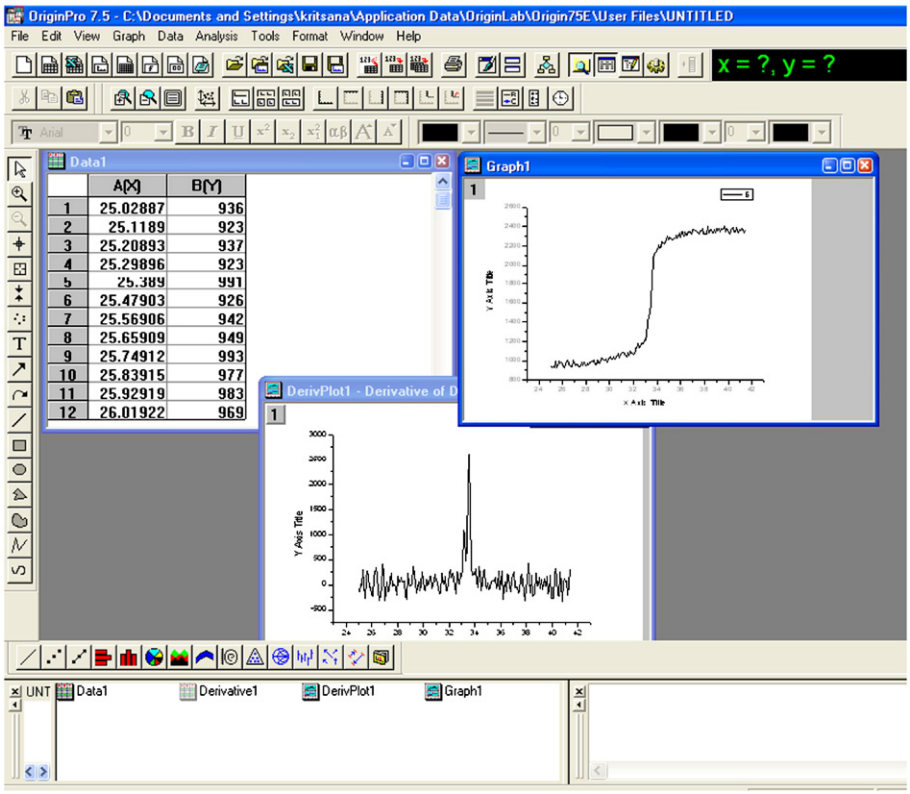

Fig. 1. Working windows of Origin.

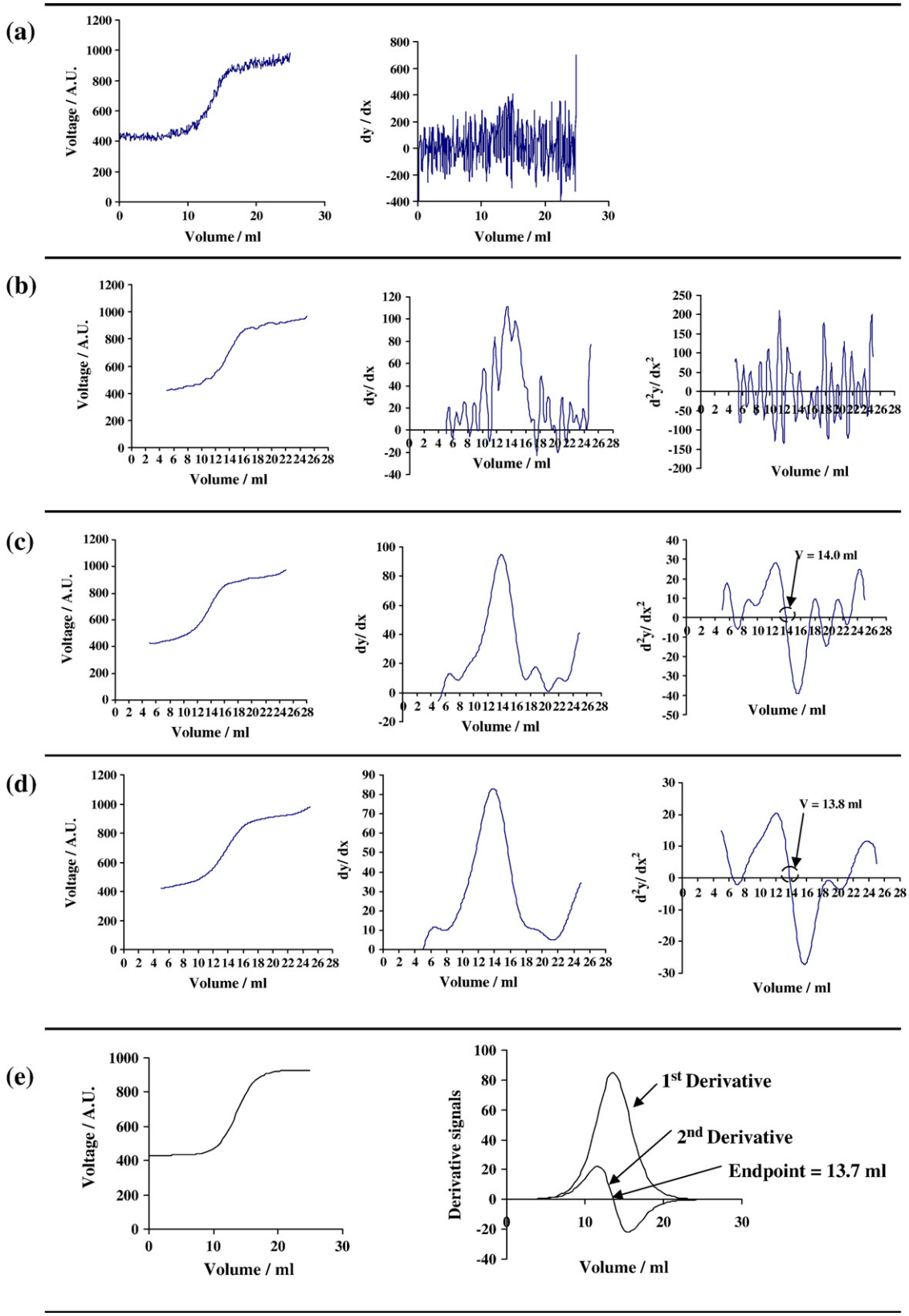

Fig. 2. Titration curve of 25.0 ml of 0.10 M HCl–0.15 M CH_3COOH mixture with 0.19 M NaOH (only the first potential break for HCl is shown here). (a), raw titration curve and its first derivative; (b), 5 data points Fast Fourier Transform (FFT) smoothing curve and its first and second derivatives; (c), 15 data points FFT smoothing curve and its first and second derivatives; (d), 25 data points FFT smoothing curve and its first and second derivatives; (e) sigmoidal-fitting curve and its first and second derivatives.

software version 2.0 with 757 VA Computrace was used to control the system. The resulting measurements were exported as TEXT files (txt).

These obtained DAT, CSV, TEXT files were able to be opened with both Excel and Origin for derivatization of signals and other data processing as well. The first derivative of analytical signals was computed by using the following equation:

$$dy/dx = 0.5^*\{[(y_{i+1} - y_i)/(x_{i+1} - x_i)] + [(y_{i+2} - y_{i+1})/(x_{i+2} - x_{i+1})]\} \quad (1)$$

where dy/dx is the first derivative data, and x_i and y_i are the ith data point. The first derivative graph was the plot of dy/dx against x. The second derivative and other derivative orders were computed by successive derivatization from the first derivative data.

Once a set of data is imported to Origin worksheet as shown in Fig. 1, we can easily plot the graph of the raw data, and also can simply click for signal derivatization to obtain simultaneously the derivative data and its graph by the built-in derivative software. Origin could perform successive signal derivatization up to the second derivative in an active working window.

Calculation of derivative data set in Excel could be also simply performed by using equation (1) in an Excel worksheet window. In this work, Excel was used for calculation of derivatization rather than using Origin because in the case of Excel, just by copying a raw data set to the pre-loaded derivative function's worksheet, the derivative data was readily obtained. These can be done several worksheets simultaneously. Therefore, it was very fast and simple to obtain derivative data if there were many raw data sets to deal with.

Excel, to our knowledge, can not be used for peak area integration and peak height measurement whereas Origin can do so simply. Hence, Origin was used for such data processing throughout this work.

3. Results and discussion

3.1. General discussion

3.1.1. Smoothing of signal: The importance of number of data points

A sigmoid titration curve is often differentiated to detect the end point. However, a noisy sigmoid titration curve obtained by titration of a HCl–CH_3COOH mixture with NaOH could not define its end point because of the noisy first derivative curve as shown in Fig. 2(a) (in Fig. 2, the second potential breaks for CH_3COOH are not shown). Therefore, signal de-noising of the original titration curves must be considered before derivatization. We have used two approaches to signal de-noising, *i.e.,* smoothing and fitting. There are several smoothing algorithms in Origin, *i.e.,* Fast Fourier Transform (FFT), Savitsky–Golay, Moving average, etc. Among them, FFT was used for smoothing of the signal depicted in Fig. 2(a) before derivatization. For smoothing, the number of data points to be considered in the calculation is important. Generally, the larger the number of data points, the smoother the signal obtained, as depicted in Fig. 2(b)–(d). As a result, the clearer derivative signals were obtained, so that the end point detection was improved. However, too large a number of data points for the calculation resulted in distortion of the shape of titration curve from the original one (Fig. 2(a)). This led to an error in the end point detection, *i.e.,* the end points obtained from the second derivative signal with 15-points (Fig. 2(c)), 25-points (Fig. 2(d)) and 45-points (not shown) are 14.0, 13.8 and 13.3 ml, respectively. The variation in the end points (*ca* 0.7 ml) was caused by the distortion of the shape of titration curve.

However, it was found that the use of sigmoidal-fitting could perform de-noising more simply and conveniently than the use of smoothing. The resulting smoothed curve and its first and second derivative curves are shown in Fig. 2(e). A very clear derivative signal was obtained, and the end point of 13.7 ml was well-defined. As advantages, Origin, allows simple "click" to adjust and change smoothing parameters, compared with the tedious manual adjustment in conventional smoothing methods. And the resulting smoothed signals are simultaneous and graphically shown. However, it should be noted that this sigmoidal-fitting algorithm should be suitable for only sigmoid-shape signals (unsuitable for non-sigmoid-shape signals).

3.1.2. Derivatization of signal: spacing of derivative

It is known that the spacing between data points used for calculation of derivatives is also important to consider since it affects the shape of the derivative signal. Large spacing causes distortion of the derivative signal. We used the pre-smoothed signal with 15 data points FFT (Fig. 2(c)) as a model of signal to be differentiated. The effect of spacing of dx is shown in Fig. 3. When the space of dx increased, the shape of the second derivative signal became broader and its amplitude decreased as well. Although the end point tended to decrease with increasing the space of dx in the range from 1 to 30, the variation of end point was not significantly large. Since the data was originally

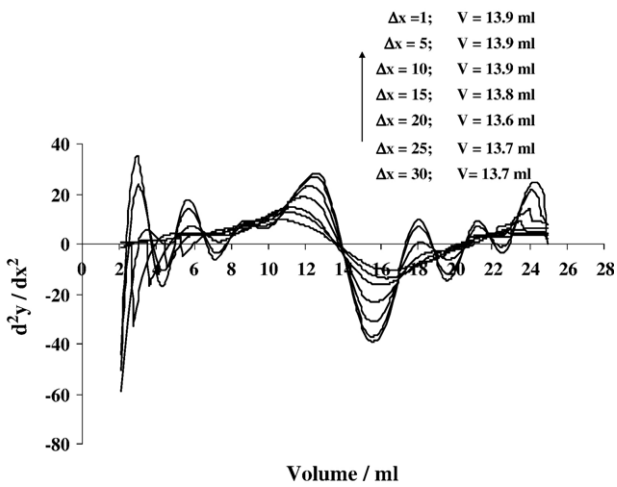

Fig. 3. Effect of spacing of dx for derivatization. The original data to be differentiated is the pre-smoothed signal with 15 data points FFT shown in Fig. 2(c).

smoothed or in some cases was de-noised, dx of 1 was used throughout for the application of derivatization.

One can set various dx values easily and simultaneously according to one's desire by using Excel, and therefore the calculation of derivatization could be performed more simply and quickly. Unlike Excel, the spacing of dx for signal derivatization calculation in Origin was fixed at 1 and could not be changed. The details for using the software packages may be obtained via their website homepages [14,15].

3.2. Detection of end point for titration

A titration curve of HCl with NaOH was performed by batchwise titration, and the first and second derivative titration curves were obtained by means of the derivative calculation mentioned above. The well-defined end point was given from the second derivative signal where it was located at the point of intersection between the second derivative signal and X-axis (became zero). The utilization of Excel for such data processing has been reported and discussed in the literature [25–27].

In the continuous flow potentiometric titration of mixture of HCl and CH_3COOH with NaOH, the titration curve is shown in Fig. 4(a) (the first potential break is shown in Fig. 2(a)). However, the titration curve was noisy, and hence the application of differentiation calculation to this signal was not feasible for obtaining the correct end points, as shown in Fig 4 (b), especially the first end point for HCl could never be found. As discussed in Section 3.1, the sigmoidal-fitting was therefore applied for de-noising. However, a limitation was encountered with performing sigmoidal-fitting over the whole curve by Origin. So, the titration curve was divided into two parts indicated by 1 and 2 in Fig. 4 before performing fitting, so that the two parts of curve could be subjected to de-noising by using Origin. The fitted curves are shown in Fig. 4(c) and (d), where their first and second derivative curves are also shown. It can be seen that the first and second derivative signals were clarified, which resulted in the easy detection of two end points. The end points for HCl and CH_3COOH were located at 13.7 ml and 33.4 ml, respectively, and those values were in good agreement with equivalence points.

3.3. Not well-resolved peaks

3.3.1. Batchwise Bradford assay for protein

Protein content was assayed by the Bradford method. The method involved the association of dye (Coomassie Brilliant Blue G-250) with protein, giving an absorption maximum at 595 nm. Under set conditions, the absorption spectra are shown

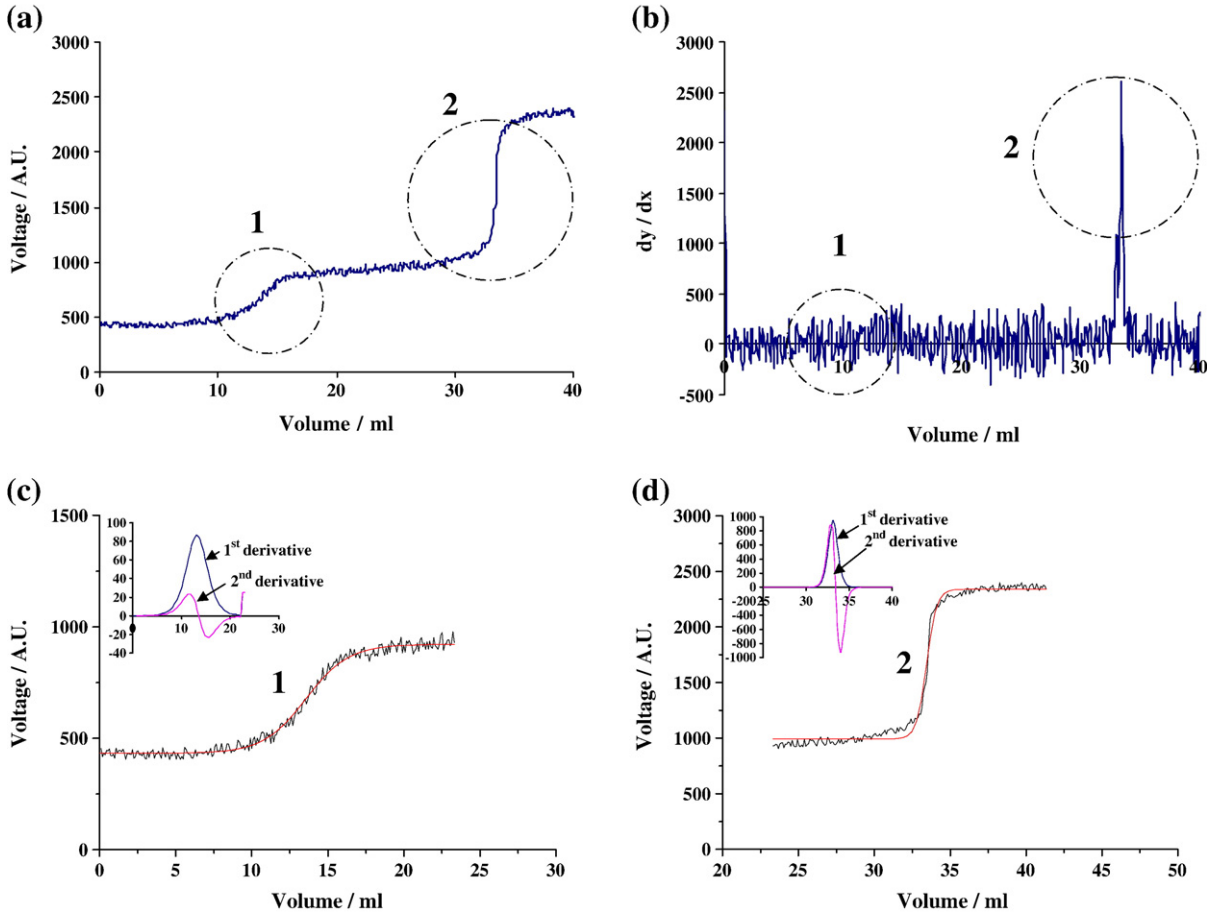

Fig. 4. Continuous flow potentiometric titration of HCl–CH_3COOH mixture with NaOH (concentration and volume as stated in Fig. 2). 1 and 2 denote the end points for HCl and CH_3COOH, respectively. Concentrations and volumes as in Fig. 2. (a), raw data of titration curve; (b) the first derivative signal; (c) sigmoidal-fitting of the first potential break and its first and second derivatives; (d), sigmoidal-fitting of the second potential break and its first and second derivatives.

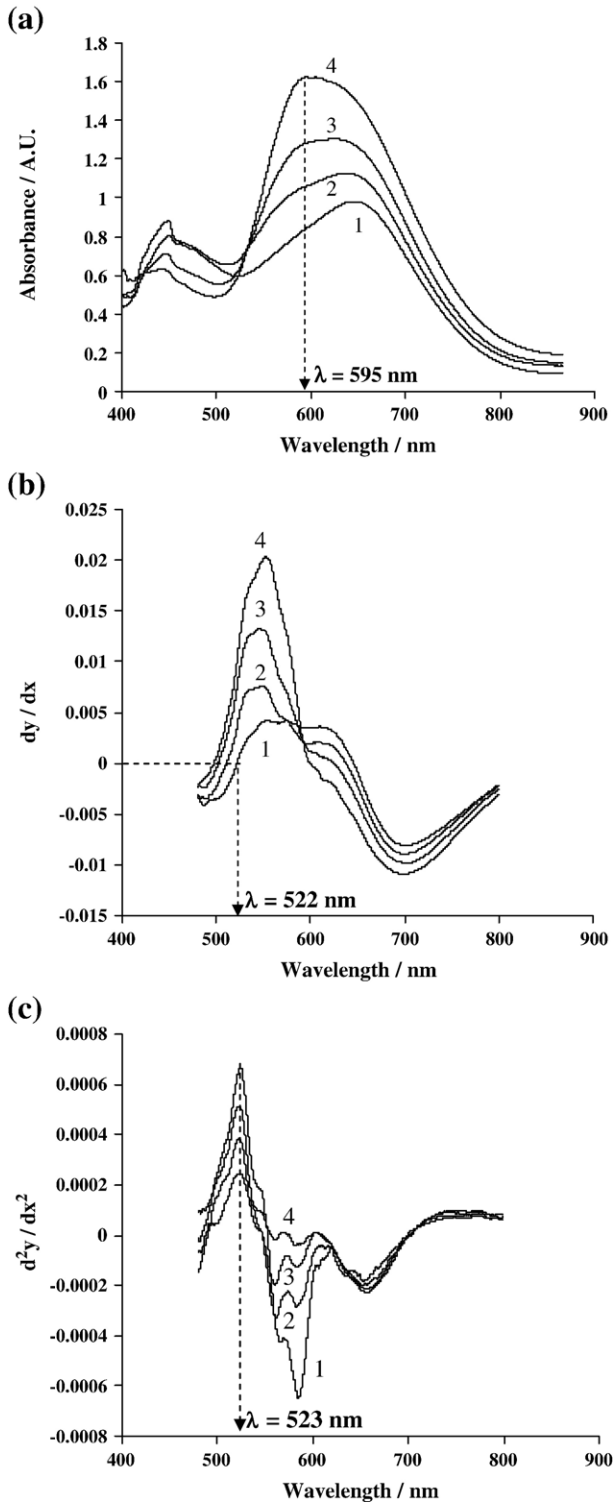

Fig. 5. Batchwise spectrophotometric determination of protein. (a), absorbance spectra; (b), the first derivative spectra; (c), the second derivative spectra. Bradford reagent: 5 ml of 0.1 g/l. Concentrations of bovine serum albumin as protein standard (mg/ml), (1), 0; (2), 0.2; (3), 0.4; (4), 0.6.

in Fig. 5(a). The absorption maximum for reagent blank was 646 nm, and that for the protein-dye ion association was 595 nm. By plotting the absorbance at 595 nm versus protein concentration, the calibration graph equation in the range of 0–0.4 mg/ml protein was $Y=1.10X+0.84$ ($r^2=0.9999$), where Y and X are absorbance and protein concentration (mg/ml), respectively. However, the dynamic range was quite short, because the analyte peak was interfered from the blank absorbance. Therefore the signal derivatization was attempted to reduce or eliminate the blank problem.

Fig. 5(b) depicts the first derivative signals. We exploited the conventional zero-crossing method which used the point

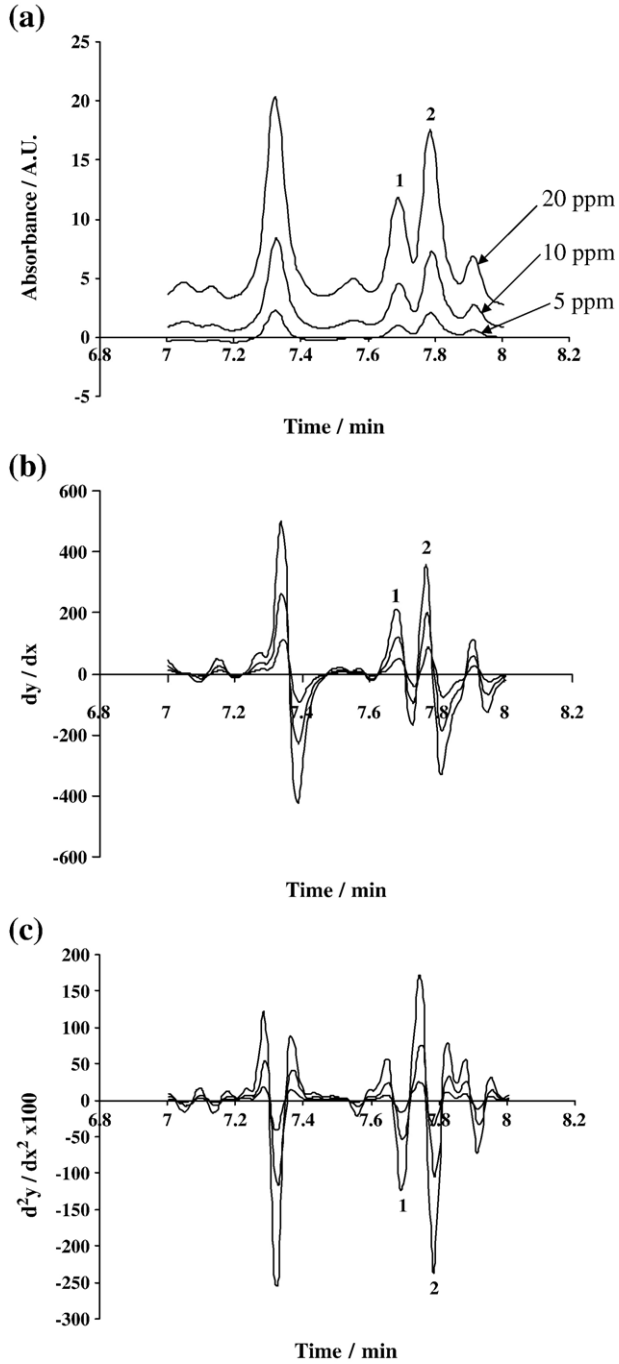

Fig. 6. Chromatographic separation of ajmaline (peak 1) and reserpine (peak 2). (a), original chromatograms; (b) the first derivatives; (c) the second derivatives. Analytical column, Luna C18 column (150×4.6 mm, 5 μm); mobile phase, 0.1% TFA and acetonitrile (gradient elution); flow rate, 1.0 ml/min; detection wavelength, 280 nm.

where blank signal becomes zero. In this case, the wavelength was at 522 nm. The calibration graph for 0–0.4 mg/ml protein was plotted by using the amplitude at this wavelength: $Y=0.0187X-8.97\times10^{-5}$ ($r^2=0.9994$), where Y and X are the first derivative amplitude and protein concentration (mg/ml), respectively. The dynamic range was not improved by using the first derivative signal. We tried to exploit the second derivative signal as another approach (Fig. 5(c)). The second derivative amplitude at 523 nm was plotted against protein concentration, and the calibration equation was $Y=0.000686X-0.000243$ ($r^2=0.9992$), where Y and X are amplitude and protein concentration (mg/ml), respectively. The dynamic range was improved to be in the range of 0–0.6 mg/ml.

3.3.2. Chromatographic separation of ajmaline and reserpine

The application of derivatives to chromatographic analysis of multicomponents was reported in the literature [28], but without any detailed information. We demonstrate here the application of a simple signal derivative procedure for chromatographic analysis of ajmaline and reserpine. These two compounds (peaks 1 and 2 in Fig. 6) were not well-resolved under the set of conditions. Quantitative analysis optimally requires good separation of the two chromatographic curves. However, optimization procedures are usually time consuming and waste many chemicals. The derivatization of the chromatogram was attempted for resolving both peaks. The relative standard deviations of peak area and peak height of derivative signals for the first and second derivatives were comparable. However, the calibration graph from the second derivative signals was more sensitive than that from the first derivative by about 50 fold. The calibration equations using the second derivative peak area and height were $Y=-21.7X+61.2$ ($r^2=0.9999$) and $Y=-720X+2.00\times10^3$ ($r^2=0.9999$) for ajmaline and $Y=-37.0X+88.3$ ($r^2=0.9999$) and $Y=-1.35\times10^3X+3.13\times10^3$ ($r^2=0.9999$) for reserpine, where Y is the peak area or height and X is the analyte concentration (ppm), respectively. The relative standard deviations of peak areas were 1.4% and 1.9% for analyzing 20 ppm ($n=3$) of ajmaline and reserpine, respectively.

Quantification of both species was feasible without further optimization of experimental conditions for resolving both species. This is very useful for fast method development. Also

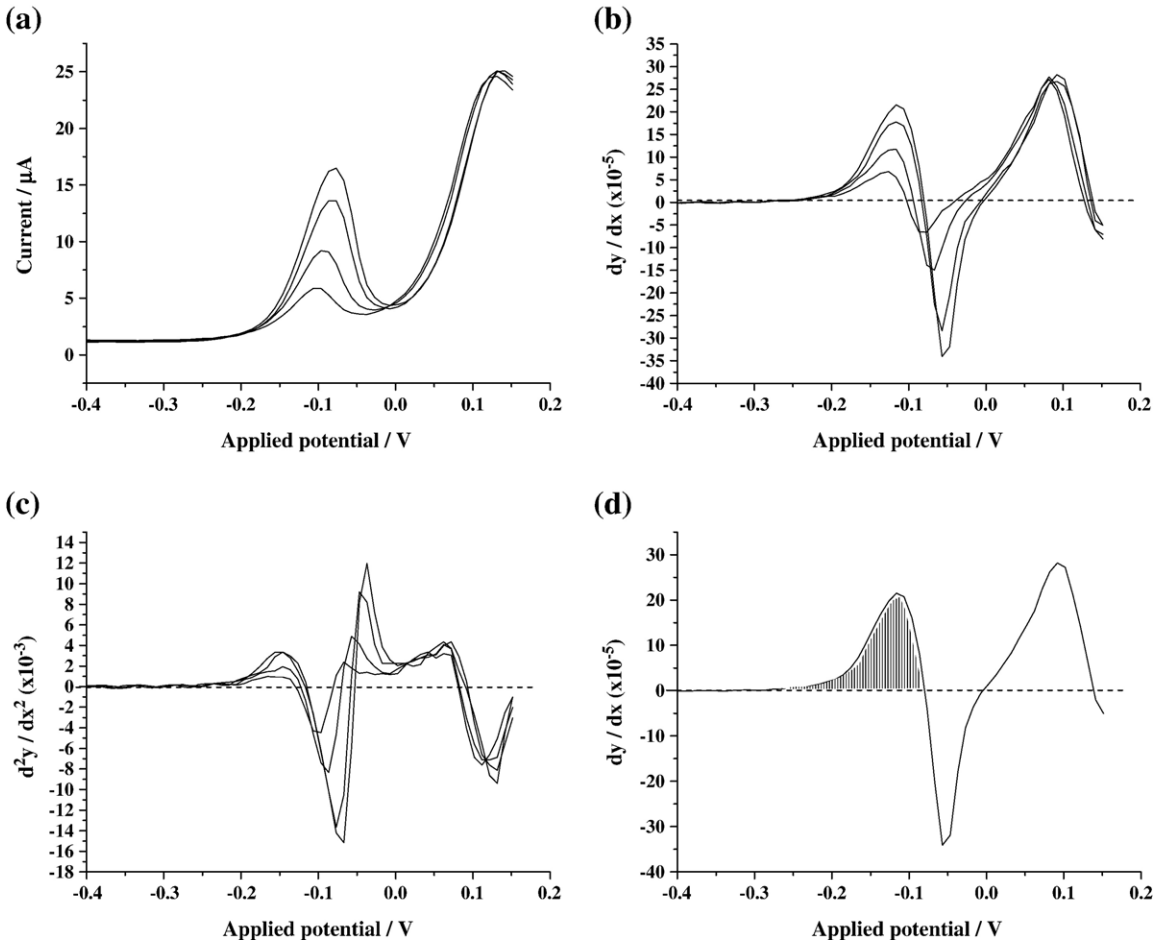

Fig. 7. Anodic stripping voltammograms for determination of Cu. (a), original; (b), the first derivatives; (c) the second derivatives; (d) the shade area denotes the peak integration area from the first derivative. Supporting electrolyte, 0.6 M acetate buffer pH 4.6; deposition potential/time, −0.8 V/87 s; scan potential, −0.9 to 0.15 V; sweep mode, square wave; step, 0.01 V; amplitude, 0.04 V; frequency, 50 Hz; sweep rate, 0.5 V/s; cleaning potential/time, 0.2 V/20 s. Cu amount (µg/ml), 0; 0.05; 0.10; 0.12.

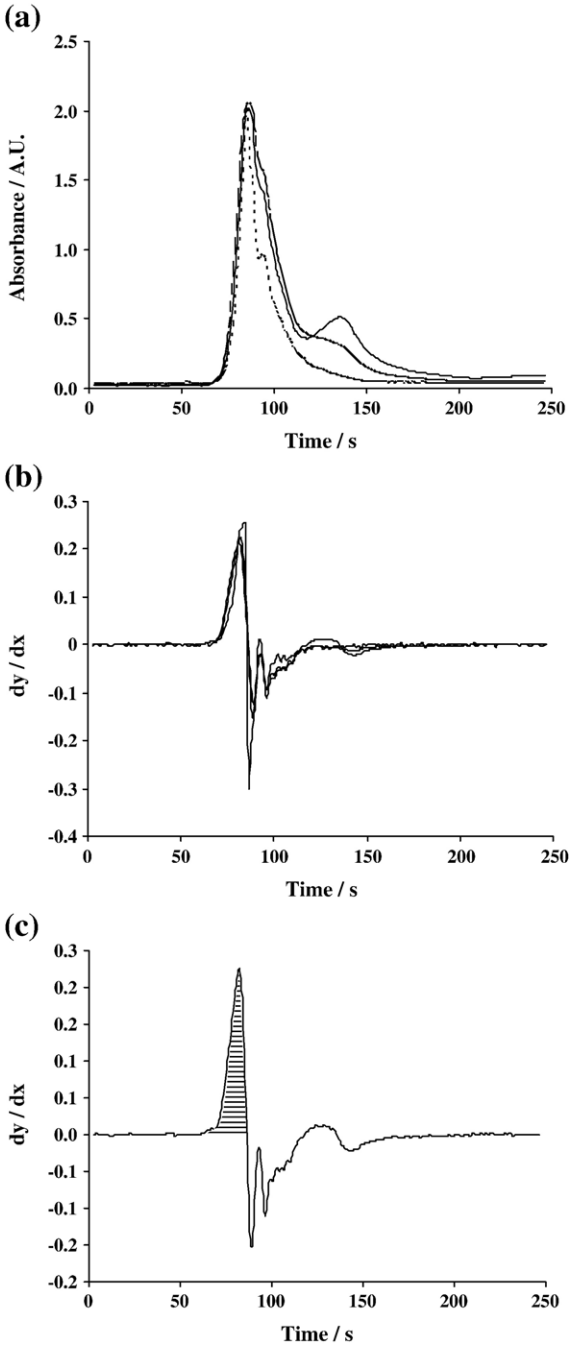

Fig. 8. SIA-grams of Bradford assay for protein (triplicate analysis). (a), original; (b), the first derivative; (c), the shade area denotes the peak integration area. Bradford reagent, 100 µl of 0.25 g/l; BSA, 100 µl (0.4 mg/ml); Carrier, 10 % (v/v) H_3PO_4/5 % (v/v) ethanol.

by applying the signal derivatization procedure, the analysis time under conditions for unwell-resolved peaks can be shortened compared with those for well-resolved peak profiles.

3.4. Unprecise peak area due to peak tailing

3.4.1. Anodic stripping voltammetric determination of Cu

The voltammogram obtained by analyzing Cu under a set of conditions is depicted in Fig. 7(a). One encounters difficulty in the selection of integration window for such a tailing peak. In this work, the voltammograms were computed to their first and second derivative signals (Fig. 7(b) and (c)). The integration window was simply defined by selecting the two outermost data points of the interested peak where the derivative signals was close to zero. An example of integration area is depicted in Fig. 7(d). The relative standard deviations of peak area and height for the first and second derivatives were comparable. However, the calibration graphs using the first derivative were better in linearity than those using the second derivative. The calibration equations from the first derivative were $Y=(8.49\times10^{-5})X+4.13\times10^{-6}$ ($r^2=0.9971$) for peak area and $Y=(1.21\times10^{-3})X+6.54\times10^{-5}$ ($r^2=0.9972$) for peak height. The relative standard deviations for sample analyses were 1.6% and 4.5% for peak area and peak height, respectively.

3.4.2. Sequential injection analysis of protein content by Bradford assay

A flow injection method using Bradford assay was proposed for the determination of protein in spiked human urine and rat liver microsome samples [29]. The recovery ranged from 89.3 to 91.9%. The results were not satisfactory for precise analysis, and Bradford assay has not been introduced to the SIA method so far. Fig. 8(a) shows the SIA-grams obtained by triplicate analysis of a standard BSA solution under a set of conditions. It can be seen that the three SIA-grams exhibit peak tailing which was caused by possibly refractive index effects. The relative standard deviation ($n=3$) in peak area was as high as *ca* 50%. Generally, this situation requires optimization of experimental conditions to reduce or eliminated the tailing of peaks. However, we adopted signal derivatization for solving such a problem. The first derivative SIA-grams are shown in Fig. 8(b). The peak area was integrated as in the shade area in Fig. 8(c). The relative standard deviation ($n=3$) of peak areas was greatly improved to as low as 3.4%. In addition, the application of derivatization to such SIA-grams was very useful because the optimization of experimental conditions for solving the problem was unnecessary, which led to fast method development similar to the discussion in Section 3.3.2.

4. Conclusion

The derivatization of signals using two commercial software packages, namely Excel and Origin, has been successfully applied to the solving of analytical problems in batch and flow-based methods such as titrimetry, spectrophotometry, chromatography, voltammetry and sequential injection spectrophotometry. Resulting derivative analytical signals by using such simple and flexible packages resulted in easy and accurate end point detection for noisy sigmoid titration curves, well-resolved absorbance spectra and chromatograms and no tailing effect in voltammograms and SIA-grams. Such signal derivatization should be attractive for not only enhancement of many chemical analyses, but also fast method development since the optimization of experimental conditions could be minimized.

Acknowledgements

The authors would like to thank The Thailand Research Fund (TRF) and The Postgraduate Education and Research Program in Chemistry (PERCH) for financial support. Also, the present work was partly supported by a grant of the Frontier Research Project "Materials for the 21st Century — Materials Development for Environment, Energy, and Information" (for 2002–2006 fiscal years) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Field-amplified sample injection and in-capillary derivatization for capillary electrophoretic analysis of metal ions in local wines

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Received 21 March 2007; received in revised form 27 March 2007; accepted 27 March 2007
Available online 1 April 2007

Abstract

In-capillary derivatization and field-amplified sample injection (FASI) coupled to capillary zone electrophoresis (CZE) was evaluated for the analysis of metals (Co(II), Cu(II), Ni(II), and Fe(II)) using 2-(5-Nitro-2-Pyridylazo)-5-(*N*-Propyl-*N*-Sulfopropylamino)Phenol (Nitro-PAPS) as the derivatizing agent. For FASI, the optimum conditions were water as sample solvent, 1 s hydrodynamic injection (0.1 psi) of a water plug, 5 s of electrokinetic introduction (10 kV) of the sample. The in-capillary derivatization was successfully achieved with zone-passing strategy in order tandem injection of Nitro-PAPS reagent (0.5 psi, 7 s), a small water plug (0.1 psi, 1 s), and metal ion introduction (10 kV, 5 s). The solution of 45 mmol L^{-1} borate pH 9.7 and 1.0×10^{-5} mol L^{-1} Nitro-PAPS containing 20% acetonitrile was used as the running buffer. The limit of detection obtained by the proposed method was lower than those from pre-capillary derivatization about 3–28 times. The recovery of the method was comparable to pre-capillary derivatization method. In-capillary derivatization-FASI-CZE was applied to analysis of metals in wine samples. The results were compared with those obtained by CZE with pre-capillary derivatization method and atomic absorption spectrometry (AAS).
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Keywords: Capillary zone electrophoresis; Field-amplified sample injection; In-capillary derivatization; Metal ions; Nitro-PAPS; Wines

1. Introduction

The analysis of heavy metals in a range of substances is of considerable interest due to potential human toxicities. There is increasing demand for simple and reliable methods for the simultaneous detection of several metal ions with low detection limits and low sample/reagent consumption. Atomic absorption spectrometry (AAS) is the most widely used method for metal analysis but simultaneous multi-element analysis is not possible. Inductively coupled plasma atomic emission spectrometry (ICP-AES) is capable of multi-element analysis, but the instrumentation is relatively expensive. The simultaneous determination of metal ions with conventional high performance liquid chromatography (HPLC) and ion chromatography (IC) have several disadvantages, such as long analysis times and consumption of large volumes of reagents. Recently, capillary electrophoresis (CE) has been shown to be a powerful separation technique for the analysis of metals in a wide variety of sample matrices [1–8] because of the significant advantages of resolution, speed, simplicity and lower sample/reagent consumption. The electrophoretic determination of metals can be easily achieved by indirect detection through the addition of appropriate species to the background electrolyte to produce UV-absorbing species [9,10], but the number of appropriate compounds for this environment is limited. The pyridylazo reagent, 2-(5-Nitro-2-Pyridylazo)-5-(*N*-Propyl-*N*-Sulfopropylamino) Phenol (Nitro-PAPS) is a synthesized chromogenic reagent which forms water-soluble chelates with giving high molar absorptivities of ca. 10^4–10^5 L mol^{-1} cm^{-1} [11–13]. Nitro-PAPS has been used as a chromogenic reagent in various analytical techniques for the determination of various metal ions. Flow-injection spectrophotometry for the determination of Fe(II) in purified salts [13] and the trace analysis of V(V) in river water [14] using Nitro-PAPS have been demonstrated. The HPLC-based determination of Cu(II), Co(II), Ni (II) and Fe(II) in rain and river waters using Nitro-PAPS has

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doi:10.1016/j.microc.2007.03.006

been reported [11]. The use of Nitro-PAPS chelates incorporating pre-capillary derivatization in CE for the determination of trace metal impurities in nickel and iron salts has been described [15].

In CE, derivatization has been accomplished by either pre-capillary [16,17], post-capillary [18] and in-capillary [1,2,19–21] methods. Pre-capillary derivatization requires large amounts of derivatization ligand, whereas post-capillary derivatization requires significant post-capillary hardware modification, and loss of efficiency during separation and peak broadening has been noted [18,22,23]. The increased popularity of in-capillary derivatization takes advantage of the fact that there is minimal sample dilution compared to pre-capillary and post-capillary derivatization. In-capillary derivatization can be performed by three modes: zone-passing, at-inlet and throughout-capillary [24–26]. Among these strategies, zone-passing is appropriate for the Nitro-PAPS derivatization since the formation of the metal complex occurs within seconds.

In order to increase the sensitivity of detection in CE, the on-line preconcentration methods have been developed with different strategies, such as large volume sample stacking [27], sweeping [28], dynamic pH junction-sweeping [29], and field-amplified sample injection (FASI) [8,30–35]. FASI is very popular since it is quite simple, requiring only the electrokinetic injection of the sample after the introduction of a small plug of high-resistivity solvent (mainly water). In FASI, samples are normally prepared in a low conductivity buffer and injected electrokinetically into a capillary containing a high-conductivity running buffer.

In this work, the feasibility of combining FASI with in-capillary derivatization of metal ions (Co(II), Cu(II), Ni(II), and Fe(II)) with Nitro-PAPS before their analysis by capillary zone electrophoresis (CZE) is demonstrated. The combination of electrokinetic injection and water or organic solvent field-amplified sample stacking is investigated. Studies of effects of diluents (aqueous versus organic) on the stacking by the electroinjection under FASI are performed. Finally, in-capillary derivatization-FASI-CZE is applied to the analysis of these metal ions in local wine samples.

2. Materials and methods

2.1. Chemicals and reagents

All the reagents used in this work were of analytical reagent grade. Nitro-PAPS was obtained from Dojindo (Japan). A 1×10^{-3} mol L^{-1} stock solution of Nitro-PAPS was prepared in deionized water. The 1000 mg L^{-1} stock atomic absorption standard solutions of metal ions including Co(II), Cu(II), Ni(II) and Fe(II), was purchased from Ajax Finechem (Australia). The working solutions of each metal were daily prepared in deionized water except for Fe(II) was prepared in acidic medium of HCl (1% v/v). Sodium tetraborate was purchased from Univar-Ajak (Australia). Water for preparation of samples and buffer solution was the deionized water obtained from a RiO_s™ Type I Simplicity 185 (Millipore-Waters, U.S.A.) with the resistivity of 18.2 MΩ cm.

2.2. Instrumentation

Experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis instrument (Fullerton, CA, U.S.A.) equipped with a photodiode array detection system. The 32 Karat software version 5.0 (Beckman) was used for instrument control, data acquisition and data analyses. Electrophoretic separations were carried out using uncoated fused-silica capillaries (Beckman) with a total length of 60.2 cm (50.0 cm effective length) and 75 μm I.D. On-capillary detections were performed at either selected wavelengths (200, 214, 250 and 570 nm) or in the scanning mode (190–600 nm). All CZE experiments were thermostated at 25 °C.

A Perkin-Elmer Instruments AAnalyst 100 atomic absorption spectrometer (U.S.A.) equipped with Perkin-Elmer single element hollow cathode lamps and an air–acetylene flame with air and acetylene flow rates of 10 and 3 mL min^{-1} was used for the determination of metals. The wavelengths (nm) selected for the determination of the analytes were as follows: Co 240.7, Cu 324.8, Ni 232.0, and Fe 248.3.

2.3. Pre-capillary derivatization (as a reference method)

The working solutions of metal ions were mixed with Nitro-PAPS solution (1×10^{-3} mol L^{-1}) in the mole ratio of ligand: metal of 2:1 at pH 6.0 at room temperature. The sample solution was introduced into the electrophoretic system by hydrodynamic injection at 0.5 psi (1 psi = 6895 Pa) for 5 s before separation. The absorbance was detected at 250 nm.

2.4. In-capillary derivatization and FASI

In-capillary derivatization of metals and Nitro-PAPS was performed by zone-passing option in the tandem mode. Briefly, the capillary was first filled with the carrier electrolyte and then a plug of Nitro-PAPS solution was introduced at 0.5 psi for 7 s. After that, FASI was performed by an introduction of a small water plug (0.5 psi, 1 s) and then the sample was introduced into the capillary with electrokinetic injection at 10 kV for 5 s. Subsequently, the separation voltage of 20 kV was applied through the capillary and the on-line reaction occurred inside the capillary when the metals mixed with the Nitro-PAPS. The complexing reaction occurs during the separation to obtain the corresponding metal-Nitro-PAPS chelates and subjected to spectrometric detection.

2.5. Electrophoretic separation

Prior to use, all new capillaries were initially pretreated with the following cycles: methanol, 0.1 mol L^{-1} HCl and 0.1 mol L^{-1} NaOH for 10 min each and deionized water for 5 min between each rinse and finally equilibrated with the buffer solution for 10 min before applying voltage of 10 kV across the capillary for 5 min. To maintain reproducible migration times, the capillary was pre-conditioned with the running buffer for 3 min before each run. After completing the separation session, the capillary was flushed with 0.1 mol L^{-1}

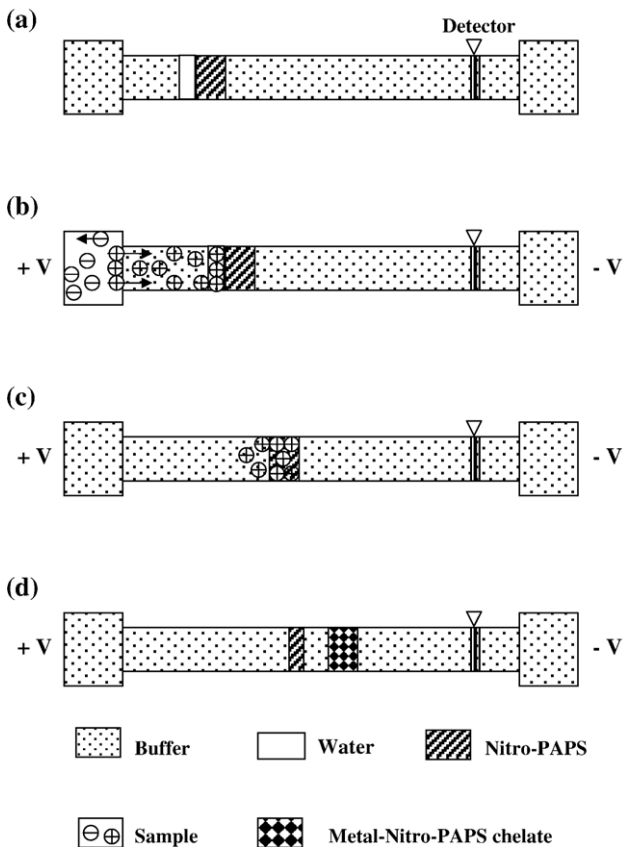

Fig. 1. The schematic reaction of metal ions and Nitro-PAPS based on in-capillary derivatization. (a) Hydrodynamic introduction of buffer, Nitro-PAPS and water plug, (b) electrokinetic injection of the sample, (c) mixing and reaction of metals with Nitro-PAPS, and (d) separation of the metal-Nitro-PAPS chelates.

NaOH for 5 min and then washed with deionized water for 3 min. The running buffer used throughout the separation was 45 mmol L^{-1} borate buffer pH 9.7 and 1.0×10^{-5} mol L^{-1} Nitro-PAPS containing 20% acetonitrile. All running buffers were filtered through a 0.45 μm membrane filter (Nylon, Chrom Tech) and degassed by sonication before use.

2.6. Wine samples

The wine samples studied included a variety of homemade red and white wines from a variety different fruits. The samples were purchased from the local supermarkets in Khon Kaen Province in Northeastern Thailand. The wines were filtered through a 0.45 μm membrane filter and were appropriately diluted about 10 times with water before derivatization and analysis. All analyses were performed in triplicate.

The AAS determinations of metals in wines were performed on standard calibration curves. The calibration curves were constructed in range 0–5 mg L^{-1} with obtained correlation coefficients higher than 0.999 for all studied metals. To obtain the results in line with the calibration curves, wine samples were appropriately diluted with water before directly analyses.

3. Results and discussion

3.1. Spectrometric properties

Although the metal ion-Nitro-PAPS complexes are normally detected at visible wavelengths, higher sensitivity and good signal-to-noise ratios were achieved with detection at 250 nm. Using Job's method, the ligand-to-metal ratio of 2:1 was found for the Co(II), Ni(II) and Fe(II) complexes, and 1:1 for the Cu (II) complex.

3.2. In-capillary derivatization and FASI

In-capillary derivatization can be achieved by successive introductions of a sample solution and the reagent solution followed by application of the specified voltages. Reaction occurs while both zones are overlapping each other in the capillary. In the present study, in-capillary derivatization of metal ions and Nitro-PAPS reagent was performed based on the zone-passing strategy. The Nitro-PAPS reagent was first introduced into the capillary by the hydrodynamic injection mode, followed by hydrodynamic injection of a small water plug and then the metal ions were injected using the electrokinetic mode. All solutions were successively introduced at the anodic end of the capillary. After the specified voltage was applied, the metal ions as positively charged species zones migrated faster than the Nitro-PAPS reagent zone toward the cathode, whereas the negatively charged Nitro-PAPS species migrated to the anode. Thus, the metal ions moved into the reagent zone and reacted to form the metal-Nitro-PAPS chelates. The mixing scheme is illustrated in Fig. 1. The parameters affecting FASI such as injection voltage and sample matrix were investigated and optimized. Preliminary studies involving metal

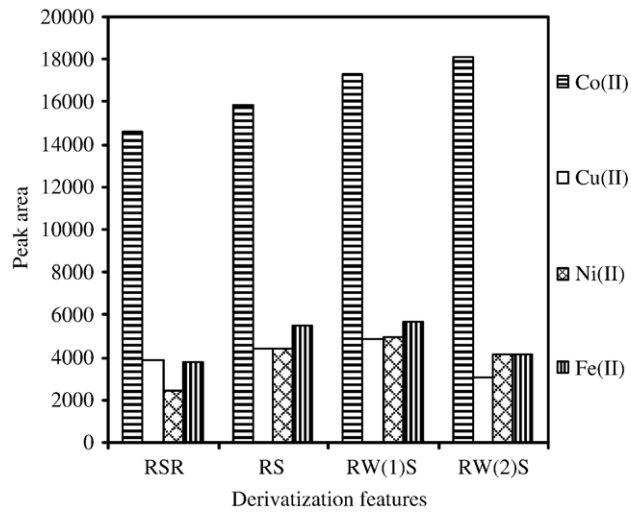

Fig. 2. Comparison of introduction order for in-capillary derivatization based on zone-passing. Conditions: metal ion standards = 3.0 mg L^{-1} each, reagent solution = 1.0×10^{-3} mol L^{-1}, reagent injection time = 7 s at 0.5 psi; sample introduction = 10 kV for 5 s; water injection time = 1 s at 0.1 psi; running buffer = 45 mmol L^{-1} borate pH 9.7 and 1.0×10^{-5} mol L^{-1} Nitro-PAPS containing 20% acetonitrile, potential = 20 kV. Segment assignment: S, Sample; R, reagent; W, water.

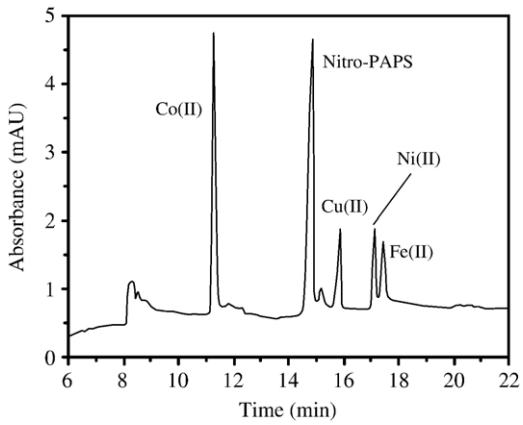

Fig. 3. Electropherogram of metal ion standards (3.0 mg L^{-1} each) obtained from in-capillary derivatization and FASI under the optimum condition as followed: 45 mmol L^{-1} borate pH 9.7 and 1.0×10^{-5} mol L^{-1} Nitro-PAPS containing 20% acetonitrile, applied voltage of 20 kV, temperature of 25 °C and on-capillary detection at 250 nm.

ions dissolved in different solvents such as 10% acetonitrile, water, and 0.4 mol L^{-1} sodium chloride showed that metal ions prepared in water gave the highest peaks for all the chelates. Although metal ions in acetonitrile provided good peak shape, the migration times of the analytes were shifted to longer times. Thus, aqueous solutions of metal ions were used for further studies.

The effect of injection voltage was studied by introducing metal ions dissolved in water at different applied voltages (7–15 kV) with a constant time interval (5 s). The peak areas increased up to 10 kV and remained nearly constant thereafter, possibly due to the competing migration directions and rates that affect local concentrations. Thus, an injection voltage of 10 kV for 5 s was chosen. Fig. 2 summarizes the results corresponding to each derivatization strategy; R, S, and W refer to the reagent, sample, and water, respectively. Among the various zone-passing introduction modes, the R-S-R configuration gave the lowest responses, except for Co(II). The R-S tandem mode gave higher sensitivities for all metal ions, compared to RSR. If the R-S tandem mode was carried out by introduction of a small water plug (at 0.1 psi for 1 s) before electrokinetic metal introduction, RW(1)S, increasing responses were observed. While a time of 2 s, designated RW(2)S, produced an increase for Co(II), the responses for other metals decreased significantly, possibly due to dilution in the reaction zone. The optimum condition is, therefore, represented by RW(1)S.

3.3. The optimization of CZE conditions

In order to achieve complete separation of metal-Nitro-PAPS chelates, the crucial parameters affecting the separation were: buffer pH, concentration of buffers, organic modifier, and separation voltage. Preliminary experiments with three common buffers, acetate (near pH 5.0), phosphate (near pH 7.0), and borate (near pH 9.0) buffers showed that acetate and phosphate did not lead to complete separation due to overlap of peaks from some metals and Nitro-PAPS, whereas borate buffer provided successful separation. Borate buffer was then investigated at the pH ranges of 9.0–10.5. Co(II), Cu(II) and excess Nitro-PAPS were well separated, but by increasing of pH, increased the resolution of Ni(II)- and Fe(II)-Nitro-PAPS could be achieved. At pH values higher than pH 10.5, excess Nitro-PAPS and Cu (II)-Nitro-PAPS peaks co-eluted. The best separation was obtained at pH 9.7.

The effect of borate concentration was then evaluated ranging from 35 to 55 mmol L^{-1}. Increasing the borate concentration resulted in decreased mobility and better resolution. However, high current in the capillary system was observed at high concentrations, so 45 mmol L^{-1} was chosen as the optimum value.

The type and concentration of organic modifier are other parameters that can improve resolution and selectivity in separations [17,20]. For the present study, acetonitrile was selected and varied over the range of 10–25% (v/v). The chelates of metals were completely separated with the running buffer containing 20% acetonitrile, so this was selected for subsequent runs.

In this study, voltages over the range of 15–22 kV showed little effect on separation. However, a poor baseline was observed when the separation was performed at the highest applied voltages, so the optimum of 20 kV was used throughout.

In all the studies, Nitro-PAPS (1.0×10^{-5} mol L^{-1}) was added into the running buffer in order to prevent the dissociation of metal-Nitro-PAPS chelates during the separation and provided good baselines [36]. No effects of Nitro-PAPS concentration changes were observed other than some baseline drift at higher values.

In summary, the optimum running buffer was 45 mmol L^{-1} borate pH 9.7 and 20% acetonitrile containing 1.0×10^{-5} mol L^{-1} Nitro-PAPS. The separation voltage was 20 kV and on-capillary detection was carried out at 250 nm. Under the optimum CZE conditions, the four metal ions was completely separated within 20 min (Fig. 3) with the migration order of Co(II)-, unreacted Nitro-PAPS, Cu(II)-, Ni(II)- and Fe(II)-Nitro-PAPS, respectively.

Table 1
The analytical characteristics of the proposed method

Complex	Linear equation, $y=ax+b$		Linearity (mg L^{-1})	R^2	Intra-day precision, RSD(%), $n=6$		Inter-day precision, RSD(%), $n=4\times3$	
	a	b			t_M	Peak area	t_M	Peak area
Co(II)-Nitro-PAPS	27032.0	−87.0	0.05–3.00	0.9982	0.28	2.00	0.35	4.00
Cu(II)-Nitro-PAPS	1476.7	472.5	0.10–3.00	0.9989	0.38	2.43	0.51	4.20
Ni(II)-Nitro-PAPS	1769.1	537.4	0.05–5.00	0.9953	0.42	2.75	0.67	3.85
Fe(II)-Nitro-PAPS	4384.0	−524.6	0.20–5.00	0.9996	0.25	2.55	0.32	4.36

Table 2
The limit of detection (LOD) and recovery for in-capillary and pre-capillary methods

Complex	LOD (mg L^{-1})			LOD factor		Recovery[b] (%), n=3	
	Pre-capillary[a]	In-capillary					
		Electrokinetic injection[c]	Pressure injection[d]	Pre-capillary/electrokinetic	Pressure/electrokinetic	Pre-capillary	In-capillary
Co(II)-Nitro-PAPS	0.20	0.025	0.30	8.0	12.0	80–102	80–95
Cu(II)-Nitro-PAPS	0.20	0.080	0.50	2.5	6.2	83–104	85–102
Ni(II)-Nitro-PAPS	0.70	0.025	1.00	28.0	40.0	80–103	82–97
Fe(II)-Nitro-PAPS	0.70	0.100	0.50	7.0	5.0	82–94	87–103

[a] Injection of complexes at 0.5 psi for 5 s.
[b] Reported as mean values for the twelve studied wine samples.
[c] Metal ion injection at 10 kV for 5 s.
[d] Metal ion injection at 0.5 psi for 5 s.

The peak of excess Nitro-PAPS (migration time 15 min) was close to the Cu(II) peak (16.0 min).

3.4. Analytical validations

To validate the quantitative aspects of the proposed method using FASI combined with in-capillary derivatization method, quality and quantity parameters using the optimized CZE method were evaluated and the results are shown in Table 1. Calibration curves based on the peak area at concentrations between 0.05 mg L^{-1} and 5.00 mg L^{-1} exhibited good linearity was obtained with R^2 values higher than 0.995. The reproducibility of the method was reported in terms of relative standard deviation (RSD) using six repeat determinations for intra-day precision and four replicate injections for inter-day comparisons. The RSD obtained from both of intra-day and inter-day precisions were lower than 0.5% and 0.7%, respectively, for migration time (t_M) whereas 3.0% and 4.5% for peak area. Limit of detection (LOD) was estimated based on signal-to-noise ratio (S/N) of 3:1 for both pre-capillary and in-capillary methods. The results are shown in Table 2. Co(II)-Nitro-PAPS has larger molar absorptivity (ε) than the other studied metal ions about 1 order of magnitude (i.e. 10^5 L mol^{-1} cm^{-1} for Co(II)-Nitro-PAPS whereas the other metal ions have ε about 10^4 L mol^{-1} cm^{-1}). Thus, a larger signal of Co(II)-Nitro-PAPS as well as its low LOD is obtained. Similar result was observed [11]. The in-capillary method with the electrokinetic injection (10 kV, 5 s) gives lower concentration detection than pre-capillary approach by a factor of 3–28. Whereas the in-capillary method with the electrokinetic injection produced lower LODs in the range of 5–40 times when compared to pressure injection (0.5 psi, 5 s). The results obtained by the proposed method can be used for the analysis of metals in sub-ppm level as lower or similar to those obtained by others [9,10,19]. To ensure that the present method can be used for the analysis of metals in real samples, the recovery was studied by spiking the metal ion standard solutions at 2.0 mg L^{-1} each into all samples and analyzed in triplicate. As shown in Table 2, the recovery obtained from in-capillary method was comparable to the pre-capillary method. The recovery ranges showed dependence on the origin of the sample and the sample matrix.

3.5. Application

The analysis of 12 wine samples was performed following the procedure described in Section 2.6. Fig. 4 shows the typical electropherograms obtained by the proposed method of Emblic fruit wine and white grape wine samples, respectively. The identification of peaks was carried out by comparing the migration time of known metal ions in a standard containing only a single metal ion with those in a metal mixture and by spiking with known metals and looking for increases in peak height. The contents of metals present in all studied wines are

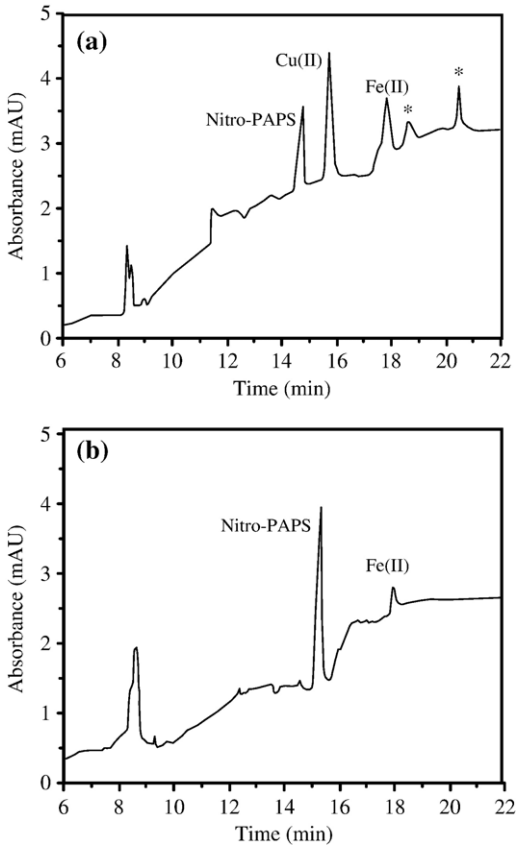

Fig. 4. Electropherograms of wine samples under the optimum conditions: (a) Emblic fruit wine and (b) white grape wine. Remarkable with asterisks (*) are unknown peaks.

Table 3
The content of metals in wine samples (mean, $n=3$)

Wine	Copper(II), mg L^{-1}			Iron(II), mg L^{-1}		
	In-capillary	Pre-capillary	AAS	In-capillary	Pre-capillary	AAS, total iron
Emblic fruit	19.53	19.96	20.36	50.89	51.37	51.80
Mangosteen	n.d.	n.d.	n.d.	7.78	7.36	7.54
Jambolan	n.d.	n.d.	n.d.	11.17	11.95	13.84
Star gooseberry	n.d.	n.d.	n.d.	12.29	12.59	11.26
Organic fruit	n.d.	n.d.	n.d.	10.03	10.28	11.30
Black galingale	5.37	5.52	5.46	15.25	15.73	15.78
Roselle	n.d.	n.d.	n.d.	19.09	20.69	19.60
Longan	n.d.	n.d.	n.d.	34.49	35.38	39.40
Bel fruit	n.d.	n.d.	n.d.	14.18	15.67	13.30
Strawberry	n.d.	n.d.	n.d.	15.86	16.71	14.52
White grape	n.d.	n.d.	n.d.	15.26	15.78	14.34
Red grape	n.d.	n.d.	n.d.	13.03	13.89	12.90

n.d.: not detected.

listed in Table 3. Only Cu(II) and Fe(II) were found in the studied wine samples whereas Co(II) and Ni(II) were not detected. Cu(II) was found in Emblic fruit and Black Galingale wines with a high level about 20 mg L^{-1} for the first sample. The levels of copper in various beverages (e.g. wines, tea, fruit juices, etc.) from some other countries have been reviewed [37]. In wines, the copper concentration was found to be less than 2 mg L^{-1}. Although copper was classified as an essential metal, excessive levels in wines are potentially toxic. The maximum acceptable level of copper in wine as established by the Office International de la Vigne et du Vin (OIV) is 1 mg L^{-1} [38]. Iron is usually present in musts and wines at a concentration range varying from 0.5 up to 20 mg L^{-1} [39]. The highest permissible concentration of iron in table wines set by the official national legislation has been determined to be 10 mg L^{-1}. Iron contents higher than 8–10 mg L^{-1} may result in turbidity and color changes [40]. In the present study, Fe(II) was found at different amounts in all wine samples. The highest value (~51 mg L^{-1}) of Fe(II) was found in Emblic fruit wine which also has the highest content of Cu(II). The Longan wine contained Fe(II) at a level of 34 mg L^{-1} and Fe(II) in Roselle wine at 19 mg L^{-1}. Fe(II) was also detected in other wine samples at levels of 16 mg L^{-1} or lower. The presence of copper and iron at these levels suggest that all wines may be at risk of cloudiness and lack of stability. To establish the reliability of the proposed method for analysis of metals in wine samples, the results obtained using the CZE method with in-capillary derivatization and FASI method have been compared to those using pre-capillary derivatization method and AAS (Table 3). The statistical significant as determined by the *t*-test at 95% confidence level showed no significant difference in the results obtained by the three methods. Comparisons presented here suggest that rapid and reliable results can be obtained in a highly automated process to establish the quality of wines. Iron contamination in homemade preparations is of particular interest.

4. Conclusion

The method and results described here demonstrate that FASI in combination with in-capillary derivatization is a useful technique for CZE determination of cobalt(II), copper(II), nickel(II), and iron(II) using Nitro-PAPS as the complexing agent. FASI of metal ions and in-capillary mixing with Nitro-PAPS were successfully obtained with zone-passing strategies using tandem injection order of Nitro-PAPS, a small water plug, and metal ions. Under the optimum condition, the separation of four metals can be achieved within 20 min. Limit of detections obtained from in-capillary derivatization with electrokinetic injection were 3–28 folds higher than those obtained from the pre-capillary method. The successful application of this method for the determination of metals in wines has been demonstrated. Only copper and iron were found at high levels in wine samples and these may influence on color and stability of the homemade wines. In-capillary derivatization and FASI were superior in terms of simplicity and ease of automation when compared to pre-capillary derivatization. Moreover, small quantities (~nL level) of reagent and sample were required. Thus, the method can be considered to be an alternative to other more complicated methods for analysis of metals in wines.

Acknowledgements

We are grateful to the Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC) and the Thailand Research Fund (TRF) for the financial support. We wish to thank Prof. Richard L. Deming (Department of Chemistry and Biochemistry, California State University Fullerton, Fullerton, U.S.A.) for his kind comments.

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ANALYTICA CHIMICA ACTA 600 (2007) 188–193

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Online assay of bone specific alkaline phosphatase with a flow injection-bead injection system☆

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ARTICLE INFO

Article history:
Received 4 October 2006
Received in revised form
20 December 2006
Accepted 31 December 2006
Published on line 13 January 2007

Keywords:
Bone alkaline phosphatase
Wheat germ
Flow injection
Bead injection

ABSTRACT

Alkaline phosphatase (ALP) has been used as one of the biomarkers for bone resorption and liver diseases. Normally, total alkaline phosphatase is quantified along with other symptoms to determine the releasing source of the alkaline phosphatase. A semi-automated flow injection-bead injection system was proposed to conveniently and selectively assay bone alkaline phosphatase (BALP) based on its specific binding to wheat germ coated beads. Amount of BALP in serum was determined from the intensity of the yellow product produced from bound BALP on the retained beads and its substrate pNPP. The used beads were discarded and the fresh ones were introduced for the next analysis. The reaction cell was designed to be opened and closed using a computer controlled solenoid valve for a precise incubation time. The performance of the proposed system was evaluated by using it to assay BALP in human serum. The results were compared to those obtained by using a commercial ELISA kit. The system is proposed to be an easy and cost effective system for quantification of BALP as an alternative to batch wise wheat germ specific binding technique.

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1. Introduction

Alkaline phosphatase (ALP) is an enzyme primarily produced in liver and bone. It is also produced in lesser amount by the placenta, intestines and kidneys. Some conditions such as bone disease, liver damage and bile duct blockage can cause the release of high amount of ALP into the bloodstream [1–3]. The chemical structures of ALP from different sources are slightly different. Therefore, the isoenzyme test of ALP can reveal its origin which relates to the malfunctioning organ. The assay of ALP in serum is normally part of a routine blood test used mainly for the diagnosis of liver and bone disorders [4]. However, the primary screening value of blood ALP is the total ALP which cannot differentiate between each isoenzyme. To make a full use of any ALP isoenzyme as a marker of a particular disease, the assay technique has to be specific for that isoenzyme. Bone alkaline phosphatase (BALP) would be a very useful biomarker because the diagnosis of most bone diseases needs a long term monitoring of bone mass which may be too late for the effective treatment.

There are a few techniques that have been used to differentiate the two main isoenzymes, bone and liver ALPs,

☆ Presented in the Flow Analysis X.

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doi:10.1016/j.aca.2006.12.056

from each other and from other isoenzymes. Some conventional techniques such as heat activation [5–7] and chemical inhibition [5,6] are simple but the assay of enzyme activity after heat and chemical exposure would not be practical for quantitative analysis for diagnosis of disease. Some other techniques require either high performance instrumentation (such as HPLC and electrophoresis) [8–11] or skilled personnel (such as immunoassay) [12,13], both of which are not always available in the regions where funding are limited and standard facilities are non-existant. Another interesting technique is called wheat germ lectin precipitation. The lectin isolated from *Triticum vulgaris* (wheat germ) is known to selectively bind to *N*-acetylglucosamine and *N*-acetylneuraminic acid residues of BALP [14–16]. This precipitation technique involves few chemicals and simple apparatus. However, BALP activity has to be measured from the precipitate which is not so convenient. Well trained personnel are still needed to perform the experiment and analyze the results. Incorporation of this specific interaction between wheat germ lectin and BALP into a more automated yet simple and cost effective system should be useful in providing health care to people living in areas located far from a modern hospital.

Flow injection-bead injection (FI-BI) is a combination of online chemical mixing and product detecting process with the use of reactive solid bead surfaces that can be introduced into and removed from the system with a flowing stream of solution. The injected beads are retained in the cell that can be designed and adapted as needed. FI-BI has been reported as an alternative to a more sophisticated sequential injection-bead injection (SI-BI). FI-BI offers some degrees of automation and improvement of precision as compared to batch wise process while minimizing the operation expense. Moreover, the closed system of FI helps to reduce the risk of direct contact between an operator and blood samples [17–19].

By employing the wheat germ coated beads with a FI-BI system, an online highly specific assay of BALP can be done easily. Reactive beads are injected into the system and trapped in place. Sample containing BALP is then passed through the bead retention cell. BALP that is captured by wheat germ on the beads can be detected with a suitable substrate. A computer controlled system for bead injection and incubation timing should help to improve the precision and reduce personal errors as compared to the batch wise wheat germ specific binding process.

As in the use of any biomarkers, an elevated BALP almost always requires other complementary testing for the decision of treatment. Therefore, the assay of BALP itself is just one of the aids in the diagnosis of bone disease or the elimination of other ALPs-related disorders. The first generation FI system is simple to set up and is cost effective. It is suitable for such tasks as the quantitative screening of BALP to aid in the prediction of bone disease or to monitor the progress of treatment. A simple spectrophotometer can be used as a detector to quantify the amount of enzyme–substrate product. The performance of the proposed system was evaluated by using it to assay BALP in human serum.

2. Experimental

2.1. Apparatus

The FI-BI system design is as shown in Fig. 1. It is composed of two peristaltic pumps (FIA Lab, USA); one for introduction of beads and carrier solution and another one for controlling the flow of buffer, sample and substrate solutions via 3-way switching valves. BALP standards or serum samples and substrate solution were injected into the system by means of

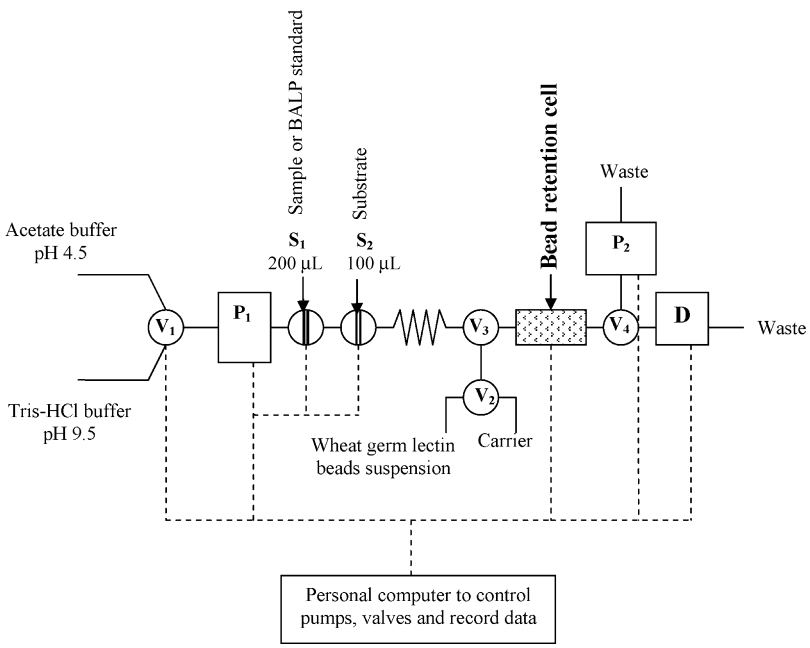

Fig. 1 – The FI-BI system for the assay of BALP. V_1, V_2, V_3 and V_4 are 3-way switching valves, S_1 and S_2 are 6-port injection valves, P_1 and P_2 are peristaltic pumps, D is a Spectronic 21, carrier is DI water and substrate is pNPP.

six-port injection valves (Pittman, USA and Metrohm, Germany). A Spectronic 21 (Spectronic Instrument, USA) with a flow through cell 1 cm, 80 μL (HELLMA, Germany) is for the detection. Two designs of bead retention cells, one based on a two-way pinch valve (Cole-Parmer, USA) and another of Perspex glass cell with solenoid valve (Guardian Electric, USA), were investigated. The pump, valves and the data were controlled and recorded via a computer with an in-house written software using LabVIEW® and Origin (Northampton, USA). The results obtained from the assay of serum samples using the FI-BI system were compared to those obtained by using the commercial 96 well BALP ELISA kit (Metra BAP EIA kit, Cat. No. 8012, Quidel, USA) [20].

2.2. *Reagents and samples*

Wheat germ lectin beads (size 200–300 μm diameter) were purchased from Sigma (Product No. L1394). Acetate buffer solution pH 4.5, 50 mM was used for bead conditioning. Tris–HCl buffer pH 9.5, 100 mM was used to create an alkaline working condition for enzyme–substrate reaction. As suggested by the manufacturer, triethanolamine pH 7.6, 0.1 M was used as a solvent for preparation of standard BALP solution from solid BALP. BALP stock standard solution was prepared at the concentration of 20,000 U L^{-1}. A liver ALP (LALP) stock standard solution of the same concentration was prepared and stored similarly to the BALP standard solution. *p*-nitrophenol phosphate (pNPP) working solution of 5 mM was prepared in Tris–HCl buffer solution. Bovine serum albumin (BSA, Sigma) was used to dilute the human serum samples that were obtained from the hospital to the required dilution factor. Fetal bovine serum (Lot. No. CN J0094, HyClone Laboratories, Inc.) was used for optimization study of the FI-BI system. Blood samples were collected at the Orthopedics Surgery Department, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University, Thailand. Details of preparation of each reagent, serum samples and dilution of serum are available in ESI.

3. Results and discussion

3.1. *Bead retention cell*

Two main purposes of the bead retention cells are the accommodation of the chemical reaction and retention of the analyte.

The first design based on a two-way pinch valve, see ESI, has limited use because of tubing deformation due to pinching pressure.

For the second bead retention cell design, the straight channel of 1/16 in. diameter was created in a Perspex glass piece. Another channel was created at 45° to the straight channel as shown in Fig. 2 to perfectly fit the solid rod (PEEK tubing) which is attached to the metal rod of a solenoid valve. When the valve was switched on, the solid rod was inserted into the flow path of beads in the straight channel, leaving the channel length of 3 cm for bead retention space. The positions of the cell and the valve were adjusted in the way that the tiny space between the cell surface and the solid rod was suitable to retain beads while the solution could flow through. By mounting the cell and the solenoid valve in place, the small space between the cell surface and solid rod can be kept unchanged. After analysis, the solid rod was pulled back and beads were pushed out with a flowing stream of solution. The movement of solid rod was controlled electronically using the LabVIEW® software for applying the voltage to the solenoid valve. There was no problem of back pressure using this cell and therefore, it was chosen for further experiments.

3.2. *The FI-BI operation*

Operation steps of the FI-BI system (Fig. 1) with the Perspex-solenoid valve bead retention cell were as follows. First, wheat germ lectin coated beads were loaded into the flow cell with a peristaltic pump P_2 through valves V_2, V_3 and V_4 out to waste. Then deionized (DI) water was pumped into the system to pack beads in the bead retention cell by switching valve V_2 to the carrier line. After that, the pump P_2 was stopped and by switching the valves V_3 and V_4, the acetate buffer pH 4.5 was allowed to flow into the system by the peristaltic pump P_1.

A BALP standard or serum sample was injected into the acetate buffer stream via the injection valve S_1 and passed into the bead retention cell. Then the pump P_1 was stopped to allow incubation of BALP with wheat germ lectin coated beads for a desired period of time. Specific lectin on the bead surfaces interacted and separated BALP isoform from human serum sample. After that, the Tris–HCl buffer pH 9.5 was pumped through the system via an operation of the valve V_1 to remove unbound species and to adjust the condition to be alkaline. pNPP was injected into the buffer stream by means of the injection valve S_2. It passed through the bead retention cell and out through the flow cell. The baseline signal of pNPP solution was recorded. Then the pump was stopped for incu-

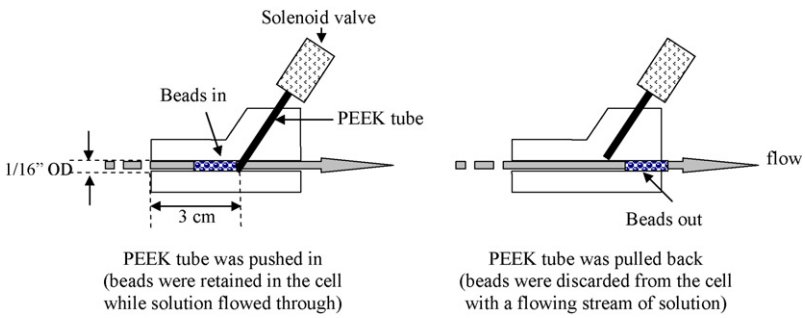

Fig. 2 – The bead retention cell based on a solenoid valve.

Table 1 – The operational conditions of the flow injection-bead injection system for the assay of BALP in human serum samples

Parameters	Selected conditions
Bead suspension	0.25 mL beads in 4 mL acetate buffer pH 4.5
Flow rate (P_2)	3.0 mL min^{-1}
Bead loading time	10 s (Approximately 30 μL of bead volume)
Bead packing	DI water 2 min
Flow rate (P_1)	1.8 mL min^{-1}
Bead conditioning	Acetate buffer 2 min
Sample volume	200 μL
Sample-bead incubation	5 min
Washing step	Tris–HCl buffer 3 min
Substrate volume	100 μL 5 mM pNPP
Enzyme-substrate incubation	5 min
Detection wavelength	405 nm

bation. The sample (200 μL) and a substrate (100 μL) would be stopped at the bead retention cell adequately, which could be controlled by the flow rate of carrier solution and the time when the pump stopped. Finally, the enzyme–substrate product, p-nitrophenol (pNP) was pushed into the flow through cell and was detected at 405 nm by pumping the buffer solution through detection unit. Used beads were discarded and replaced by fresh ones. This helps to reduce the risk of bead contamination, bead surface deterioration and to cut down the analysis time by eliminating the bead regeneration step.

3.3. *Operational conditions*

Various parameters such as sample volume, flow rate, incubation time and concentration of substrate were optimized to obtain the most suitable operational conditions. Since human serum samples were limited, all experiments in this section employed fetal bovine serum as a source of BALP to mimic matrices of real serum samples which will be used in the future.

The summarization of the selected operational conditions of the FI-BI system is shown in Table 1. It should be noted that after incubation of BALP with pNPP, enzyme–substrate product pNP was pushed into the detector. Two repeated enzyme–substrate incubation and detection steps were able to be performed for each sample on the same injected beads. Therefore, the total analytical time for the assay of BALP was approximately 30 min for 2 replicated enzyme–substrate product measurements.

A 5 min incubation time was chosen to shorten the assay process. Although it may not reach equilibrium, the advantage of the FI enables the reproduced timing for the measurement. Sample throughput may be improved by flowing the solution through the reactor slowly rather than stopping it during incubation steps. A more sensitive detector such as fiber optic may be needed to improve performance.

3.4. *Calibration curve*

The selected operational conditions of the FI-BI system were employed to obtain a calibration graph. The BALP activities

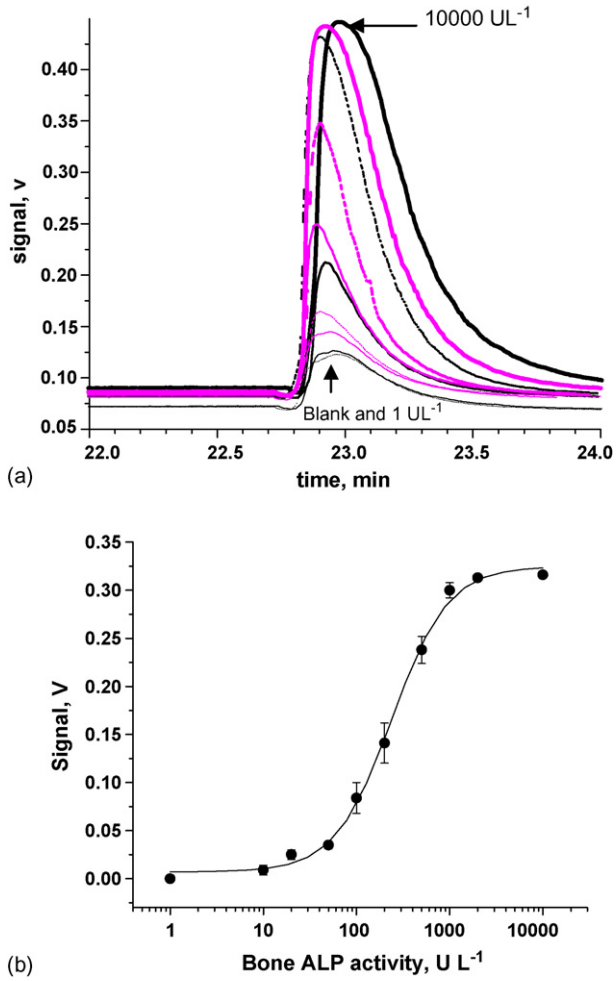

Fig. 3 – (a) The profile of the analytical signals of standard BALP at various concentrations and blank. Peak heights are increased in order with the increase of the BALP activities that are varied from 1 to 10, 20, 50, 100, 200, 500, 1000, 2000 and 10,000 U L^{-1}. (b) Calibration curve of the BALP activities.

were investigated in the range of 1–10,000 U L^{-1} to cover the amount of BALP usually found both in healthy people and bone disease patients. The profile of the analytical signals of BALP activities is shown in Fig. 3(a). The blank signal was found to be the same as and superimposed with the signal obtained from BALP of 1 U L^{-1}. The corresponding calibration curve, plotted between the differences of analytical signals from the background signal and concentrations of BALP, is presented in Fig. 3(b). The calibration curve is the sigmoidal (logistic) fit, see ESI. From this calibration curve, the working range was estimated to be 10–1000 U L^{-1}. This can cover the range of BALP levels reported to be found in healthy people (35–95 U L^{-1}) [16] and at various elevated levels in patients with bone diseases such as osteoporosis.

3.5. *Precision and accuracy*

Within-run precision or repeatability [21] was determined by repeated assaying BALP in a single fetal bovine serum 10 times within one day. The percentage of relative standard deviation

Table 2 – Percent differences of spiked BALP in normal serum samples

Sample	Found BALP ($U L^{-1}$)	Expected BALP ($U L^{-1}$)	%Difference[a]
Normal serum	16	–	–
Normal serum + 20 $U L^{-1}$ std. BALP	43 ± 5	36	19 ± 16
Normal serum + 100 $U L^{-1}$ std. BALP	94 ± 7	116	19 ± 6
Normal serum + 500 $U L^{-1}$ std. BALP	379 ± 16	516	27 ± 3
Normal Serum + 1000 $U L^{-1}$ std. BALP	687 ± 79	1016	32 ± 8

[a] Accuracy was expressed as the percentage of difference between the calculated activities of spiked BALP (expected value) and the measured amount of BALP found by using the FI-BI system: %Difference = [(found value − expected value)/(expected value)] × 100.

(%R.S.D.) was 6. Between-run precision or reproducibility [21] was determined by 10 assays of BALP of a single fetal bovine serum on different days (1 run per day) and the R.S.D. was found to be 5%.

Accuracy of the method and working range of the calibration curve were investigated by spiking various BALP activities (100, 500 and 1000 $U L^{-1}$) into a normal serum sample. The results in Table 2 indicate that accuracy of working range (10–1000 $U L^{-1}$) of the calibration curve is decreased when BALP activity is higher than 100 $U L^{-1}$ (more than 20% difference). This may be due to limited bead surfaces available for accommodation of high amount of BALP. The practical working range should actually be in the range of 10–100 $U L^{-1}$ (less than 20% difference). Therefore, dilution of serum sample is necessary for the serum sample with BALP activity near or higher than 100 $U L^{-1}$.

3.6. *Cross reaction*

Because both BALP and LALP exist in the tissue-nonspecific ALP gene locus, they have similar structures. Even though wheat germ lectin has been reported to be 90% specific toward BALP [22], the LALP which is an abundant isoenzyme of ALP in human serum may have cross-reactivity with the wheat germ lectin. From the experiment, see ESI, it was found that low amount of LALP (50–100 $U L^{-1}$) does not have detectable cross reactivity with the wheat germ lectin. LALP at 200 $U L^{-1}$ showed 3% cross reactivity while 500 and 1000 $U L^{-1}$ showed the same level of cross reactivity of 7%. These results are similar to those reported by the commercial BALP ELISA kit (5–8% cross reactivity with LALP). This low cross activity of LALP should not interfere with the assay of BALP.

3.7. *Estimation of the amount of BALP in human serum*

The FI-BI technique was used to estimate BALP in human serum samples. The results obtained from the proposed FI-BI system were compared to those obtained from a commercial ELISA kit based on monoclonal antibody against BALP. The two methods gave a regression equation of $y = 1.76x - 3.83$ with a correlation coefficient (r) of 0.74 at the significance level of 0.001 ($p = 0.001$), when plotting the results obtained by the FI-BI system as y-axis and that of the commercial ELISA kit as x-axis. By average, BALP activities obtained from the FI-BI assay are almost twice higher than those found when using a commercial ELISA kit. This could be due to higher area of solid surfaces of beads as compared to the surface of the wall of micro-well. The actual cause of unequal analytical signals of the two methods is still unknown and further studies will be useful. Nevertheless, higher sensitivity obtained from the FI-BI system should be a great benefit.

In this study, comparison of average BALP activities in the normal group and the osteoporosis group from the FI-BI assay indicates that the levels of BALP in these two sample groups are significantly different. The trends were with that by the commercial ELISA kit. The FI-BI system showed average BALP of 46 and 18 $U L^{-1}$ for osteoporosis and normal serum samples, respectively while the ELISA kit showed average BALP of 24 and 15 $U L^{-1}$ for the same osteoporosis and normal serum samples, respectively. The significance of the difference of the average BALP activities in normal and osteoporosis groups were calculated in terms of p-value. The p-value of 1.0×10^{-4} (significance level of 0.0001, 99.99% confidence level) was found with the FI-BI assay and p-value of 6.4×10^{-3} (significance level of 0.01, 99% confidence level) was found with ELISA kit. The difference in BALP levels of the two sample groups found when using the FI-BI system is higher than that found when using the ELISA kit which is the consequence of the higher sensitivity of the FI-BI system, as mentioned earlier. The FI-BI assay should provide better differentiation of normal from patient groups.

4. Conclusions

A cost effective FI-BI system for the assay of bone alkaline phosphatase (BALP) in human serum samples was developed. Wheat germ coated beads were employed to isolate BALP form human serum, followed by the enzyme–substrate reaction for detection. The simple FI-BI system can be used to perform online assay of BALP. As compared to the ELISA kit, the proposed system offers an assay run with good analytical characteristics, comparable precision and cross activity, simpler operation with less requirement for a highly trained personnel, less direct contact by the operator to blood sample, and lower cost. Referring to a sample throughput, it should also be noted that although the ELISA 96 well procedure can assay 96 samples in 4 h of one run but the proposed FI-BI system provides much shorter analysis time per run (30 min as compared to 4 h). Therefore, this FI-BI system is useful for a small to medium number of samples. Another advantage of the FI-BI system includes its tendency to better differentiate between groups of people with and without bone disease as compared to the ELISA kit. Therefore, this developed system would have a high potential of being used as an alternative

method to screen and monitor for bone disorders. Further investigation for a higher degree of automation and studies to determine a more precise cut-off value using more samples would be useful.

Acknowledgements

Financial support from The Thailand Research Fund (TRF), Commission on Higher Education (CHE), The Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC), Ministry of Education and The National Research Council of Thailand (NRCT, to PK) are gratefully acknowledged. We thank Dr. Jaroon Jakmunee for some useful contributions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2006.12.056.

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ISSN 0911-775X CODEN: JFIAEA

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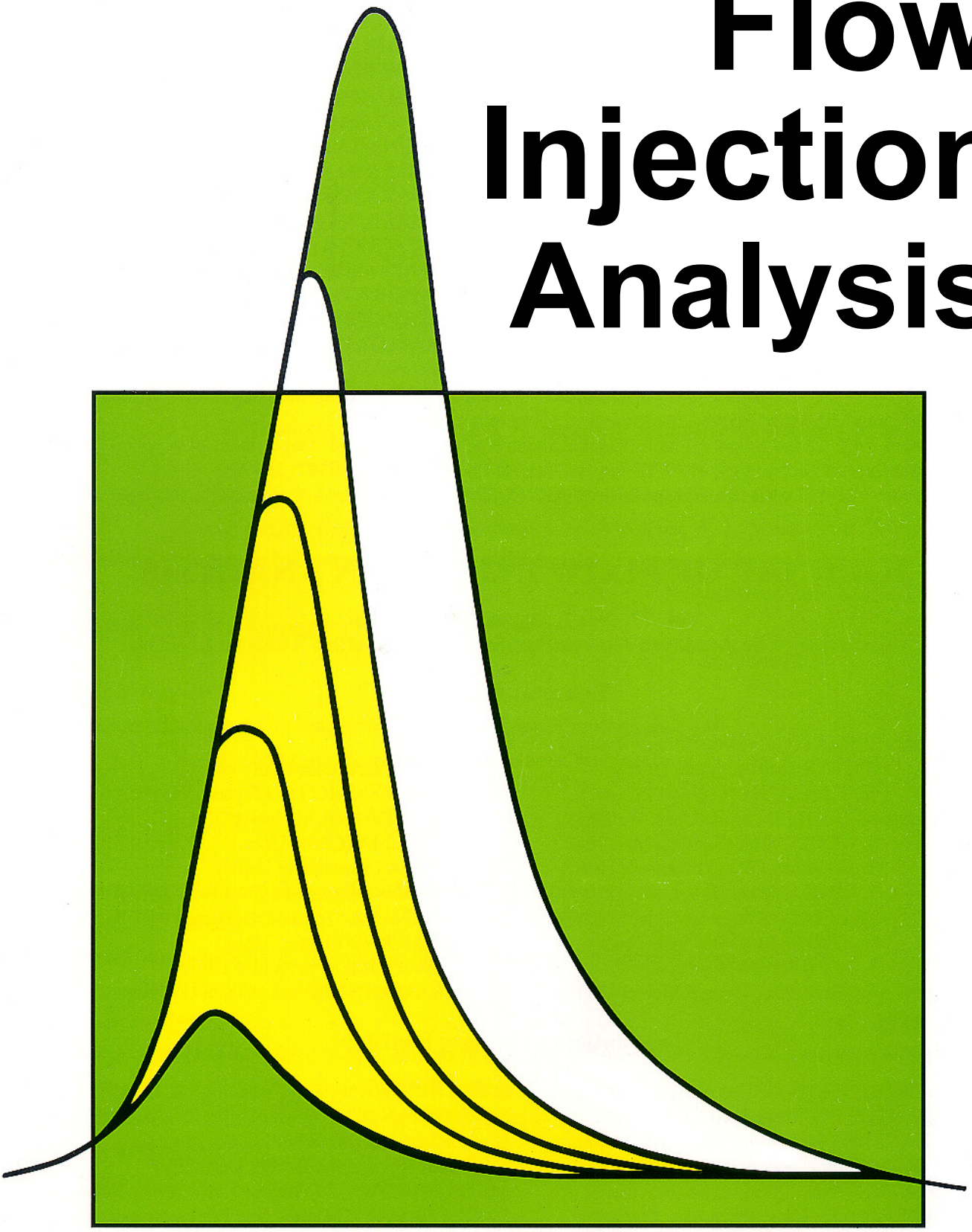

FIA 研究懇談会会誌

J. Flow Injection Anal., Vol. 24, No. 1 (2007) 23–26

Spectrophotometric Flow Injection Analysis of Protein in Urine Using Tetrabromophenolphthalein Ethyl Ester and Triton X-100

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Abstract

A sensitive, rapid and accurate determination of protein in patient urine was carried out by flow injection analysis (FIA) using tetrabromophenolphthalein ethyl ester (TBPE·H) and Triton X-100 at pH 3.0. The detection system was based on the ion association formation between human serum albumin (HSA) and TBPE·H in the micelle formed by Triton X-100. The calibration graph was linear in the range of 0.15 – 12 mg/dL HSA with R^2 = 0.998. The 3σ limit of detection of the proposed FIA method was 0.05 mg/dL at 610 nm. The relative standard deviation (n = 10) of 3.0 mg/dL HSA was 1.2% and the sample throughput was 30 h^{-1}.

Keywords Protein determination, Urine, Tetrabromophenolphthalein ethyl ester, Flow injection analysis; Ion association formation

1. Introduction

Of proteins, albumin is very important as an active substance in our body and is synthesized in the liver. In the liver disease, the amount of albumin in the blood decreases. And also, protein is excluded as a renal proteinuria in nephropathy and diabetic nephropathy. Especially, the measurement of protein amount excluded in the urine is an important indicator to the diabetes diagnosis. In the clinical laboratory, the test paper impregnating the dyestuff is commonly used for the rapid examination of renal disease. The visible detecting technique using the test paper is simple and easy to deal with, however, the detection of protein is semi-quantitative and the measurable concentrations are classified as follows; (–) < 15 mg/dL, (±) 15 – 30 mg/dL, (+) > 30 mg/dL, (++) > 100 mg/dL, (+++) > 300 mg/dL.

Accordingly, simple and accurate spectrophotometric determinations of urinary albumin and micro-albuminuria assay using bromophenol blue [1,2] have been reported. And, Fujita et al. have reported highly sensitive spectrophotometric methods for urinary protein with dye-metal complexes and micelle media [3–7]. Huang et al. have reported the determination of albumin and globulin with α-, β-, γ-, δ-tetrakis(4-sulfophenyl)porphine [8]. Bromocresol green and bromocresol purple were used for the serum albumin determination [9] and bromothymol blue has been proposed the determination of serum albumin [10]. And also, the chemical equilibrium of protein-dye binding for protein error was studied [11,12] and the interactions of dye and/or complexes with protein were reported [13,14]. On the other hand, Yoshimoto et al. have proposed the visual, simple and sensitive analytical method for the protein detection [15,16]. Recently, simultaneous determination of human albumin, globulin and glucose by near-infrared spectroscopy [17] and fluorescence quenching method [18] of bovine serum albumin by CdS nanoparticles have been reported. And, Hashimoto et al. reported the protein assay with tetrabromophenol blue [19]. However, these methods mentioned above have been performed by the batchwise technique and the procedure needs much time and large reagent consumption.

In the clinical laboratory, the routine and accurate quantification of albumin in urine is very important for a clinical evaluation of patients with renal disease. Flow injection analysis (FIA) developed by Ruzicka and Hansen is a versatile and practical technique [20]. The technology has been widely used in environmental, clinical and pharmaceutical analyses because the method permits automatic, rapid and sensitive analysis compared with the batchwise method. FIA with Coomassie brilliant blue G-250 was used to determine rapidly protein in urine [21] and also, high-sensitivity flow method with micro-flow plunger pumps [22] and capillary stream sensor [23] were demonstrated for the serum albumin determination. However, the sample throughput was only 15 h^{-1}. The albumin in biological fluids was analyzed using FIA-CL technology, however, the derivatization reaction was carried out off-line [24].

We found that an insoluble TPBE·H formed around at pH 3.8 was dissolved in the presence of Triton X-100. In this paper, a simple two channel FIA system using TBPE·H with Triton X-100 is proposed as a rapid and precious sensor of urinary protein.

2. Experimental

2.1. Reagents

All reagents used were of analytical-reagent grade, and deionized water purified by an Advantec GSH-210 apparatus was used throughout.

A TBPE stock solution (1.0×10^{-3} M) was prepared by dissolving 0.07 g of tetrabromophenolphthalein ethyl ester potassium salt (MW: 700, Tokyo Kasei) in 100 mL of ethanol.

A Triton X-100 solution (0.5%) was prepared by dissolving 0.5 g of *t*-octylphenoxypolyethoxyethanol (Sigma Chemical) in 100 mL of water.

A human serum albumin standard (HSA, 100 mg/dL) was prepared by 0.1 g of human serum albumin (MW: 66,000, Seikagaku Kogyo) in 100 mL of water.

Working solutions were prepared by suitable dilution of the stock solutions with water.

Buffer solution was prepared by mixing 0.1 M potassium hydrogen phthalate and 0.1 M hydrochloric acid.

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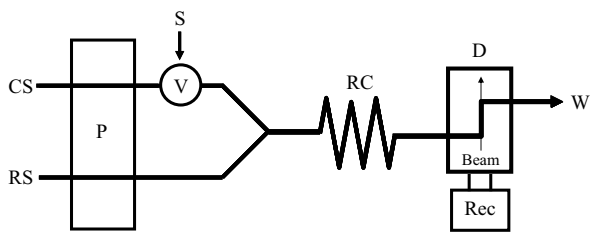

Fig. 1 Schematic diagram of the flow system. CS, water; RS, TBPE·H (1.2×10^{-5} M) + TritonX-100 (0.02%) + buffer (potassium hydrogen phthalate-hydrochloric acid, pH 3); P, pump (0.9 mL/min); V, valve; S, sample (200 μL); RC, reaction coil (ϕ 0.25 mm × 5 m); D, detector (610 nm); Rec, recorder; W, waste.

2.2. Apparatus

A JASCO, Model V-550, double beam spectrophotometer with 10 mm cell and a Horiba, Model F-22, pH meter were used for the batchwise method.

The manifold of the flow injection system is shown in Fig. 1. The dual micro-pump (F·I·A Instruments, Model PD-2000) was used to deliver carrier (CS) and reagent solution (RS). An LED visible spectrophotometer with an interfering filter (610 nm) (F·I·A Instruments, Model KCM-0306) equipped with a micro flow cell was used for the measurement of protein associate.

2.3. Procedures

2.3.1. Batchwise method

To the HSA standard solution, 5 mL of 1.0×10^{-4} M TBPE solution, 5 mL of 0.1% Triton X-100 and 2 mL of buffer solution (pH 3.1) were added in a 25 mL volumetric flask and the mixture was diluted to the mark with water. After mixing, absorbance was measured at 610 nm against water.

2.3.2. FIA method

The two channel flow system as shown in Fig. 1 is assembled. CS (water) and RS (a mixture of 1.2×10^{-5} M TBPE, 0.02% TritonX-100 and buffer (pH3.0)) were pumped at the rate of 0.9 mL/min. An aliquot (200 μl) sample solution is injected into the CS stream by a six-way injection valve. After mixing in the 5 m reaction tube (inner diameter 0.25 mm), absorbance was monitored at 610 nm.

3. Results and discussion

3.1. Batchwise study

3.1.1. Effects of TBPE and Triton X-100 concentrations

TBPE concentration was varied from 1.0×10^{-5} M to 8.0×10^{-5} M and Triton X-100 concentration was varied in the range of 0.005 – 0.1% in the absence of HSA. TBPE (blue) dissolved in ethanol was converted to yellow TBPE·H molecule in an aqueous media below pH 3.5 because the dissociation constant, $pK_{a,TBPE}$, was 4.2 [25]. A yellow TBPE·H was insoluble in water, however, TBPE·H was soluble in the presence of Triton X-100. Absorbance of the reagent blank increased with increase of TBPE concentration. This could be due to that TBPE·H in the micelle produced a dissociated TBPE ion slightly. In this study, 0.02% Triton X-100 and 2.0×10^{-5} M TBPE were chosen.

3.1.2. Effect of pH for ion association formation

The effect of pH on the formation of TBPE-protein associate was investigated. pH was varied from pH 2.9 to 4.2. The 2.0×10^{-5} M TBPE and 0.02% Triton X-100 solutions were used for 2.4 mg/dL HSA. With increasing pH, absorbance of the associate increased, however, absorbance of the reagent blank increased gradually. However, the calibration curve at pH 3.4 gave $y = 0.21x + 0.05$, $R^2 = 0.956$ (y = absorbance, x = HSA concentration in mg/dL, R^2 = correlation coefficient) and that at pH 3.1 gave $y = 0.17x + 0.03$, $R^2 = 0.980$ in the range of 0 – 3.0 mg/dL HSA. Consequently, pH 3.1 was chosen in this study.

Fig. 2 shows absorption spectra at pH 3.1 and 3.8 for various HSA concentrations. At pH 3.1, the reagent blank gave a low absorbance at 610 nm and absorbance of the associate increased in proportion to the HSA concentration. And also, the isosbestic point appeared at 490 nm. The larger absorbance was obtained at pH 3.8, however, the absorbance of the reagent blank became larger and the absorbance of the associate was not proportional to the HSA concentration, because TBPE·H in the micelle dissociated into TBPE anion at higher pH, and the ion associate formation was not stoichiometric.

3.1.3. Binding ratio of protein-TBPE associate

The binding ratio of the protein-TBPE associate was investigated by the continuous variation plots. The result is represented in Fig. 3. According to the continuous variation plots, it can be assumed that the binding number of TBPE to HSA is over 18. And also, it was simulated to obtain a presumed binding number as given below. When 3.6×10^{-7} M HSA was added, the absorbance was 0.417 at 610 nm. Hence, the molar absorptivity

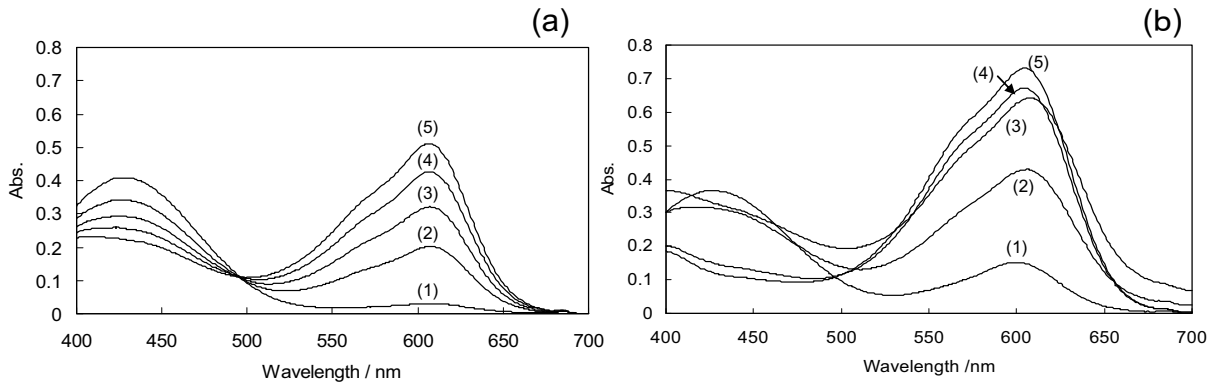

Fig. 2 Absorption spectra at different pH by batchwise method. (a), pH 3.1; (b), pH 3.8. HSA concentrations (mg/dL): (1), 0; (2), 0.8; (3), 1.6; (4), 2.4; (5), 3.2. TBPE·H, 2.0×10^{-5} M; Triton X-100, 0.02%.

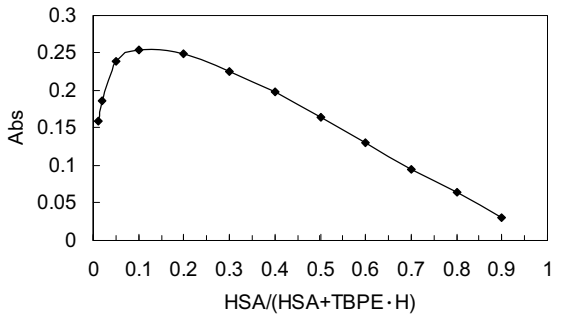

Fig. 3 Continuous variation plot of TBPE-HSA associate. $C_{HSA} + C_{TBPE} = 4\times10^{-5}$ M.

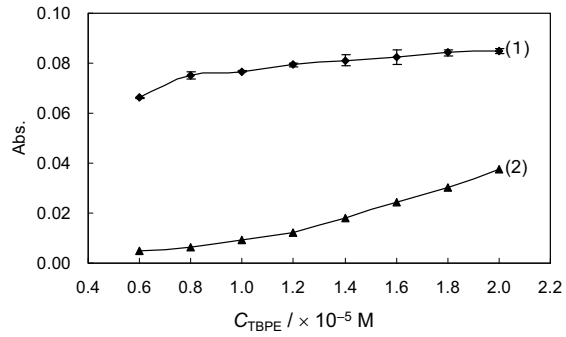

Fig. 4 Effects of TBPE concentration in FI method. (1) HSA (3 mg/dL) and (2) baseline absorbance against water. pH 3.0; Triton X-100, 0.02 %; RC: ϕ 0.25 mm×5 m; flow rate, 0.9 mL/min; sample loop, 200 μL. Error bar denotes standard deviation in tripricate.

for 3.6×10^{-7} M HSA, $\varepsilon_{HSA\cdot(TBPE)x}$ (x denotes the binding number of TBPE), was 1.2×10^{6} L mol^{-1} cm^{-1}. In this study, ε_{TBPE} in the presence of Triton X-100 was 5.7×10^{4} L mol^{-1} cm^{-1}. The ε_{TBPE} multiplied by 21 gives the same value of 1.2×10^{6} L mol^{-1} cm^{-1} as the $\varepsilon_{HSA\cdot(TBPE)x}$. Consequently, we estimated the binding ratio of TBPE to HSA was 21:1. Hence, the chemical equilibrium of the association reaction and its micelle extraction constant ($K_{ex(m)}$) can be expressed as:

$$21(\mathrm{TBPE\cdot H})_m + \mathrm{HSA}^{21+} \rightleftharpoons (\mathrm{HSA}^{21+}\cdot(\mathrm{TBPE}^{-})_{21})_m + 21\mathrm{H}^{+} \quad (1)$$

$$K_{ex(m)} = \frac{[\mathrm{HSA}^{21+}\cdot(\mathrm{TBPE}^{-})_{21}]_m[\mathrm{H}^{+}]^{21}}{[\mathrm{TBPE}\cdot\mathrm{H}]_m^{21}[\mathrm{HSA}^{21+}]} \quad (2)$$

where $[\ ]_m$ is the concentration of chemical species in the micelle. Motomizu et al. have reported micelle extraction constants of ion associate formed between quaternary ammonium salts (quaternary phosphonium salts) and TBPE·H in the presence of Triton X-100 [26,27]. However, in this study, it was difficult to determine the micelle extraction constant of TBPE-HSA because HSA is a large and hydrophilic molecule.

3.1.4. Calibration graph and interference study

A calibration graph with a good linear relationship (R^2 = 0.980) was obtained up to 3.0 mg/dL of HSA. The 3σ limit of detection (LOD) and 10σ limit of quantitation (LOQ) were 0.05 and 0.17 mg/dL, respectively.

Sodium chloride and creatinine concentrations in normal urine are 1000 mg/dL and 100 mg/dL, respectively. The interferences on sodium chloride and creatinine were studied. As the results, sodium chloride did not interfere up to 30 mg/dL and 18 mg/dL creatinine did not give the interference. Accordingly, the coexisting sodium chloride and creatinine in urine did not give the interference on the determination of HSA in this method because the urine sample was diluted with water to 62.5-fold before measurements.

3.2. FIA study

3.2.1. Effect of pH

The effect of pH for 3.0 mg/dL HSA was investigated in the range of pH 2.7 – 3.3. With increasing pH, absorbance increased gradually. However, over pH 3.1, absorbance of the baseline also increased. In this study, pH 3.0 was selected.

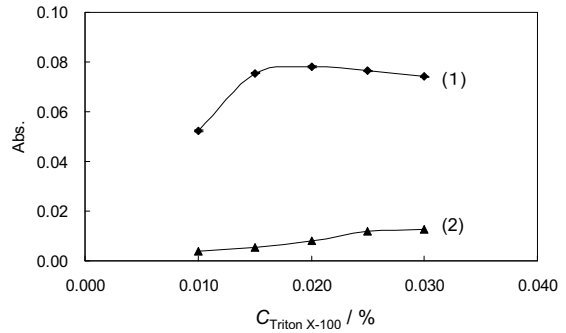

Fig. 5 Effects of Triton X-100 concentration in FI method. (1) HSA (3 mg/dL) against reagent blank and (2) baseline absorbance against water. TBPE, 1.2×10^{-5} M; pH 3.0; RC, ϕ 0.25 mm×5 m; flow rate, 0.9 mL min^{-1}; sample loop, 200 μL. Error bar denotes standard deviation in tripricate.

3.2.2. Effects of TBPE and Triton X-100 concentrations

TBPE·H concentration was varied in the range from 0.6×10^{-5} to 2.0×10^{-5} M (Fig. 4). Over 1.2×10^{-5} M TBPE·H, absorbance increased slightly. However, over 1.4×10^{-5} M TBPE·H, absorbance of the baseline increased drastically. A higher TBPE·H concentration gave the large reagent blank in a similar manner as mentioned in the batchwise study. In this study, 1.2×10^{-5} M TBPE·H was used.

The effect of Triton X-100 concentration was varied from 0.01 to 0.03%. At the 0.01% solution, the absorbance of the associate was very low because the dissolution of TBPE·H into the micelle was not sufficient. Over 0.015%, the largest and constant absorbance was obtained as shown in Fig. 5. The 0.02% concentration was chosen.

3.2.3. Other variables

The reaction coil (tubing of 0.25mm ID) length was varied in the range of 0.5 to 5 m using 3 mg/dL HSA. The constant and largest absorbance was obtained even below 2 m. However, the correlation coefficient (R^2) of the calibration curve was 0.977 at 2 m and R^2 at 5m was 0.998 in the range of 4–8 mg/dL HSA. As a result, a 5-m tubing was chosen.

Table 1 Determination of protein in human patient urine

Sample No.	Test paper[a]	Batchwise method /mg/dL[b]	FIA method /mg/dL[b]
1	++	50.1±2.4	43.2±1.3
2	++	82.3±1.9	83.7±4.5
3	++	58.3±2.3	53.2±1.2
4	++	89.7±2.2	88.0±2.2
5	++	70.6±2.0	66.3±1.5
6	++	89.8±3.0	81.8±0.8
7	++	108.8±1.2	109.7±1.0

a. Semi-quantitation by Albustix purchased from Bayer Medical Ltd., The symbol (++) shows > 100 mg/dL protein
b. Dilution ratio for sample determined was 62.5.

The effect of flow rate was investigated in the range of 0.5 to 1.3 mL min^{-1}. Over 0.7 mL min^{-1}, the constant and largest absorbance was obtained and the baseline became lower.

The sample volume was varied from 50 μL to 300 μL. With increasing the volume, absorbance increased gradually. The 200 μL sample volume was used.

3.2.4. Calibration graph

The calibration graph was linear up to 12 mg/dL of HSA with $R^2 = 0.998$ and the graph passed through the origin: $y = 0.026x$ (y = absorbance, x = HSA concentration in mg/dL). The LOD and LOQ of the proposed FIA method were 0.05 and 0.15 mg/dL, respectively. The sample throughput was 30 h^{-1}. The relative standard deviation ($n = 10$) was 1.2% for 3 mg/dL.

3.2.5. Interferences

Interference by sodium chloride and creatinine was studied on the determination of 3 mg/dL HSA by the proposed FIA method. Over 60 mg/dL sodium chloride, absorbance decreased. However, sodium chloride less than 20 mg/dL did not interfere. And, 10 mg/dL creatinine did not give any interference. Since urine sample was diluted with water to 62.5-fold in this study, the influence from sodium chloride and creatinine could be negligible.

3.3. Application to urinary protein and serum protein analysis

The urinary protein contents in samples collected from nephropathy and diabetic nephropathy patients were determined by a paper test kit (commercially available Albustix by Bayer Medical), batchwise method and the proposed FIA method. Each urine sample was centrifuged at 3000 rpm and the supernatant was diluted with water to 62.5-fold. The results obtained by the three methods are summarized in Table 1. The concentrations by the test paper showed around 100 mg/dL for all samples because the judged concentration range was rough and not accurate as described above. The correlation coefficient (R^2) between batchwise method and FIA method was 0.954, and the slope was 1.03. The experimental t-value was 2.42 and the theoretical t-value was 2.447 for $n = 7$. Accordingly, the proposed FIA using TBPE·H and Triton X-100 was useful by offering some advantages on low reagent blank, rapidity, simplicity, accuracy and less reagent consumption.

In conclusion, two channel flow injection system using TBPE·H dissolved in Triton X-100 was proposed for the determination of urinary protein collected from patients. The calibration range was 0.15 – 12 mg/dL with RSD of 1.2%. The sample throughput was 30 h^{-1}. This could be an alternative method in the clinical laboratory.

Acknowledgement

The author (T.S.) gratefully acknowledges the financial support of this study by Grants-in-Aid for Scientific Research No. 17550087 from Japan Society of the Promotion of Science. This work was partly supported by Grant-in-Aid for Scientific Research No. 18750067 (N.T.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. K.W. and K.G. acknowledge The Thailand Research Fund (TRF) and The Postgraduate Education and Research Program in Chemistry (PERCH) for financial support.

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(Received April 20, 2007)
(Accepted May 7, 2007)

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Original Paper

High-performance liquid chromatography with sequential injection for online precolumn derivatization of some heavy metals

HPLC was coupled with sequential injection (SI) for simultaneous analyses of some heavy metals, including Co(II), Ni(II), Cu(II), and Fe(II). 2-(5-Nitro-2-pyridylazo)-5-[*N*-propyl-*N*-(3-sulfopropyl)amino]phenol (nitro-PAPS) was employed as a derivatizing reagent for sensitive spectrophotometric detection by online precolumn derivatization. The SI system offers an automated handling of sample and reagent, online precolumn derivatization, and propulsion of derivatives to the HPLC injection loop. The metal–nitro-PAPS complexes were separated on a C_{18}-μBondapak column (3.9×300 mm^2). Using the proposed SI-HPLC system, determination of four metal ions by means of nitro-PAPS complexes was achieved within 13 min in which the parallel of derivatization and separation were processed at the same time. Linear calibration graphs were obtained in the ranges of 0.005–0.250 mg/L for Cu(II), 0.007–1.000 mg/L for Co(II), 0.005–0.075 mg/L for Ni(II), and 0.005–0.100 mg/L for Fe(II). The system provides means for automation with good precision and minimizing error in solution handling with the RSD of less than 6%. The detection limits obtained were 2 μg/L for Cu(II) and Co(II), and 1 μg/L for Ni(II) and Fe(II). The method was successfully applied for the determination of metal ions in various samples, including milk powder for infant, mineral supplements, local wines, and drinking water.

Keywords: HPLC / Metal ions / Nitro-PAPS / Online precolumn derivatization / Sequential injection

Received: May 18, 2007; revised: June 29, 2007; accepted: July 2, 2007

DOI 10.1002/jssc.200700215

1 Introduction

Metal ion analysis is of great importance in various fields, including food, pharmaceutical, medical, and environmental sciences. Numerous analytical techniques have been reported for the determination of heavy metals. The most widely used instruments, *e.g.*, atomic absorption [1–3] and inductively coupled plasma atomic emission spectrometers [4, 5], used for metal determination suffer from both spectral and chemical interferences and involve instrumentation complexity. A common UV–Vis spectrophotometry [6] is often simpler and less expensive than atomic spectroscopic methods but does not allow a multielemental analysis and requires derivatization process for improving the sensitivity and selectivity. HPLC [7–10] and ion chromatography [11] have been widely recognized as versatile methods for multielement and sensitive analyses of metal ions using either pre- or postcolumn derivatization. In a typical postcolumn procedure, a chromogenic reagent is mixed with column eluent in a postcolumn mixer and the derivatives formed are detected. To be useful, postcolumn reactions must be rapid and generate low background signals. Dead volume associated with connecting tubing, detector, and mixing devices must be minimized to avoid unnecessary peak dispersion. The precolumn derivatization procedure ensures that the excess of reagent is completely separated from the chelates and does not actually contribute to the increase in baseline signals at the peak positions of the chelates. This fact indicates that the attainable sensitivity is determined only by the inherent S/N of the detector. The potential of this approach is realized in the RP HPLC partition mode, including the ion-pair type. Ion-pair RP HPLC (IP-RPHPLC)

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Abbreviations: IP-RPHPLC, ion-pair RP HPLC; **Nitro-PAPS,** 2-(5-nitro-2-pyridylazo)-5-[*N*-propyl-*N*-(3-sulfopropyl)amino]phenol; **SI,** sequential injection

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offers both sensitivity and selectivity owing to the large degree of flexibility in the separation parameters. Several chelating agents have been successively used as a derivatizing reagent such as 4-(2-pyridylazo)resorcinol (PAR) [10], 5-(2-hydroxy-5-nitrophenylazo)thiorhodamine [12], 2,4-dihydroxybenzylidenethiorhodamine [13], 1-(4-aminobenzyl) ethylenediamine-*N*,*N*,*N*′,*N*(-tetraacetate [14], 2-(5-bromo-2-pyridylazo)-5-(*N*-propyl-*N*-(3-sulfopropylamino)aniline (5-Br-PSAA) [15], and 2-(5-nitro-2-pyridylazo)-5-[*N*-propyl-*N*-(3-sulfopropyl)amino]phenol (nitro-PAPS) [16].

Precolumn derivatization is normally performed offline (batch) because of instrumentation simplicity. But offline manipulation is proved to be laborious, time consuming, especially if a number of samples is to be processed, and may result in low precision. On the other hand, online derivatization offers automation, ease and high sampling rate, providing an attractive alternative [17]. Some developments have involved the coupling of flow injection (FI) [10, 18–20] or sequential injection (SI) [17, 21] with HPLC for online precolumn derivatization. FI involves an inexpensive hardware and a simple operational basis, while SI offers high potential for higher degree in laboratory automation with less reagent consumption.

In this work, attempts were made for coupling IP-RPHPLC with SI for online precolumn derivatization and determination of some heavy metals, including Co(II), Cu(II), Ni(II), and Fe(II). Nitro-PAPS was used as the derivatizing reagent for a sensitive spectrophotometric detection. Application to a variety of samples was investigated. The method would provide simple, low consumption of sample and reagent, and easy operation with higher degree of automation. The chromatographic system could be an alternative for simultaneous analysis of some heavy metals.

2 Materials and methods

2.1 Chemicals and reagents

All the reagents used were of analytical reagent grade. Deionized water was used throughout the experiments. The atomic absorption standard solutions (1000 mg/L) of copper (II) nitrate and nickel (II) nitrate were obtained from Ajax Chemicals (Australia). Stock standard solution (1000 mg/L) of cobalt (II) was prepared using cobalt (II) chloride hexahydrate in water. Stock standard solution (1000 mg/L) of iron (II) was prepared by dissolving iron (II) sulfate heptahydrate in 10^{-4} mol/L hydrochloric acid. Working standards were freshly prepared by diluting the stock solution with water to obtain appropriate concentrations. Nitro-PAPS was obtained from Dojindo Laboratories (Japan). Tetrabutylammonium bromide (TBABr) was purchased from Fluka (Switzerland). ACN was of HPLC grade. All HPLC mobile phases were filtered through 0.45 μm membrane filters and sonicated prior to use.

2.2 Instrumentation

The SI-HPLC system used is schematically depicted in Fig. 1a. The SIA part consists of a 2.5 mL syringe pump (Cavro, USA), a 10-position selection valve VICI with a microelectric actuator (Valco Instruments, USA), and a holding coil (220 × 0.07 cm id PTFE tubing). Instrumentation control was manipulated *via* the FIAlab software (FIAlab Instruments, USA). The HPLC part consists of a Waters 6000A dual pump, a Rheodyne injector with a 50 μL injection loop, and a Waters 484 absorbance detector (Waters, USA) operated at the wavelength of 570 nm. The SI system was connected to the HPLC injection port by means of the 37 × 0.07 cm id PTFE tubing. Separations were performed on a 3.9 × 300 mm^2 C_{18}-μBondapak column with 5 μm particle size (Waters). The CSW 32 software (Waters) was used for data acquisition.

2.3 Procedure

Effective separation of the studied metal–nitro-PAPS complexes was achieved through the IP-RPHPLC mode using the mobile phase containing ACN, buffer and ion pairing agent. The column was equilibrated with the mobile phase being used for 25 column-volumes before start.

The SI-HPLC operation steps were as follows: the manifold lines were washed with water, and all the reagents were filled into the ports of the selection valve. Then, suitable volumes of the reagents were sequentially aspirated and stacked as zones in the holding coil. By flow reversal of the syringe pump, the reaction mixture, metal–nitro-PAPS complexes in this work, was propelled to the HPLC injection loop and injected into the HPLC system. A mobile phase flow rate of 1.0 mL/min was used throughout.

3 Results and discussion

3.1 IP-RPHPLC of metal–nitro-PAPS chelates

To achieve adequate separation for the studied chelates within a minimum elution time, optimization of the HPLC separation was performed by offline derivatization of metal–nitro-PAPS chelates. Nitro-PAPS chelates of Co(II), Ni(II), Cu(II), and Fe(II) were formed by mixing metal ion solutions and the nitro-PAPS solution with the concentration ratio of 1:2 and was manually injected in the HPLC system. It has been known that the composition of mobile phase plays an important role in IP-RPHPLC. The mobile phase components include concentration of buffer, pH, amount of organic modifier, and

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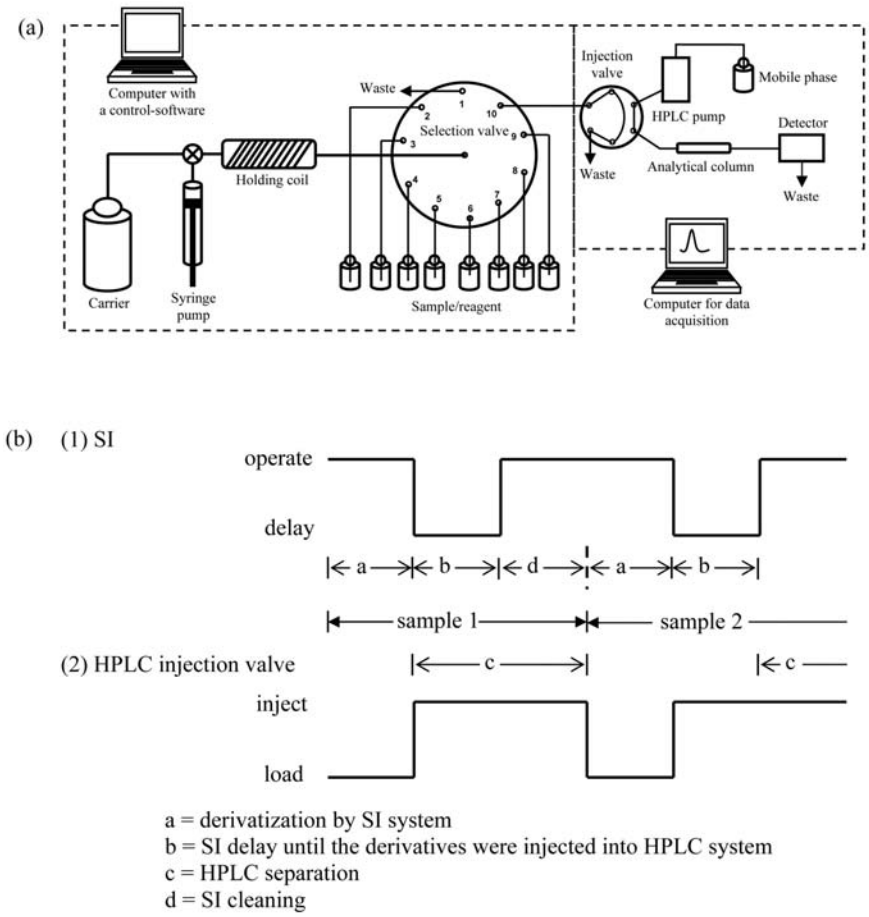

Figure 1. (a) Schematic diagram of the SI-HPLC system, (b) diagram of operational sequence of the SI-HPLC system.

concentration of ion pairing agent. The selectivity of the IP-RPHPLC was susceptible to the content of organic modifier in the mobile phase due to the variation in polarity and hydrophobicity which lead to different strengths and selectivity. The role of buffer is to control the pH of the chromatographic system, while the role of the ion pairing agent is to provide the exchange sites for the target analytes. Therefore, some degrees of variation in the retention behavior and chromatographic selectivity are expected by addition of ion pairing agent in IP-RPHPLC. The mobile phase was selected from our preliminary studies and was found to be 30% ACN in 10.0 mmol/L acetate buffer of pH 5.0 and 3.5 mmol/L TBABr. Separation of four metal–nitro-PAPS chelates and removal of excess nitro-PAPS reagent was achieved within 13 min with the elution order of Cu(II)-nitro-PAPS, Co(II)-nitro-PAPS, excess nitro-PAPS reagent, Ni(II)-nitro-PAPS, and Fe(II)-nitro-PAPS, respectively. The conditions were adopted for the coupling of SI to HPLC.

3.2 Online coupling of SI to HPLC

Using the SI-HPLC system shown in Fig. 1a, the experimental parameters affecting the sensitivity of the determination were considered. First, it must be ensured that the most concentrated part of the reaction mixture will be injected into the separation column. Therefore, after forming derivatives by the SI system, the dispensing volumes of the syringe pump ranging from 240 to 380 μL were optimized and the peak areas were compared. The dispensing volume (at a constant flow rate) determined the time interval between the start of dispensing of the derivative zone by the syringe pump of the SI system and the actual injection into the HPLC system. Second, the sequence orders of aspiration together with the volumes of sample and reagent and the number of flow reversals were examined. The operational sequence in which the sample and reagent were aspirated as small segments gave a higher sensitivity, while the mixing by several flow reversals was unnecessary. The metal–nitro-PAPS derivatives could be formed immediately and the sensitivity was enhanced by mixing during the transportation to the HPLC loop. However, the connection between the SIA part and the HPLC injection loop should be shortened to prevent further dispersion of the product zone. It has been known theoretically that increase in the sample and/or reagent volumes results in increase in the sensitivity but it must be compromised between the volumes

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Table 1. The operational sequence of the SI-HPLC system for online derivatization of metal–nitro-PAPS complexes

Sequence	Port no.	Flow rate (μL/s)	Volume (μL)	Description
1	3–9	50	70	Aspiration of sample into HC[a)]
2	2	50	25	Aspiration of nitro-PAPS into HC
3	3–9	50	70	Aspiration of sample into HC
4	2	50	25	Aspiration of nitro-PAPS into HC
5	3–9	50	60	Aspiration of sample into HC
6	10	50	300	Propulsion of metal–nitro-PAPS derivatives to HPLC loop
7	10	–	–	HPLC analysis

a) HC = holding coil.

Table 2. Analytical characteristics of the proposed system

Metal–nitro-PAPS	Linear range (mg/L)	Linear equation	r^2	LOD[a)] (μg/L)
Cu(II)	0.005–0.250	$y = 1652.6x - 72.5$	0.9928	2
Co(II)	0.007–0.100	$y = 2479.5x - 3.2$	0.9968	2
Ni(II)	0.005–0.075	$y = 1607.8x - 1.5$	0.9945	1
Fe(II)	0.005–0.100	$y = 3018.0x - 4.9$	0.9938	1

a) S/N = 3.

of derivative product and the volume of the HPLC injection loop. The derivative should be formed such that it is just enough for injecting into the HPLC loop with adequate sensitivity and precision. In addition, the peak shape of the obtained chromatogram must be considered. On increasing the injection volume, a wider peak will be obtained and leads to overloading of the column. The selected operational sequences as well as the volumes of sample and reagent are summarized in Table 1 and Fig. 1b. Finally, the chemical parameter, the concentration of nitro-PAPS reagent, was varied to increase the sensitivity and minimize the excess of reagent concentration. The optimum results were obtained using a nitro-PAPS concentration of 1×10^{-4} mol/L. The sensitivity determined by the peak areas of all metal complexes remained constant and the peak of excess nitro-PAPS reagent was resolved from the derivative peaks. A typical chromatogram is presented in Fig. 2. Baseline disturbance was observed under an elution time of approximately 3–5 min from the time when the blank solution was injected, and this could be due to the excess of the reagent. Quantification of peak area using peak-to-peak baseline correction was carefully performed.

3.3 Analytical characteristics

Under the selected SI and HPLC conditions, quantitative features of the proposed system were evaluated by examining the linear range, precision, and LOD. Calibration graphs were constructed by plotting the peak area against the concentration of each metal ion. The detection limits were calculated at a signal level of three times

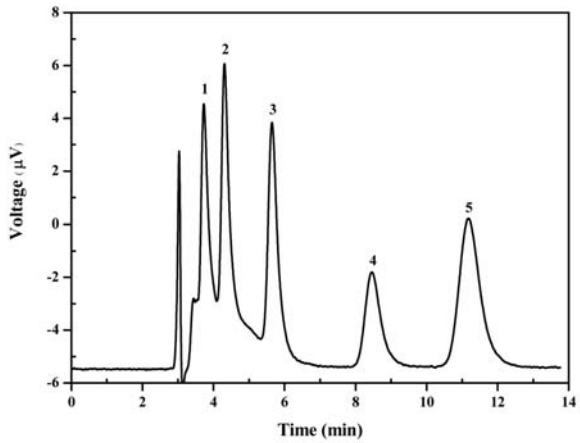

Figure 2. Typical chromatogram of metal–nitro-PAPS complexes: 1, 0.100 mg/L Cu(II); 2, 0.050 mg/L Co(II); 3, excess nitro-PAPS; 4, 0.075 mg/L Ni(II); 5, 0.075 mg/L Fe(II).

the baseline noise (S/N = 3). The equations of the obtained calibration graphs, the linear ranges, and the detection limits are given in Table 2. The RSDs of the peak area and retention time of each analyte were less than 6%. The effects of interferences on the SI-HPLC of metal–nitro-PAPS chelates were studied by adding the metal ions at different concentrations into a mixture of 0.060 mg/L Co(II), 0.010 mg/L Cu(II), Fe(II), and Ni(II). No interference, *i.e.*, no signal change within ±5%, was observed from common ions with the maximum concentrations studied up to 20 mg/L of Na^+, Ca^{2+}, and Cl^-; 25 mg/L of CO_3^{2-}, 30 mg/L of SO_3^{2-}, NO_2^-, and NO_3^-; and 50 mg/L of Cd^{2+}, Pb^{2+}, and Zn^{2+}.

3.4 Analysis of real samples

The applicability of the SI-HPLC system was investigated by determining the metal ions in various samples, including modified milk powder for infants, mineral supplements, local wines, and drinking water. For milk powder and supplement samples, fine powder samples were weighed, dissolved in water, and filtered before determination. For wines and drinking waters, the sam-

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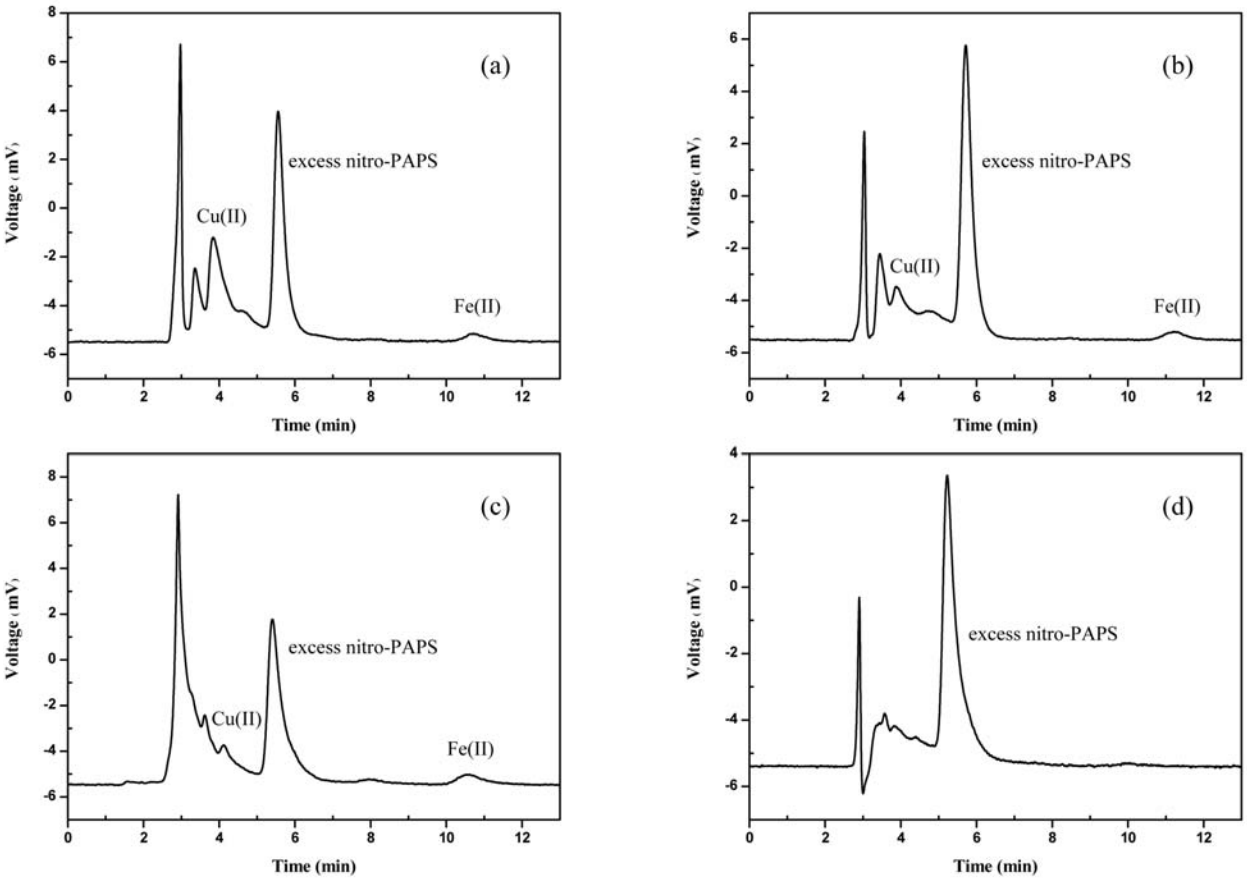

Figure 3. Chromatograms obtained from sample determination: (a) milk, (b) supplement, (c) wine and (d) drinking water.

Table 3. Analysis of real samples and recovery studies

Sample	Cu(II)			Co(II)			Ni(II)			Fe(II)		
	This work[a)] (mg/L)	Labeled (mg/L)	Recovery (%)	This work (mg/L)	Labeled (mg/L)	Recovery (%)	This work (mg/L)	Labeled (mg/L)	Recovery (%)	This work[a)] (mg/L)	Labeled (mg/L)	Recovery (%)
Milk 1	273.7 ± 2.1	312.0	91	–	–	99	–	–	99	5.8 ± 0.2	7.0	97
Milk 2	314.0 ± 3.6	340.0	95	–	–	98	–	–	96	5.5 ± 0.1	6.0	95
Milk 3	552.5 ± 1.8	580.0	97	–	–	99	–	–	99	7.2 ± 0.1	7.8	99
Supplement 1	1.6 ± 0.4	2.0	93	–	–	96	–	–	98	8.7 ± 0.1	10.0	97
Supplement 2	2.1 ± 0.1	2.5	90	–	–	99	–	–	99	12.9 ± 0.1	15.0	94
Wine 1	1.0 ± 0.2	1.4	102	–	–	94	–	–	105	1.5 ± 0.1	1.8	98
Wine 2	5.2 ± 0.6	6.8	93	–	–	97	–	–	102	1.8 ± 0.1	2.1	93
Wine 3	–	–	95	–	–	95	–	–	98	0.8 ± 0.2	1.0	105
Wine 4	–	–	106	–	–	101	–	–	96	0.2 ± 0.1	0.4	101
Water 1	–	–	93	–	–	99	–	–	99	–	–	98
Water 2	–	–	90	–	–	99	–	–	99	–	–	99

– Not detected.
Supplement 1 ingredients (tab^{-1}): Cu ($CuSO_4$) 2 mg, Fe ($FeSO_4$) 10 mg.
Supplement 2 ingredients (tab^{-1}): Cu ($CuSO_4$) 2.5 mg, Fe (ferrous gluconate) 15 mg.
[a)] $n = 3$.

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ples were analyzed by the system without any further pretreatment except filtration and dilution. Typical chromatograms of sample determination are shown in Fig. 3. The results, as shown in Table 3, were in agreement with the labeled values (for milk powder and mineral supplement samples) and those obtained by AAS methods (for wines and drinking water), evaluated by *t*-test at 95% confidence level. It was not surprising that some metal ions such as Co(II) and Ni(II) were not detectable in these samples and all metal ions in drinking water samples were lower than the LODs. However, the recoveries, assessed by experiment of known amounts of metal ions (0.020 mg/L each) spiked in the real samples, obtained were satisfactory (90 – 106%).

4 Concluding remarks

Coupling HPLC with SI offers an online precolumn derivatization system for analyses of some heavy metal ions. The derivatives can be formed online and subsequently injected and analyzed in the HPLC system. This leads to possibility in automation. By parallel operation of derivatization and separation, advantages of less consumption of sample/reagent, as well as better analysis time could be gained. It has been demonstrated that HPLC hyphenated with SI could be an alternative for simultaneous analyses of some heavy metal ions in various samples.

We thank the Thailand Research Fund (TRF) and the Commission on Higher Education (CHE). This work was partly supported by the Postgraduate Education and Research Program in Chemistry (PERCH). Thanks are due to Professor T. Sakai for supply of the reagent (Nitro-PAPS) which was under the Frontier Research Project – Materials for the 21st Century (Materials Development for Environment, Energy and Information).

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Anal Bioanal Chem (2007) 388:1839–1846
DOI 10.1007/s00216-007-1361-5

ORIGINAL PAPER

Exploiting flow injection system with mini-immunoaffinity chromatographic column for chondroitin sulfate proteoglycans assay

Supaporn Kradtap Hartwell · Kanokphan Pathanon · Duriya Fongmoon · Prachya Kongtawelert · Kate Grudpan

Received: 9 March 2007 / Revised: 8 May 2007 / Accepted: 10 May 2007 / Published online: 20 June 2007
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Abstract A flow injection (FI) system with a mini-immunoaffinity chromatographic column was used to perform on-line assays of specific proteoglycans. The 300-μL mini-column contained beads coupled with monoclonal antibodies against the specific sulfation pattern of chondroitin sulfate proteoglycans, which have been reported to be a potential biomarker for cancer. The amount of these proteoglycans present was estimated indirectly from their protein content using the Bradford assay, which is an alternative to a direct carbohydrate assay. The system developed was tested by assaying for chondroitin sulfate proteoglycans in sera from patients with various cancers and comparing the results to those obtained for sera from healthy people. The results indicated that this approach could be used as a cost-effective alternative system for determining the amount of these specific biomarker proteoglycans. The column could be reused at least 90 times, with each run consisting of 200 μL of serum sample diluted twofold; an analysis rate of 30 min per run was achieved, as compared to 4 h for a batch procedure.

Keywords Flow injection · Immunoaffinity · Proteoglycans · Chromatographic column · Chondroitin sulfate · Cancers

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Introduction

Some diseases, such as cancers, can be difficult and expensive to diagnose at an early stage, which has led to the study and use of biomarkers by many researchers [1–7]. A good tumor marker should have the following characteristics: it should be cancer-specific; it should not exhibit elevated levels in a normal person; it should be easy to detect during the earliest stages of cancer; the marker signal should increase in strength as the cancer progresses [8–11]. Even though a cancer marker with all of the above characteristics is yet to be found, some tumor biomarkers have still proved useful. Examples of cancer biomarkers currently in use are carcinoembryonic antigen (CEA) [12, 13], prostate-specific antigen (PSA) [14, 15], and alpha fetoprotein (AFP) [16]. Many other new biomarkers are currently being studied [3, 4, 6, 8, 13]. Among them, certain proteoglycans have been studied as biomarkers of liver, joint, and cancer diseases in clinical research [17–20].

There are many modern biochemical techniques that have been used to study such biomarkers; for example, DNA and gene expression technology [2, 21], PCR [1, 13], immunoassay [16], as well as chromatography and mass spectrometry, such as matrix-assisted laser desorption/ionization (MALDI) and surface-enhanced laser desorption/ionization (SELDI) [10, 22, 23]. However, these standard high-performance techniques are simply not available in many rural areas of developing countries since they lack adequate facilities and funding. A simple and low-cost system that minimizes the need for well-trained operators would be very useful since it could be used to provide health care to people living in remote places where poverty and inadequate transportation often prevent access to a modern hospital.

Flow injection (FI) is a cost-effective and well-established technique that utilizes the continuous flow of solutions in a closed system. The chemical reactions and physical interactions of reagents and samples take place on-line, and the resulting product can be monitored simultaneously when coupled with a suitable detector [24–32]. The FI technique has been applied in many study areas, including environmental, food, industrial, and clinical studies [33–40]. Immunoaffinity chromatography has been used for the purification of substances from complex biological matrices [41, 42], and for the removal of small amounts of biological materials from large amounts of contaminating substances [43].

The work presented here was conducted to develop a simple and low-cost system to assay for chondroitin sulfate proteoglycans (proteoglycans that contain the specific sulfation pattern of chondroitin sulfate), which have been reported to be a potential biomarker for ovarian cancer [44–48]. The FI system allowed on-line isolation of the specific proteoglycans from nonspecific ones in a mini-immunoaffinity chromatographic column that was packed with specific monoclonal-antibody-coupled beads. Quantification of the specific proteoglycans was done indirectly by determining the amount of protein in the proteoglycans using a Bradford protein assay [49, 50], which gave a colored complex that could be quantified spectrophotometrically. Serum samples from patients with different types of cancer were studied to evaluate the potential of the proposed system as an alternative approach to assaying for the proteoglycans biomarker of interest. The system may provide an alternative and inexpensive method of on-line operation. This may reduce the need for experienced personnel during batchwise operation or an expensive automated system for the precise timing of incubation/washing steps and the precise manipulation of small volumes of reagents used in more complicated techniques such as a batchwise ELISA procedure.

Materials and methods

Reagents and apparatus

All chemicals used were of analytical grade unless specified otherwise. Monoclonal antibodies against the specific sulfation pattern of chondroitin sulfate were prepared in the laboratory. This is an IgM that can bind to A-1 shark proteoglycans. Its specificity was checked by ELISA, using an A-1 shark-coated well plate, peroxidase-conjugated mouse anti-IgM, and *O*-phenylenediamine substrate. The antibodies were coupled onto Sepharose 4B beads (Pharmacia Biotech, Piscataway, NJ, USA) through cyanogen-bromine (CNBr) activation. Bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) was used as a standard protein.

Instruments used in this work include a B 5050 incubator (Heraeus, Hanau, Germany), an RS-60 tube rotator (BioSan, Riga, Latvia), a Mettler HK 160 autobalance (Mettler Instruments A.G., Greifensee, Switzerland), a 5301 concentrator (Eppendorf, Hamburg, Germany), a FOC-1 lyophilyzer (Martin Christ, Osterode, Germany), a Gast 0211-V45M-0230CX pump (Benton Harbor, MI, USA), a Mini-PROTEIN II electrophoresis cell and a model 200/2.0 Mini Trans-Blot electrophoretic and power supply (Bio-Rad, Hercules, CA, USA), a Vortex-Genie k-550-GE (Springfield, MA, USA), a UV-VIS spectrophotometer (UV 1200, Shimadzu, Kyoto, Japan), a pH meter (Beckman Coulter, Fullerton, CA, USA), a model 400 peristaltic pump (Watson–Marlow Alitea Co., Ltd., Stockholm, Sweden), a six-port bulkhead-mounted injection valve and four-way switching valve (Upchurch Scientific, Oak Harbor, WA, USA), a Spectronic 21 (Milton Roy Company, Ivyland, PA, USA) with a flow-through cell (Hellma, Plainview, NY, USA), BDS software for integration (BarSpec Ltd., Rehovot, Israel), and a Titertek Multiscan MCC/340 microplate reader (Labsystems Flow Laboratories, VA, USA).

Flow injection mini-immunoaffinity chromatographic column system

The flow manifold used in this work is represented diagrammatically in Fig. 1. The 0.1 M Tris-HCl buffer containing 0.15 M NaCl, pH 7.4, was pumped by a peristaltic pump to equilibrate the column for 2 min. Another peristaltic pump was used to pump the Bradford dye reagent (Bio-Rad) diluted in 30% v/v ethanol. Serum sample solution was injected into the column via a six-port injection valve with a 200-μL sample loop. 0.1 M acetate buffer with 0.5 M NaCl, pH 2.0, was used as the eluting buffer.

Results and discussion

The reagents and the beads

The monoclonal antibody used in this study is called WF6, which has been proved, as described in the international patent No. WO 2005/118645 A1 and previous reports [51, 52], to selectively bind to the specific sulfation pattern of chondroitin sulfate containing chondroitin-6-sulfate (CS-C). It was raised in mice and produced from serum-free media for hybridoma cell cultures.

Purification of monoclonal antibody using a thiophilic-gel (T-gel) column (Pierce Biotechnology, Rockford, IL, USA) was carried out according to the manufacturer's

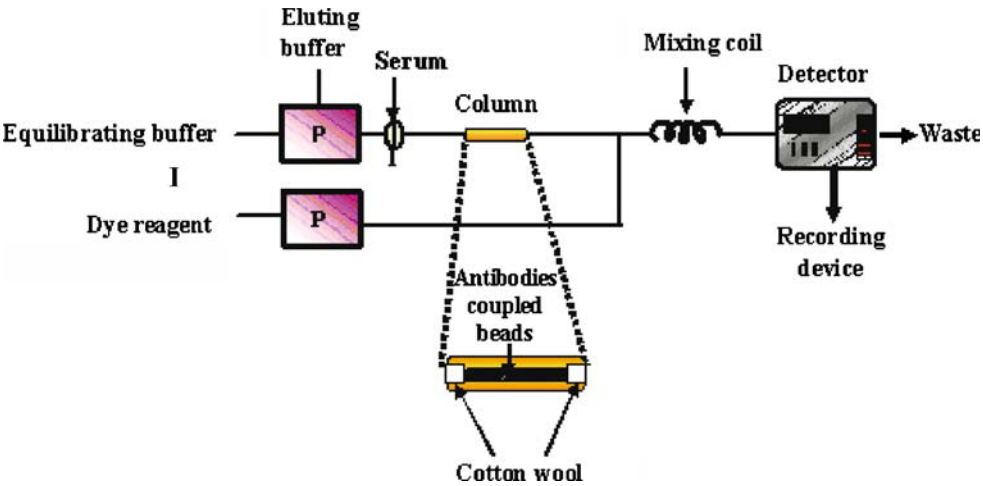

Fig. 1 Diagram of the FI system with a mini-immunoaffinity chromatographic column used to assay for specific proteoglycans. *P*, a peristaltic pump; *I*, six-port injection valve; *S*, sample; equilibrating buffer: 0.1 M Tris-HCl containing 0.15 M NaCl; eluting buffer: 0.1 M acetate buffer containing 0.5 M NaCl; dye reagent: Bradford. Pump tubing was Tygon® tubing with 1/16″ ID; all other tubing was made of Teflon with 1/32″ ID

instructions [53, 54]. Thiophilic adsorption is based on the ability of immunoglobulins to bind to an immobilized ligand that contains a sulfone group. Sodium phosphate buffer solutions with and without potassium sulfate were used to elute unbound and bound proteins, respectively. At a low salt concentration, i.e., without potassium sulfate added to the buffer, the disulfide bonds were broken and the bound proteins were released. The molecular weight of the monoclonal antibody from the bound fraction was identified using electrophoresis, and its specific activity was confirmed by ELISA prior to use.

Coupling of antibodies onto the beads was based on the standard CNBr method, as suggested by the manufacturer of Sepharose 4B (Phamacia Biotech) [55–58]. Sepharose 4B gel was first activated with cyanogen bromine (CNBr). At a high pH of 11–12, CNBr reacts with the –OH groups on the agarose beads to form reactive electrophilic cyanogen ester groups on the bead surfaces. This cyanogen ester reacted with the nucleophilic groups, e.g., $–NH_2$, –SH and –OH of the antibodies in the coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). After washing away the excess antibodies, the remaining reactive cyanogen ester groups were blocked to prevent nonspecific binding by incubating beads in 0.2 M glycine, pH 8.0, for 2 h at 25 °C. Finally the beads were washed in 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0, followed by 0.1 M Tris-HCl buffer containing 0.5 M NaCl, pH 8.0. Antibody-coupled beads were maintained at 4 °C before use.

Separation of specific proteoglycans in the FI system

The target chondroitin sulfate proteoglycans in serum were isolated from other nonspecific proteoglycans by means of the FI system with the mini-immunoaffinity chromatographic column. The affinity column was packed with beads coupled with monoclonal antibody. When the serum samples were passed through the affinity column, nonspecific proteoglycans and other serum proteins were not retained by the antibody-coupled beads and were thus eluted out first with the equilibrating buffer (0.1 M Tris-HCl containing 0.15 M NaCl, pH 7.4). They formed a colored complex when mixed with the Bradford reagent, and gave a first peak (unbound peak) at 590 nm when detected with a Spectronic 21. After the baseline became constant, indicating that the nonspecific proteoglycans and other serum proteins had been removed, the eluting buffer (0.1 M acetate buffer containing 0.5 M NaCl, pH 2.0) was introduced to elute the specific proteoglycans, resulting in a second peak (the bound peak). The profile of the separation of proteoglycans into two groups, nonspecific and specific, is illustrated in Fig. 2, where A is the unbound peak and B is the bound peak, respectively. The column was cleaned with

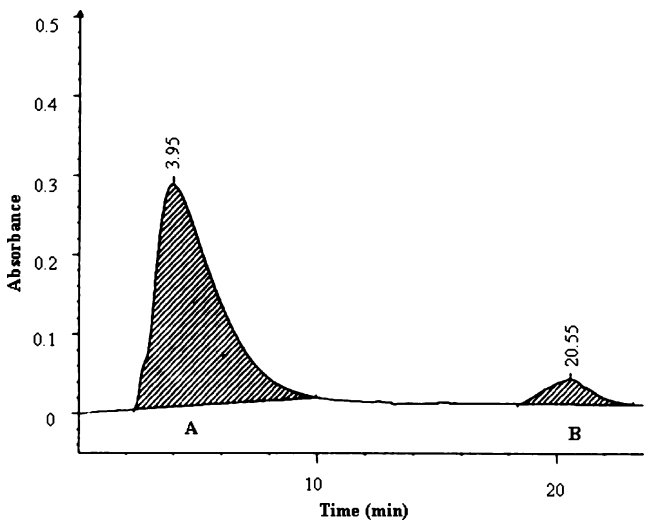

Fig. 2 Absorbance profile of the amount of protein in proteoglycans obtained using a Bradford dye assay after separating the specific proteoglycans (peak B) from the nonspecific proteoglycans (peak A) in normal serum sample using the FI system with a mini-immunoaffinity chromatographic column. Peak areas were integrated and the bound peak (peak B) percentage was calculated based on the total peak area (sum of the areas of peaks A and B)

approximately five times the column volume of the equilibrating buffer before the next sample analysis was started.

Using electrophoresis [59] and Western blotting [60, 61] techniques, the bound portion that was thought to contain the proteoglycans of interest was investigated to estimate the range of molecular weights of these proteoglycans and to confirm their specific activity, and then compare these properties to those of the unbound portion. The results showed that the amount of proteoglycans associated with the bound peak of cancer serum was greater than that associated with the bound peak from normal serum (seen at 70 kDa MW protein, results not shown). The unbound peak also contained specific proteoglycans but the amount associated with this peak was smaller than the amounts associated with the bound peaks in both the normal and cancer sera. The specific proteoglycans detected in the unbound peak may be proteoglycans that were hindered by other proteins or proteoglycans in serum and thus did not bind to the beads. Only the levels of specific proteoglycans associated with the bound peaks were used when analyzing serum samples.

Indirect estimation of the amount of specific proteoglycans from the amount of protein

The proportion of the total proteoglycans that were chondroitin sulfate proteoglycans was calculated based on a comparison of the protein content in the proteoglycans. Protein content was determined using the peak areas obtained from the Bradford protein assay, since this assay is much simpler to perform than a direct carbohydrate assay.

A possible limitation of this indirect protein assay is that the free chondroitin sulfate chains or fragments might not be detected. However, these fragments should not be found in serum in significant quantities due to the covalent nature of the bond between the protein core and the glycans. Even if free chondroitin sulfate is produced during the enzyme reactions and released into the serum, it should be bound to other serum proteins. In addition, the WF6 antibody exhibits specificity toward the pattern of chondroitin sulfate [51, 52], so other serum proteins without chondroitin sulfate should not interfere with an assay for the specific proteoglycans.

The performance of the proposed system was evaluated by determining the amount of protein in the specific proteoglycans in sera from patients with various cancers as compared to those from healthy people. It was assumed that the sum of the areas under peaks A and B (see Fig. 2) corresponds to the total protein. The amount of protein in the specific proteoglycans was estimated from the ratio of the area under peak B to the sum of the areas under peaks A and B. The ratio was converted into milligrams (mg) via a calibration plot constructed from a series of BSA standards that formed colored products when they react with Bradford reagent. BSA was chosen as the standard protein since it is widely available.

Optimization of the FI mini-immunoaffinity chromatographic column system

There are a few parameters of the FI mini-immunoaffinity chromatographic column system that need to be optimized. These include the concentrations of Bradford reagent and the solvent, the flow rate, the gel volume, the sample dilution ratio, the length of the mixing coil, and the pH of the buffer solutions. Optimization was performed using pooled serum samples from nine healthy people. This was to ensure an adequate number of samples for the optimization process.

It was observed that the Bradford dye could stick to the flow line and the wall of the flow-through cell when only water was used as a solvent. This caused the baseline to drift upwards over time, which made it difficult to integrate the peak area. Although the Bradford reagent is soluble in methanol [62], ethanol (which is less toxic) was tried as a solvent to eliminate the problem of the dye sticking to the tubing wall. The concentration of aqueous ethanol was varied from 10 to 40% (v/v), and a concentration of 30% (v/v) was chosen in order to conserve the reagent as much as possible while effectively preventing the colored Bradford reagent from sticking to the tubing wall.

The concentration of the Bradford reagent was also studied in order to save reagent. Various dilutions of the Bradford reagent with 30% (v/v) ethanol (1:10, 1:25, 1:50 and 1:100) were tried, and the 1:25 ratio was ultimately chosen.

The flow rate of the buffer solution greatly affected the performance of the system. When the flow rate was too high it led to a high back-pressure that caused leakage. When the flow rate was too low it increased the analysis time and lowered the sample throughput. Flow rates of 0.5, 1.0, 2.0, and 3.0 mL/min were investigated. A flow rate for the buffer and the Bradford reagent solution of 1.0 mL/min was selected.

The gel volume used also affected the results. Packing too much gel into the column caused high back-pressure, while too little gel led to the ineffective isolation of proteoglycans. The gel volume was varied by repacking the column with different volumes of gel. The optimum amount of gel, 300 μL, was chosen after testing the system with 200, 300, 400, and 500 μL of gel.

The highest concentration of sample that could be studied was related to the capacity of the column (i.e., the amount of gel used). However, if the sample concentration was too high, the specific proteoglycans would pass through the column without retention and would be considered to be nonspecific proteoglycans. To prevent such a false negative result, all samples introduced into the

column need to be diluted to the same dilution factor. In this study, dilution factors of 1:8, 1:4, and 1:2 were investigated and compared to the results obtained using the undiluted sample. A dilution factor of 1:2 was selected since it gave the best sensitivity.

The mixing coil is also an important part of this system. A coil that is too short will not provide enough time for the reaction to occur, while a coil that is too long will increase the analysis time and the dispersion of the product. The effect of varying the length of the mixing coil of 1/32″ ID (lengths of 25, 50, 100, 150 and 200 cm were studied) was evaluated, and a length of 150 cm was eventually chosen.

A poor choice of buffer solution pH would cause ineffective retention and separation of the proteoglycans, and could also damage the solid matrix. The effects of varying the pH of the equilibrating buffer (pH values of 6.5, 7.4, and 8.5 were tested) and the pH of the eluting buffer (2.0, 3.0 and 4.0 were tested) were investigated. pH values of 7.4 and 2.0, respectively, were selected, since these led to the best performance during the separation process and enabled the column to be reused.

These optimized parameters were employed during assays of chondroitin sulfate proteoglycans in serum samples taken from healthy people and cancer patients.

Performance of the system

Using the selected set of conditions described above, the same pooled serum sample from nine healthy people was analyzed 11 times consecutively. The average level of protein in the specific proteoglycans calculated from the 11 measurements was 1.87 mg in 100 mg total protein. The repeatability, expressed as %RSD, was calculated from the relative peak area of the bound proteoglycans, and was found to be 2.3. The lifetime of the column was estimated by performing the analysis with the same column over and over again until there was a significant reduction in the signal, as determined by the RSD of the system. Again, the same pooled serum was used in this study. The same column could be used more than 90 times without a significant reduction in signal. This indicates that the system gives satisfactory reproducibility. Each analytical run took about 30 min, including the washing time between each sample run. The sample volume of the twofold-diluted serum needed for each run was 200 μL.

Application of the system

The applicability of the FI system with the mini-immunoaffinity chromatographic column developed here to real-world samples was evaluated by using it to assay for chondroitin sulfate proteoglycans in serum samples from cancer patients (provided by hospitals). All serum samples were diluted twofold with 0.1M Tris-HCl, pH 7.4, prior to injection into the system.

These serum samples were catagorized into six groups, namely: 1) normal; 2) CEA or sera that had tested positive when screened for cervical cancer using the carcinoembryonic antigen tumor marker; 3) PSA or serum that had tested positive when screened for prostate cancer using the prostate specific antigen tumor marker; 4) prostate or sera from patients who were diagnosed of having prostate cancer; 5) cervix or sera from patients diagnosed with cervical cancer, and; 6) ovary or sera from patients diagnosed with ovarian cancer.

The relative amount of protein in the specific proteoglycans per 100 mg total protein was plotted for each sample from each group, as shown in Fig. 3. The average (and the standard deviation of this value) relative amount of monoclonal antibody specific proteoglycans was calculated for each sample group, and the results were compared with those for the normal samples using the *t*-test (statistical tests performed using Origin 7.0). It was found that the levels of the specific proteoglycans from the normal group were significantly lower than those of the other groups, with $P < 0.05$ (P values in all cases were no higher than 0.0005, or a confidence of level of at least 99.95%).

Various cutoff values were trialed when plotting the receiver operator characteristic (ROC) curve. The resulting curve showed that a relative amount of protein in specific proteoglycans of 2 mg per 100 mg total protein was the most suitable cutoff value. The numbers of true and false responses obtained for each cancer group when this cutoff value was used are summarized in Table 1. Among all

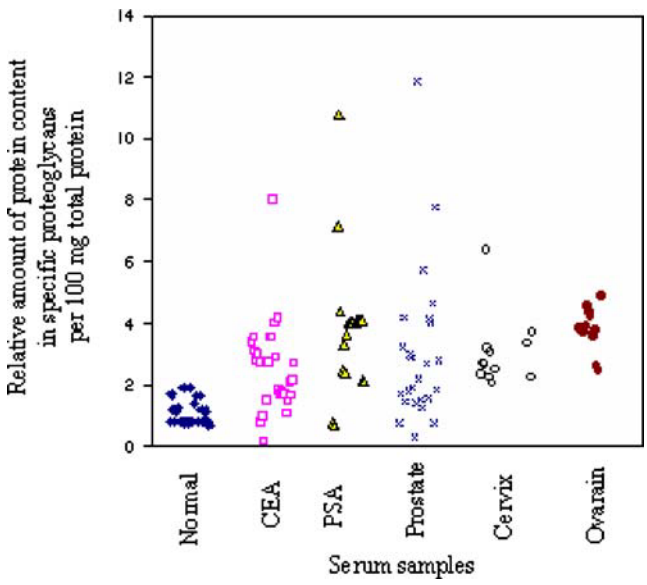

Fig. 3 Comparison of the amount of specific proteoglycans (based on the protein content) in sera from patients with different types of cancer

Table 1 Summary of the numbers of true and false responses and the sensitivity and specificity of the system obtained when 2 mg protein in specific proteoglycans per 100 mg total protein is chosen as a cutoff value

Sample group	No. of samples	Avg. % protein in specific proteoglycans*	TP*	FN*	TN*	FP*	SV*	SP*	Prob. positive*
Normal	25	1.26±0.64	-	-	25	0	-	1.00	-
CEA	27	2.64±1.48	16	11			0.59		1.00
PSA	21	3.18±2.53	13	8			0.62		1.00
Prostate	25	3.01±2.52	13	12			0.56		1.00
Cervix	12	3.03±1.17	12	0			1.00		1.00
Ovarian	14	3.79±0.66	14	0			1.00		1.00

* *Avg. % protein in specific proteoglycans* is the average relative amount of protein in specific proteoglycans per 100 mg total protein, calculated based on the area of the bound peak and using BSA as a standard protein
TP is true positive or the number of diseased patients that test positive
FN is false negative or the number of diseased patients that test negative
TN is true negative or the number of nondiseased patients that test negative
FP is false positive or the number of nondiseased patients that test positive
The same TN, FP, and SP were used for all cancer cases because they are based on the same normal group.

SV is sensitivity or $\frac{\text{number of diseased patients with positive test}}{\text{number of diseased patients}} = \frac{TP}{TP+FN}$
SP is specificity or $\frac{\text{number of nondiseased patients with negative test}}{\text{number of nondiseased patients}} = \frac{TN}{TN+FP}$
Prob. positive is probability of disease if the test is positive $= \frac{TP}{TP+FP}$

groups, the ovarian cancer group showed the highest average amount of specific chondroitin sulfate proteoglycans. The test showed very good sensitivity and specificity for the ovarian and cervical cancer groups, while lower sensitivities were obtained for other groups. The CEA, PSA and prostate groups showed high percentages of false negative results, which indicate that assays for this particular proteoglycan biomarker may not be suited to these three sample groups. The ROC plot also revealed that the ovarian group yielded higher sensitivity and specificity for all cutoff values than the other cancer groups. These results agree with previously reported studies which found that an elevated amount of chondroitin sulfate proteoglycans is associated with the existence of cancer and other disorders of the ovary [44–48]. The same trends were seen in a preliminary study performed by our group using batchwise competitive ELISA with A-1 shark, which showed that the average amount of chondroitin sulfate proteoglycans in samples from people with ovarian cancer was significantly higher than it was in samples from healthy people. However, the results in Fig. 3 also show that the levels of these specific proteoglycans obtained for various cancers overlap. Therefore, it would be necessary to consider the level of some other complementary biomarker in addition to the level of these specific proteoglycans in order to predict the specific type of cancer. In addition, a larger number of samples would be needed for an in-depth evaluation of the method and to determine a more precise cutoff value. A future study with increased numbers of samples from both the normal and each of the cancer groups is planned in order to validate the reliability of this method for routine analysis.

Nevertheless, this preliminary study illustrates the ability of the proposed system to assay for the amount of specific proteoglycans, which is a candidate biomarker for ovarian cancer. The results obtained here show the same trends as seen in the results of previous reports [44–48]. The proposed simple semi-automatic system showed good performance and yielded results that agree with those obtained using the more complicated batchwise ELISA process. The main advantages of this system are its relatively low cost, its relatively short analysis time per run, and the reduced need for highly trained personnel to perform the test in a batchwise procedure. This system would be beneficial in cases where a batch of a small-to-medium number of samples need to be tested, as well as in situations where modern medical facilities are not available.

Conclusion

A FI system with a mini-immunoaffinity chromatographic column was untilized to assay for chondroitin sulfate proteoglycans, a candidate biomarker for ovarian cancer. This approach involves indirectly quantitating the specific proteoglycans using the Bradford protein assay. The performance of the system developed here was evaluated by performing assays of chondroitin sulfate proteoglycans in human serum samples. The column could be reused over 90 times, even when the assays involved blood matrices.

The flow system offers a semi-automated capability in which the analyte can be isolated and monitored on-line. This economical system has the potential to be developed further for use as an alternative routine screening test for some proteoglycan-based biomarkers.

Acknowledgements We acknowledge the Thailand Research Fund (TRF), the Commission on Higher Education (CHE), the National Research Council of Thailand (Drugs and Chemicals Project), and the Center for Innovation in Chemistry:Postgraduate Education and Research in Chemistry(PERCH-CIC) for their support. We also thank Prof. Robert Kennedy, Prof. Jonathan Sweedler, Dr. Pises Pisespongsa, M.D., Dr. Jaroon Jakmunee, and Dr. Somchai Lapanantnoppakhun for their valuable comments.

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ANALYTICA CHIMICA ACTA 604 (2007) 139–146

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Successive determination of urinary protein and glucose using spectrophotometric sequential injection method

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ARTICLE INFO

Article history:
Received 15 August 2007
Received in revised form
5 October 2007
Accepted 8 October 2007
Published on line 13 October 2007

Keywords:
Sequential injection
Spectrophotometry
Successive determination
Urinary protein and glucose
Ion association with tetrabromophenolphthalein ethyl ester
Catalytic reaction
Screening for diabetic

ABSTRACT

A new sequential injection (SI) system with spectrophotometric detections has been developed for successive determination of protein and glucose. The protein assay is based on ion-association of protein with tetrabromophenolphthalein ethyl ester (TBPE) in the presence of Triton X-100 at pH 3.2. The blue product is monitored for absorbance at 607 nm. For glucose, hydrogen peroxide, generated by the oxidation of glucose in the presence of glucose oxidase immobilized on glass beads packed in a minicolumn, is monitored using iron-catalyzed oxidation reaction of *p*-anisidine to form a red colored product (520 nm). The SI procedure takes advantage in performing the protein assay during the incubation period for glucose oxidation. Linear ranges were up to 10 mg dL^{-1} human serum albumin (HSA) with a limit of detection (LOD) (3σ) of 0.3 mg dL^{-1}, and up to 12.5 mg dL^{-1} glucose with LOD of 0.08 mg dL^{-1}. R.S.D.s ($n = 11$) were 2.7% and 2.5% (for 1 mg dL^{-1} and 5 mg dL^{-1} HSA) and 1.4% (9 mg dL^{-1} glucose). Sample throughput for the whole assay of both protein and glucose is 6 h^{-1}. The automated system has been demonstrated for the successive assay of protein and glucose in urine samples taken from diabetic disease patients, with good agreement with the other methods. This developed SI system is an alternative automation for screening for diabetic diagnosis.

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1. Introduction

Proteins synthesized in the liver are building blocks for all body parts. Usually, most proteins are too big to pass through kidneys' filters into urine unless the kidneys are damaged. Therefore, appearance of proteins in urine is an important indicator to proteinuria. Over 30% of patients who started undergoing artificial dialysis in Japan in 2000 were the result diabetic nephropathy [1], and diabetes is closely related to kidney failure. Hence, urinary glucose, which also is an important indicator to diabetes, should be monitored for diagnosis of renal disease.

For semi-quantitative assay of protein and glucose in the clinical laboratory [2], a strip of specially treated paper with dyestuff is commonly used for convenient and rapid examination. The color appearance on the urine test paper relates to

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0003-2670/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2007.10.010

the amount of abnormal values in urine. However, it does not offer accurate analytical values and the determinable range is rough. Therefore, simultaneous and automated protein and glucose quantification in clinical diagnosis requires a method that is more accurate for screening.

Protein dye-binding assays are simple, rapid and becoming increasingly popular for the clinical determination of the total protein. Bromophenol blue was proposed for a simple spectrophotometric determination of urinary albumin [3,4]. Fujita et al. reported highly sensitive spectrophotometric methods for urinary protein with dye-metal complexes and micelle media [5–9]. Simple and rapid analytical methods with bromochlorophenol blue were proposed for protein detection [10,11]. However, the methods mentioned above are manual and consume large amounts of reagents.

Flow injection analysis proposed by Ruzicka and Hansen is a versatile and convenient technology for clinical and pharmaceutical analysis [12]. A highly sensitive flow injection method was proposed for the determination of proteins using Coomassie Brilliant Blue G-250 (CBB) [13]. Zaia et al. found that total protein obtained by using the Bradford method was not statistically different ($p > 0.05$) from the results obtained by the Biuret standard method [14]. However, a short linear range for the calibration graph was offered by this method.

Sakai et al. have reported that tetrabromophenolphthalein ethyl ester (TBPE) anion has a larger molar absorptivity (10^5 L mol^{-1} cm^{-1}), comparing with bromophenol blue and bromocresol green [15]. Fortunately, it was found that TBPEH dissolved in the micelle formed by adding Triton X-100. Consequently, it is possible to use TBPEH as a chromogenic reagent for the protein–TBPE associate formation. In a previous paper [16], TBPEH in the micelle was applied to the FIA procedure for the protein determination at less than 1 mg dL^{-1} level.

Glucose can be determined using *o*-toluidine by batchwise [17] and/or on-line procedure [18], but it requires heating. Glucose oxidase is widely used for quantitative analysis of glucose in body fluids [19,20] due to selectivity, but it is a tedious method.

Flow injection determination of glucose using glucose oxidase (GOD) immobilized on controlled porosity glass (CPG) was reported [21–23]. Hydrogen peroxide generated by the oxidation of glucose in the presence of GOD was monitored using an iron-catalyzed oxidative coupling of 4-aminoantipyrine with *N*,*N*-dimethylaniline [23]. While FIA shows some advantages for practical analyses, because of the continuous flow of the reagents it still consumes relatively high amounts of reagents, resulting in higher amount of laboratory waste in comparison to the second generation, sequential injection analysis (SIA).

We recently found that vanadium acted as a catalyst for the oxidation of *p*-anisidine by bromate [24]. Furthermore, preliminary experiments in this paper reveal that iron has a catalytic effect on the oxidation of *p*-anisidine by hydrogen peroxide and that this catalytic reaction is useful for an alternative indicator reaction of glucose. As a result, we propose here a new automated sequential injection method for successive determination of protein with TBPE and glucose based on the new catalytic reaction by iron in urinary samples. This should be useful to diagnostic screening for diabetic disease.

2. Experimental

2.1. Reagents

All chemicals used were of analytical-reagent grade and were used without further purification. The DI water used throughout the experiments was purified by an Advantec GSH-210 apparatus.

Stock standard solutions were of human serum albumin (HSA) (100 mg dL^{-1}) using MW: 66,000, Seikagaku Kogyo and glucose (180 mg dL^{-1}) prepared by dissolving D-(+)-glucose (Nakarai Chemicals Co., Osaka) in water with standing for at least 12 h before use, to ensure mutarotation equilibrium. Working standard solutions were obtained by mixing the HSA and glucose stock solutions for appropriate concentrations.

Stock solutions of TBPE (1.0×10^{-3} M) and Triton X-100 (0.5%, w/v) were prepared by dissolving tetrabromophenolphthalein ethyl ester potassium salt (MW: 700, Tokyo Kasei, Tokyo) in ethanol and *t*-octylphenoxypolyethoxyethanol (Sigma Chemical) in water, respectively.

A *p*-anisidine solution (0.1 M) was prepared by dissolving *p*-anisidine (Wako Pure Chemical Co., Japan) in water.

An iron(II) stock solution (0.1 M) was obtained by dissolving iron(II) sulphate (MW: 278.01, Wako Pure Chemical Co.) in 0.005 M sulfuric acid.

Buffer solutions were prepared by adding acetic acid in 0.5 M sodium acetate solution to adjust pH values to be 3.2 and 4.5 for protein and glucose assay, respectively.

Controlled porosity glass beads (200–400 mesh size, 182 Å mean pore size) were obtained from Sigma Chemical Company. Glucose oxidase (E.C.1.1.3.4) from *Aspergillus sp.* (200 U mg^{-1}) was obtained from Roche Diagnostics, USA. A working solution of glucose oxidase was obtained by dissolving the enzyme in phosphate buffer (4 °C, pH 7.0) and with suitable dilution. Aqueous aminoalkylating agent (10%) was prepared by adding 5.2 mL of 3-aminopropyltriethoxysilane (99%, Acros organics) to 50 mL of water and the pH was carefully adjusted to 3.45 with hydrochloric acid. The cross-linking agent (2.5%) was prepared by adding 2.5 mL of glutaraldehyde (50%, Kanto Kagaku Co., Japan) to 50 mL of water.

2.2. Apparatus

A schematic diagram of the SI system for successive analysis of protein and glucose in urine samples is shown in Fig. 1. The flow lines were of Teflon tubings (0.5 mm inner diameter). Flows of the carrier and reagent were controlled by a syringe pump (FIAlab instruments, USA) and a 10-port selection valve (C25-3180EMH, Valco instruments Co. Inc.). The enzymatic column was immersed in circulating thermostated water bath at 37 °C (Thermo minder SM-05, Taitec, Japan). Absorbance was monitored at 607 nm for protein determination and at 520 nm for glucose analysis. The detection unit consisted of a tungsten halogen light source (LS-1, FIAlab Instruments, USA) and a spectrometer (USB2000, Ocean Optics, USA) and fiber optic cables (P200-2-UV/Vis, FIAlab instruments, USA) and Z-cell (home made, path length 10 mm). The FIAlab software was used to control the system and some signal processing. Peak evaluation was made by using the Origin software with man-

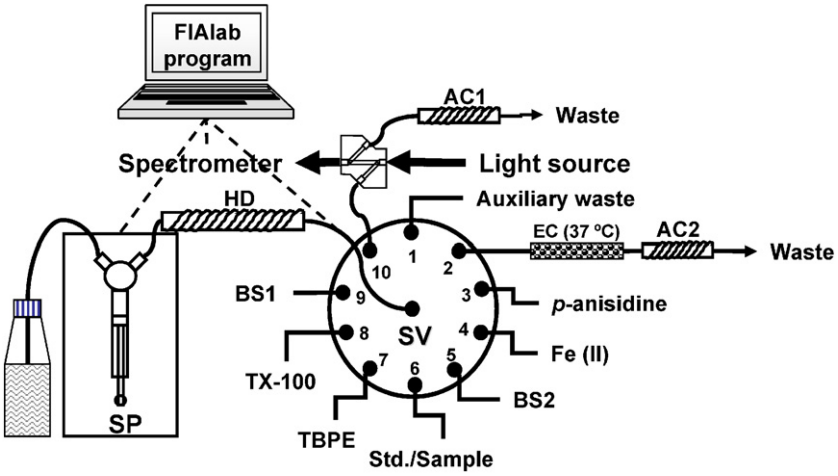

Fig. 1 – Manifold of the SI system for successive determination of protein and glucose in urine samples. HD: holding coil, EC: enzyme column (4.5 cm × 2 mm), AC: auxiliary coil, SV: 10-port selection valve, SP: syringe pump, BS1: 0.5 M acetate buffer pH 3.2, BS2: 0.5 M acetate buffer pH 4.5, TX-100: 0.1% Triton X-100, TBPE: 1 × 10^{-4} M tetrabromophenolphthalein ethyl ester, Fe(II): 5 × 10^{-3} M iron(II), *p*-anisidine: 0.1 M *p*-anisidine.

ual operation for each peak: peak area for protein while peak height for glucose.

2.3. *Procedure*

2.3.1. *Immobilization of enzyme on glass beads*

The immobilization of enzyme was performed by following procedures described in previous reports [21–23]. A 5 g amount of CPG was boiled in 300 mL of 5% nitric acid for 30 min. The CPG was washed with water and dried in an oven at 95 °C for 12 h. After that, 10% of 3-aminopropyltriethoxysilane solution (pH 3.45) was added to the dried glass beads with a ratio of 20 mL to 1.0 g of the glass. The mixture was left at 75 °C in a water bath for 2.5 h. The chemically modified alkylamino glass was washed and dried in an oven at 115 °C for 8 h. After that, the alkylamino glass (1 g) was added to 5 mL of an aqueous glutaraldehyde solution (2.5%) and was kept under reduced pressure for 30 min and then kept under atmospheric pressure for 2 h. The activated glass beads were washed with water. Glucose oxidase (100 mg) was dissolved in a cold phosphate buffer (0.1 M, pH 7.0, 4 °C) and added to the activated glass beads. The mixture was kept at 4 °C for 2.5 h. The immobilized enzyme derivative was washed with cold phosphate buffer and cold water to remove unlinked enzymes that remained

Table 1 – Operation sequence of SI system for successive determination of protein and glucose (see full protocol in ESI)

No.	Operation sequence of the SI method	Remark
1	Aspiration of buffer and glucose solution	Incubation of glucose
2	Aspiration of 0.5 M acetate buffer pH 4.5	
3	Aspiration of stand/sample solution	
4	Aspiration of 0.5 M acetate buffer pH 4.5	
5	Dispensation of all reagents to the enzymatic column	
6	Aspiration of 0.5 M acetate buffer pH 3.2	Determination of protein
7	Aspiration of 0.1% Triton X-100	
8	Aspiration of 1 × 10^{-4} M TBPE	
9	Aspiration of 0.5 M acetate buffer pH 3.2	
10	Flow several process for mixing	
11	Aspiration of protein standard/sample	
12	Flow reversal process for mixing	
13	Monitoring of product	
14	Cleaning the holding coil and flow cell	Cleaning the system
15	Aspiration of 0.5 M acetate buffer pH 4.5	Determination of glucose
16	Aspiration of 5 × 10^{-3} M iron(II)	
17	Aspiration of 0.1 M *p*-anisidine	
18	Aspiration of 0.5 M acetate buffer pH 4.5	
19	Flow reversal for mixing	
20	Aspiration of peroxide solution from the enzymatic column	
21	Flow reversal for mixing of reagents	
22	Monitoring of product	
23	Washing the holding coil, flow cell and enzymatic column	Cleaning the system

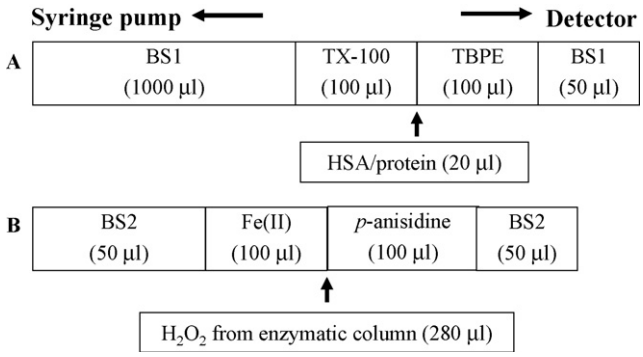

Fig. 2 – The sequence of reagents for protein and glucose determination. The concentrations of all reagents are defined in Fig. 1. (A) for protein determination, (B) for glucose determination.

on the glass beads. The immobilized glass beads were packed into a Teflon tube (4.5 cm × 2 mm). The two ends of the column were plugged with cotton filters and stored at 4 °C in water. The activity of enzyme can be kept for at least 1 month.

2.3.2. *Procedure for the successive determination of protein and glucose*

Using the manifold shown in Fig. 1, the system was filled with water as a carrier. The enzymatic column was controlled at 37 °C (temperature in human body). The home made Z-cell (10 mm path length) was connected to port#10 of a selection valve with 5 cm tubing to minimize dispersion of product zone. The auxiliary coils 1 and 2 were set to protect from waste and air flow to the flow cell and the enzymatic column, respectively. Sequences for SI operation are summarized and illustrated in Table 1 and Fig. 2.

To start the glucose determination process, 20 μL of 0.5 M acetate buffer solution of pH 4.5 (BS2), 280 μL of glucose standard solution or sample and 50 μL of 0.5 M acetate buffer solution (pH 4.5) were dispensed to the enzymatic column through port#2 for incubation during the protein analysis process. The protein determination started with aspirating the following into the holding coil (Fig. 2A): 1000 μL of 0.5 M acetate buffer solution of pH 3.2, 100 μL of 0.1% Triton X-100, 100 μL of 1×10^{-4} M TBPE and 50 μL of 0.5 M acetate buffer solution of pH 3.2. Flow reversal was made to promote mixing of the reagents via the flow cell port of the selection valve. Then, the standard human serum albumin (HSA) or sample solution was inserted into the micelle mixture solution. The blue color product zone that occurred after the second-flow reversal process was transferred to detection unit for absorbance monitoring at 607 nm. At the end of this process, the system was washed with water. Then, the glucose determination step restarted again by aspirating 0.5 M acetate buffer (pH 4.5), 5×10^{-3} M iron(II) and 0.1 M *p*-anisidine solutions as the sequence in Fig. 2B. Flow reversal was made before inserting the peroxide produced from the enzymatic column into the center of mixture. The red product formed was then transported to the detector.

3. Results and discussion

3.1. *Detection reactions*

For protein, as proposed by Sakai et al. for the micelle extraction of TBPE-protein into Triton X-100 at pH 3.0 [16], the color development occurs according to the following Eqs. (1) and (2).

$$\underset{\text{Yellow}}{\text{TBPE}\cdot\text{H}} + \underset{\text{colorless}}{\text{TX-100}} \rightleftharpoons \underset{\text{Yellow}}{(\text{TBPE}\cdot\text{H})_m} \quad (1)$$

The protein in acid solution (HSA^{x+}) would be associated into the micelle:

$$\underset{\text{Yellow}}{x(\text{TBPE}\cdot\text{H})_m} + \text{HSA}^{x+} \rightleftharpoons \underset{\text{Blue}}{(\text{HSA}^{x+}\cdot(\text{TBPE}^-)_x)_m} + x\text{H}^+ \quad (2)$$

The maximum absorption wavelength of the TBPE–protein associate was found to be at 607 nm.

The detection reactions for glucose determination by the enzymatic method are as follows:

$$\text{D-glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{GOD}} \text{D-glucono-1, 4-lactone} + \text{H}_2\text{O}_2 \quad (3)$$

$$\text{H}_2\text{O}_2 + p\text{-anisidine} \xrightarrow{\text{Fe(II)}} \text{Red color product} + \text{H}_2\text{O} \quad (4)$$

GOD promotes the oxidation of D-glucose by molecular oxygen to D-glucono-1,4-lactone [25]. Hydrogen peroxide is produced simultaneously. The hydrogen peroxide obtained from the oxidation of glucose oxidizes *p*-anisidine to a red color compound in the presence of iron(II) used as a catalyst.

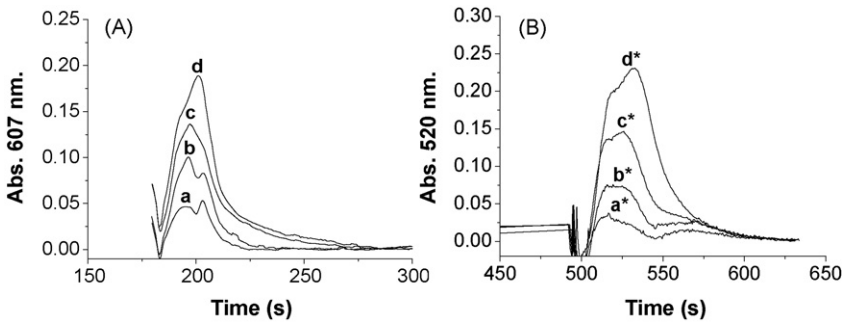

Fig. 3 – Peak profiles due to (A) protein, HSA: (a) blank, (b) 0.5 mg dL^{-1}, (c) 1.0 mg dL^{-1}, (d) 2.0 mg dL^{-1}; (B) glucose: (a*) blank, (b*) 1.8 mg dL^{-1}, (c*) 5.4 mg dL^{-1}, (d*) 12.6 mg dL^{-1}.

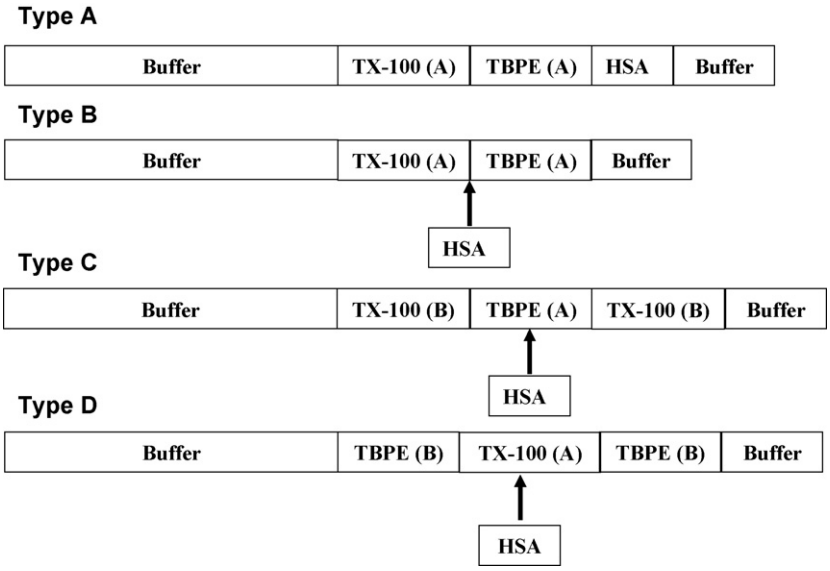

Fig. 4 – The sequences of the reagents for protein assay by the SI system. Buffer: 500 μL of 0.5 M acetate buffer at pH 3.2, TX-100(A) and TX-100(B): 50 and 25 μL of 0.1% TX-100, respectively, TBPE(A) and TBPE(B): 50 and 25 μL of 1×10^{-4} M TBPE, respectively, HSA: 20 μL of human serum albumin standard solution.

The maximum absorption wavelength of the red color product was at 520 nm.

3.2. *Peak profiles*

According to the two detecting steps in the successive determination of protein and glucose, first, the protein–TBPE associate was formed and monitored at 607 nm (Fig. 3A). Then in the steps for glucose determination, the oxidized form of *p*-anisidine was monitored at 520 nm (Fig. 3B). The detection steps for protein and glucose can be performed independently with the ability of the spectrometer to measure absorbances of the monitored products simultaneously, even in two different wavelengths.

3.3. *Variables in the determination of protein*

3.3.1. *Aspiration sequence*

The aspiration sequence of reagents into the SI system is important for mixing and consequent reaction steps. Four different aspiration sequences of reagents (types A, B, C and D) were tried, as illustrated in Fig. 4. Experiments using HSA (0–8 mg dL^{-1}), 20 μL; 1×10^{-4} M TBPE, 50 μL; 0.1% TX-100, 50 μL and the buffer solution (pH 3.2) were performed. Type B sequence was selected as it yielded small standard deviation, reproducibility and a good linear calibration graph.

3.3.2. *Effect of TBPE concentration*

The effect of TBPE concentration was examined in a range of 8×10^{-5} M to 1.5×10^{-4} M. It was found that the slope of the calibration graph was practically constant for TBPE concentrations in a range of 1×10^{-4} to 1.5×10^{-4} M. The slope was found to be less when TBPE concentration was less than 1×10^{-4} M. TBPE of 1×10^{-4} M was selected for further study.

3.3.3. *Effect of Triton X-100 concentration*

The effect of TX-100 concentration (0.05–0.13 %, w/v) on the sensitivity of the system for the protein determination was studied. A concentration of 0.09% (w/v) was suitable for the detection.

3.3.4. *Effect of pH of buffer*

In this study, pH of the 0.5 M of acetate buffer was optimized for protein detection. The effect of pH in the range of 2.7–4.8 on the 5 mg dL^{-1} HSA is presented in Fig. 5. Signals increased slightly when pH of the buffer increased. However, the blank signals also increased when pH increased. The net signals (subtraction for blank) were considered. It can be seen that the net signals become constant starting from pH 3.2. Therefore, the suitable pH for protein-detected reaction should be 3.2.

3.3.5. *Flow reversal*

For the protein determination, there are two steps employing flow reversal, one before and one after protein solution was

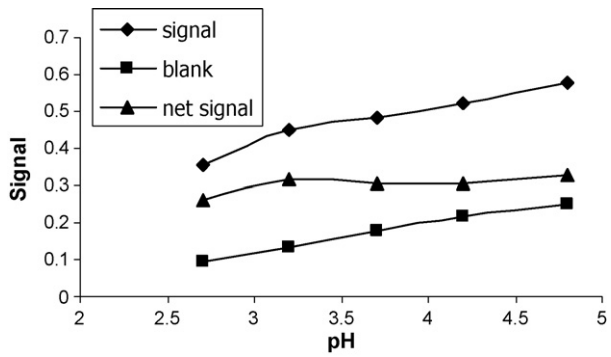

Fig. 5 – Effect of pH of 0.5 M acetate buffer on protein determination, 5 mg dL^{-1} HSA 20 μL, (■) blank signal, (♦) 5 mg dL^{-1} HSA, (▲) net signal, other conditions as in Fig. 1.

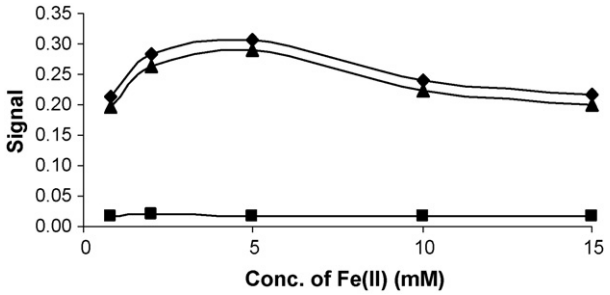

Fig. 6 – Effect of iron(II) concentration on glucose assay. 80 μL of 8 × 10^{-4} M H_2O_2 and 100 μL of iron(II) at various concentration. (■) Blank signal, (♦) 8 × 10^{-4} M H_2O_2, (▲) net signal. Other condition as in Fig. 1.

aspirated into the system. It was found that flow reversal for both the steps did not enhance signals. So, only one direction of flow should be implemented.

3.4. *Variables on the determination of glucose*

3.4.1. *Effect of iron(II) concentration*

Iron(II) solution was used as a catalytic reagent for the oxidative reaction of *p*-anisidine and hydrogen peroxide. The effect of concentration of iron(II) (8 × 10^{-4} M to 1.5 × 10^{-2} M) was studied. The results in Fig. 6 indicate that 5 × 10^{-3} M iron(II) should be chosen.

3.4.2. *Effect of p-anisidine concentration*

The effect of concentration of *p*-anisidine (0.02–0.15 M) was studied. The absorbance increased with increasing concentration of *p*-anisidine until 0.1 M and became constant over 0.1 M. Therefore, a 0.1 M of *p*-anisidine was selected.

3.4.3. *Effect of pH of buffer solution*

The effect of pH on the formation of colored product was investigated in the range pH 4.0–5.7 using 0.5 M acetate buffer. It was found that the slope of calibration obtained by using buffer of pH 4.5 was the highest. Hence, pH 4.5 was chosen.

Table 2 – The recoveries of successive determination of 3 mg dL^{-1} HSA and 5.4 mg dL^{-1} glucose in a real sample urine using various dilution factors.

Dilution factor	%Recovery	
	Protein	Glucose
10	89	52
20	100	55
30	97	74
50	98	90
60	101	100

3.4.4. *Standard/sample volume*

Sample volume loaded into the enzymatic column was studied over the range of 40–320 μL. The peak height for glucose increased with increasing sample volume up to 280 μL. The peak height became constant for a volume of 280 μL or over. Therefore, a sample volume of 280 μL was selected.

3.5. *Analytical characteristics*

Calibration graphs for protein and glucose were established under the proposed conditions: [*peak area*] = 0.747 [*mg dL*$^{-1}$ *HAS*] + 0.673, r^2 = 0.999 for 0–10 mg dL^{-1} HSA with LOD (3σ) of 0.3 mg dL^{-1} and R.S.D.s (*n* = 11) were 2.7% and 2.5% for 1 and 5 mg dL^{-1} HSA, respectively; and [*peak height*] = 0.0106 [*mg dL*$^{-1}$ *glucose*] + 0.0275, r^2 = 0.998 for 0–12.5 mg dL^{-1} glucose with LOD (3σ) of 0.08 mg dL^{-1} and R.S.D. of 1.4% (9 mg dL^{-1} glucose; *n* = 11). The sample throughput was six samples h^{-1} for successive analyses.

3.6. *Interference study*

For the determination of protein and glucose, various amounts of foreign compounds were added and their interference was examined. Sodium chloride, creatinine and urea were considered as they are commonly coexisting in urine. The average concentration levels of Na^+, Cl^-, creatinine and urea in an ordinary person's urine were reported to be 294 mg dL^{-1} (128 mEq L^{-1}), 478 mg dL^{-1} (134 mEq L^{-1}), 196 mg dL^{-1} and 1820 mg dL^{-1}, respectively, although the values may be differ-

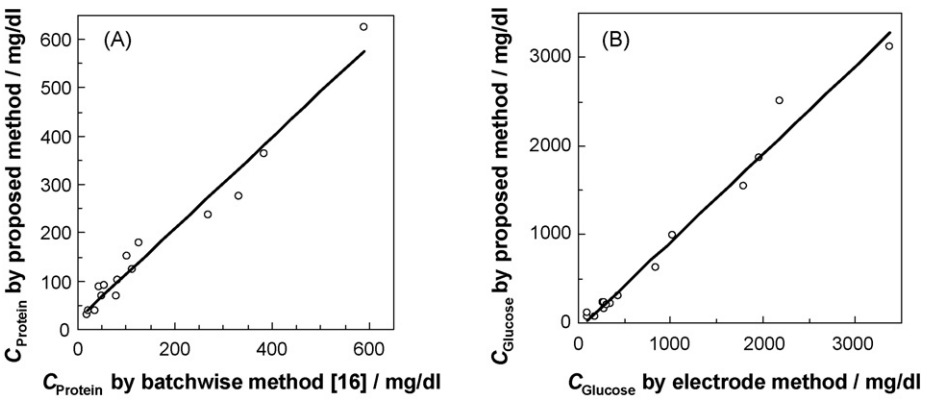

Fig. 7 – Correlations between the results obtained by the proposed method and other methods (see the text) for the determinations of (A) urinary protein and (B) glucose. (A) y = 0.942x + 20.6, r^2 = 0.967 (*n* = 15); (B) y = 0.991x − 74.3, r^2 = 0.980 (*n* = 15).

ent from person to person and/or condition to condition [26]. Sodium chloride and ceatinine did not interfere in the protein determination up to 75 mg dL^{-1} (29 mg dL^{-1} for Na^+ and 46 mg dL^{-1} for Cl^-) and 100 mg dL^{-1}, respectively, when defining as a maximum concentration of foreign species that causes a deviation of less than ±5%. Urea up to 1820 mg dL^{-1} did not interfere. For enzymatic glucose determination, uric acid and ascorbic acid were reported to compete with a reduced chromogen as hydrogen donors [27,28]. Uric acid and ascorbic acid are usually found to be about 42 mg dL^{-1} (2×10^{-3} M) and 3.7 mg dL^{-1} (2.1×10^{-5} M) in normal urine. It was found that uric acid up to 2 mg dL^{-1} (1.2×10^{-4} M) and ascorbic acid up to 0.25 mg dL^{-1} (1.4×10^{-5} M) did not interfere for the determination of 9 mg dL^{-1} glucose. In order to overcome the possible interferences discussed above, dilution of urine sample should be made so that the concentration of interfering species would become lower than their limits that would interfere.

A urine sample containing 3 mg dL^{-1} HSA and 5.4 mg dL^{-1} glucose with various dilution factors was studied for recovery to verify the approach. The results (Table 2) indicate that dilution of at least 60 folds would overcome the possible interference from the species. The R.S.D.s of this manipulation were higher than that of the standard solutions of the same concentrations without dilution.

3.7. *Applications to real samples*

The proposed SI method was applied to determine successively protein and glucose in 15 urine samples taken from diabetic disease patients in a group of different ages. The samples were filtered by filter paper (Whatman#1) and were diluted at least 60-fold with water before aspirating directly into the proposed SI system. Concentrations of protein and glucose were evaluated from the calibration equations. The results are shown in Fig. 7. The protein contents obtained by the proposed SI method agree with those by the batch-wise method [16] with correlation of $y = 0.942x + 20.6$, $r^2 = 0.967$. The correlation of glucose concentration obtained by the proposed SI method and that of a routine assay using a commercial Glucoroder GXR with an oxygen electrode (A&T Co., Yokohama) was: $y = 0.991x - 74.3$, $r^2 = 0.980$. The experimental *t*-values between the two couples of methods were 0.291 for protein and 1.77 for glucose. These *t*-values were less than the *t*-value (2.145) for 14 degrees of freedom at the 95% confidence level.

For medical checkup, protein is generally assayed in the range from 15 to 1000 mg dL^{-1} while the range for glucose is from 50 to 2000 mg dL^{-1}. Healthy subjects give concentration levels of protein and glucose less than 15 and 50 mg dL^{-1}, respectively. The proposed method would be employed for the assays of protein and glucose with suitable dilution.

4. Conclusions

A new automatic sequential injection system with spectrophotometric detection for successive determination of protein (0–10 mg dL^{-1}) and glucose (0–12.5 mg dL^{-1}) was developed. The protein assay is based on ion-associate formation with TBPEH, while the glucose assay is based on the detection of hydrogen hydroxide produced from glucose oxidase, using *p*-anisidine with iron-catalyst. With automation using SI, protein assay can be performed during the glucose oxidation incubation period. The developed system has been demonstrated for the assay of protein and glucose in diabetic patient urine samples with good agreement with the other methods. This new automated SI system should be an alternative for clinical routine assay for diabetic disease screening.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research No. 17550087 (to T.S.) from Japan Society of the Promotion of Science. K.W. and K.G. acknowledge The Thailand Research Fund (TRF) for the Royal Golden Jubilee Ph.D. program and The Commission on Higher Education for the Research Group Grant and The Postgraduate Education and Research Program in Chemistry: Center of Innovation in Chemistry (PERCH-CIC) for financial support. We thank Professor Gary D. Christian for editing the English.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2007.10.010.

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Topics in Chemical Instrumentation

edited by
David Treichel
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Illustrating Some Principles of Separation Science through Gravitational Field-Flow Fractionation

W

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Field-flow fractionation (FFF) is an elution-based separation method analogous in many ways to liquid chromatography. The FFF concept was proposed in 1966 by the late J. Calvin Giddings *(1)* and the principles were outlined in an article in this *Journal* in 1973 *(2).* FFF has been extensively used for separating and sizing particles and macromolecules *(3–7)* and has been applied to characterize a wide variety of samples including polymers *(8),* soils *(9),* river suspended matter *(10),* bacteria *(11),* starch *(12),* proteins *(13),* blood *(14),* and milk *(15).* One significant application for FFF is to determine the size-based element speciation of particulate samples *(16–21).* This involves determining the element concentration distribution across the size range of the particles.

FFF is an emerging analytical technique as evidenced by the fact that recently about 50–60 articles have been published each year in the scientific journals. There have also been several detailed reviews written on FFF aimed at researchers and there have been two books on the subject, the most recent being the *Handbook of FFF (22).* However, since Giddings's article in this *Journal* in 1973 *(2)* there have been no articles specifically aimed at educators. There are also few textbooks that mention the technique, notable exceptions being Hunter's *Introduction to Modern Colloid Science (23)* and the text *Fundamentals of Analytical Chemistry* by Skoog, et al. *(24).*

FFF separations are based on a combination of physical principles involving the force induced on particles by an applied field or gradient and hydrodynamic laminar flow in a thin unpacked channel. Different fields and gradients can be employed in FFF such as centrifugal, electrical, magnetic, fluid crossflow, thermal gradient, and acoustic standing waves. In addition back diffusion away from the accumulation wall in response to the concentration gradient may also be important. In the theory and experiments described in this article the earth's gravitational field will be utilized and the particles will be too large for concentration diffusion, which causes particle transport along a concentration gradient by Brownian motion, to be significant.

Commercial FFF equipment is available but, like HPLC and GLC, it is relatively expensive *(25, 26).* In this article we outline the construction of a simple gravitational FFF (GrFFF) system that can be made for a few hundred dollars. Many of the parts and components required are similar to those utilized for simple cost effective flow injection analysis experiments *(27).* Thus it is possible to introduce this new technique fairly simply and inexpensively. There is a disadvantage: this simple apparatus is only applicable for a limited particle diameter range of about 1–50 μm.

A major objective of the theory and laboratory exercises described in this article is to illustrate some general concepts of physical chemistry and separation science through gravitational field-flow fractionation. A secondary objective is to introduce the emerging technique of FFF to the undergraduate curriculum. The article outlines material suitable for presenting in the classroom and suggests laboratory exercises that can be given as a standard practical exercise or an expanded project. A tested undergraduate experiment along with an instructor's manual are available in the Supplemental Material.[W]

The FFF Separation Principle

FFF Instrument

The architecture of an FFF instrument is similar to liquid chromatography (LC) with the essential components being a pump, sample injection port, FFF separation channel, detector, and chart recorder or computer (Figure 1). The difference between FFF and LC is the mechanism of separation occurring in the channel. In most forms of LC differential migration of sample components is caused by chemical interaction with a stationary phase. Exceptions include size-exclusion chromatography (SEC) and hydrodynamic chromatography (HDC). In this respect FFF is more similar to SEC and HDC as selective migration in the channel is purely due to physical effects.

FFF Channel

The FFF channel is a thin open (i.e., unpacked) rectangular ribbon with triangular inlet and outlet end pieces as illustrated in Figure 2D. Typical dimensions are 20–50 cm × 1–2 cm × 0.1–0.5 mm. The method of construction is illustrated in Figure 2A–C and is described in the section on GrFFF instrumentation below.

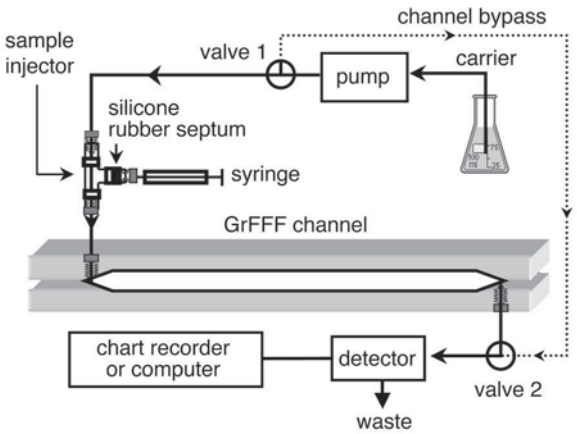

Figure 1. Schematic diagram of a GrFFF system showing the channel and the auxiliary equipment. Arrows indicate the flow directions in different positions.

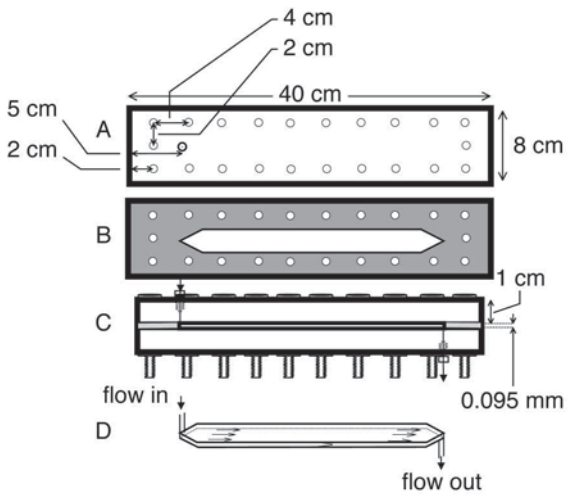

Figure 2. Construction of a gravitational FFF channel (A) lucite blocks, (B) plastic spacer sheet, (C) assembled channel, and (D) schematic representation of the flow channel

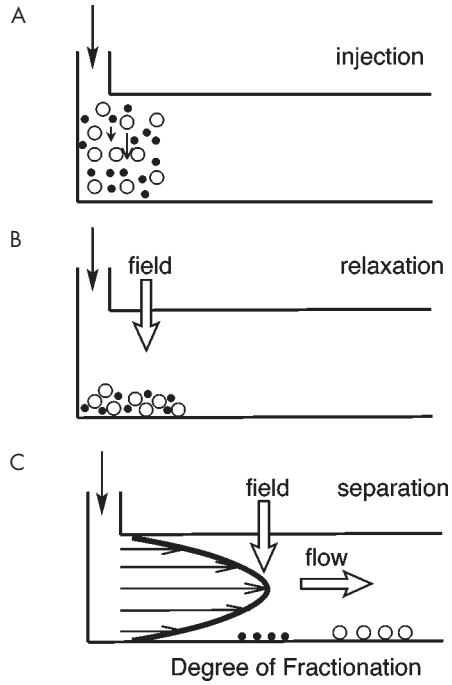

Figure 3. Cross-section view of the inlet end of the GrFFF separation channel showing the steps involved (A–C) in the separation mechanism.

Field Relaxation of the Sample

After injection of a sample suspension at one end of the channel the flow is usually turned off while the field is on. This allows the field to force the sample particles across the channel to the accumulation wall in a process referred to as sample relaxation (Figure 3). It may take only a few seconds or much longer (up to 1 hour) depending on the field strength and its interaction with the particles through the appropriate properties of the particles. For larger particles the relaxation time may be small compared to the sample run time and the relaxation step may be omitted from the experimental procedure.

Sample Relaxation Time

The relaxation time (t_{relax}) must be sufficient for the smallest particles in the sample starting at the top wall to settle to the bottom wall (i.e., a distance equivalent to the channel thickness). By transposing the Stokes settling velocity equation for a gravitational field we obtain

$$t_{relax} = Uw = \frac{18\eta w}{g\Delta\rho d^2} \quad (1)$$

where U is the settling speed, w is the channel thickness, η is the viscosity of fluid, g is the gravitational acceleration constant, $\Delta\rho$ is the density difference between the particle and the fluid, and d is the equivalent spherical particle diameter.

Laminar Fluid Flow in the FFF Channel

Following the relaxation period the carrier flow is initiated. Owing to the thin channel, non-turbulent laminar flow results and the velocity profile is parabolic across the channel, which is shown in Figure 3C. The fluid has maximum velocity at the center of the channel and is essentially zero at each wall. The fluid velocity profile is given by the quadratic equation

$$v(x) = 6\langle v\rangle\left(\frac{x}{w} - \frac{x^2}{w^2}\right) \quad (2)$$

where $v(x)$ is the velocity at distance x from the lower accumulation wall and $\langle v\rangle$ is the mean velocity of the fluid.

For the region very close to the channel wall the second term can be neglected (i.e., $(x/w)^2 << (x/w)$), thus for $x << w$ equation 2 becomes

$$v(x) \approx \frac{6\langle v\rangle x}{w} \quad (3)$$

It can be shown that for flow in a thin rectangular channel

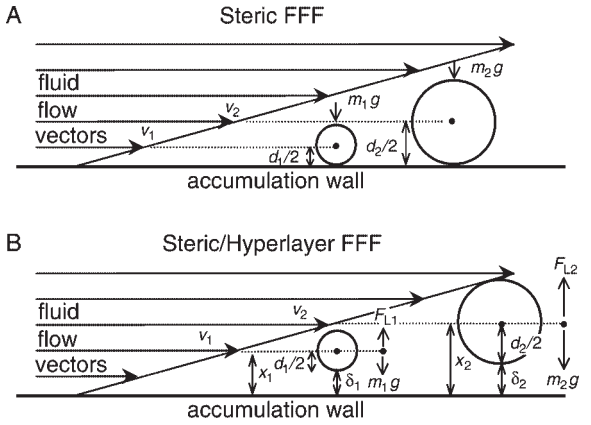

Figure 4. Two different modes of separation mechanism in GrFFF: (A) hypothetical steric FFF mechanism and (B) actual steric/hyperlayer on lift mode of FFF.

that the mean linear velocity is $\langle v \rangle$ related to the volumetric flow rate $\dot{V}$ and channel dimensions by,

$$\langle v \rangle = \frac{\dot{V}}{b\,w} \tag{4}$$

where b is the channel breadth and bw is thus the cross sectional area. (Note in SI units $\dot{V}$ should be expressed in $m^3\ s^{-1}$ but it is more common to record it as mL min^{-1}. If we use units of cm and mL min^{-1} then the linear velocity will be expressed in cm s^{-1}.)

Steric FFF Separations

After relaxation the particles are pushed close to the accumulation wall as shown in Figure 3B. For particles in the micrometer size range, back diffusion effects can be ignored. A simplified model for the separation of particles according to size is illustrated in Figure 4A. As an approximation the particles can be imagined to be pushed along by the fluid velocity vector acting at its center (i.e., at a distance $d/2$ above the accumulation wall). Thus from eq 2 this velocity will be

$$v_r = 6\langle v \rangle \left(\frac{d}{2\,w} - \frac{d^2}{4\,w^2} \right) \tag{5}$$

or using eq 3, then

$$v_r \approx \frac{3\langle v \rangle d}{w} \tag{6}$$

This indicates that the retention volume or time required for a given component to elute should decrease as the particle size increases (Figure 4A).

Unfortunately this simple model is not accurate owing to a number of additional effects not taken into account. These perturbing influences include wall attractive and repulsive forces, near wall viscous drag, and hydrodynamic lift forces *(28–30)*. This can be accounted for empirically by including a correction factor γ yielding

$$v_r = \frac{3\langle v \rangle \gamma\, d}{w} \tag{7}$$

Processes pushing the particles away from the wall result in faster elution and $\gamma > 1$, whereas attractive wall forces and additional drag slows the particle migration along the channel and will result in $\gamma < 1$.

Hydrodynamic Lift Forces

When particles in the close vicinity of the accumulation wall are pushed along the channel by the fluid flow they experience an upward lift force owing to the increased pressure in the liquid film between the particle and the wall. This hydrodynamic lift force has been discussed in more detail by Williams et al. *(29, 30)* and interested students could be referred to this more advanced treatment. The lift force (F_L) decreases as the distance between the particle and the wall increases *(30)*. At equilibrium the particles are elevated a distance δ above the wall, where the gravitational force (mg) is balanced by the lift force (F_L) as illustrated in Figure 4B.

Steric-Hyperlayer or Lift Mode of FFF

The separation mechanism depicted in Figure 4B is referred to as the steric-hyperlayer or lift mode of FFF. The particles are forced into equilibrium hyperlayers and are swept down the channel by the corresponding fluid flow vectors.

As the channel flow increases, the particles become more elevated because the magnitude of the lift force increases. As discussed above the particle velocity, and hence the retention time and volume, will depend on the distance of the particle center above the wall, which for particles in these hyperlayers will be at position x_{eq} given by

$$x_{eq} = \delta + \frac{d}{2} \tag{8}$$

where δ is the distance of closest approach between the particle surface and the accumulation wall. Thus the retention volume of a given particle is not constant but decreases with increasing flow rate. The exact relationship involves the complex hydrodynamic lift force. Consequently eqs 5 or 6 cannot be used for quantitative determination of particle diameter from the measured peak position and calibration with size standards of the same particle density is required at each flow rate as will be discussed later.

Retention Ratio

As with chromatography, retention of a sample is usually expressed in terms of the dimensionless retention ratio, R,

$$R = \frac{v_r}{\langle v \rangle} = \frac{t^\circ}{t_r} = \frac{V^\circ}{V_r} \tag{9}$$

where v_r is the migration velocity of a retained sample component, t° is the void time or elution time for an unretained component, V° is the void volume or channel volume, t_r is the retention time for a sample component, and V_r is the retention volume for sample. From eq 7,

$$R = \frac{3\gamma\, d}{w} \tag{10}$$

However, as indicated above, γ is an empirical factor that incorporates several phenomena.

Particle Elevation Parameters x_{eq} and δ

Transposing the approximate expression for $v(x)$ (eq 3) and substituting eq 9, the elevation of the particle center above the wall is

$$x_{eq} = \frac{w\,v(x)}{6\,\langle v\rangle} = \frac{wR}{6} \qquad (11)$$

Note that when dealing with particle migration if we ignore fluid slip effects, the fluid velocity $v(x)$ in eq 3 becomes approximately the particle velocity (v_r). Thus the measured retention ratio can be used to estimate approximate value of x_{eq} from eq 11 and if the particle diameter is known, δ is obtained through eq 8.

This simplified theory will not apply when the particles are forced very close to the channel wall as attractive and repulsive interactions can influence the exact position of the hyperlayer. In addition near wall viscous drag effects can cause fluid slip and retard the particle motion along the channel resulting in longer elution times than predicted by these approximate equations. In this case even negative values of δ can be obtained, which is obviously an unrealistic result.

Gravitational FFF Instrumentation

A brief summary is given here and details can be found in the Supplemental Material.[W]

Channel Construction

Two 1 cm thick lucite blocks (approximate size 40 × 8 cm) are cut and two holes were drilled and tapped to take a standard flange-free nut for connecting 1/16 inch o.d. polymeric tubing to the inlet and outlet of the channel. A thin plastic sheet is cut as shown in Figure 2B (≈0.1–0.5 mm thick) and clamped between the two lucite blocks to the channel. An overhead transparency sheet is suitable and generally thinner channels result in better resolution. For the channel used to obtain the results given here w = 0.095 mm and $V°$ = 0.57 mL.

Ancillary Equipment

HPLC pumps and UV–vis detectors are ideal for FFF although cheaper options are available *(31).* Simple septum injector systems can be used in place of more expensive LC injection valves *(27, 32).* Data capture is achieved with a chart recorder or a computer.

Samples

Various particle samples are suitable for GrFFF experiments for student exercises. Perhaps the best are monodisperse silica obtained from used HPLC columns. Other samples used in this article are polydisperse silica and silica gel, which can be fractionated into desired size ranges by gravitational settling (detailed methods are in the Supplemental Material[W]).

In the experiments described in this article, normal phase monodisperse chromatographic silica was obtained from used HPLC columns (CN packing from Partisphere RTF Columns, Whatman, United Kingdom). The 5 μm particles were spherical and the 10 μm particles had somewhat irregular shapes. A particle suspension was prepared in deionized water at a concentration of about 2 mg mL^{-1}.

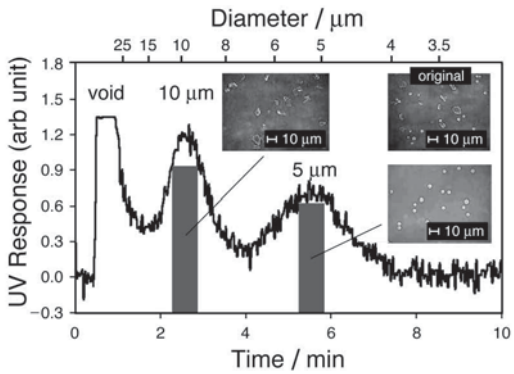

Figure 5. GrFFF fractogram of a mixture of 5 μm and 10 μm silica particles and the optical microscope pictures of the original mixture and fractions collected from the shaded regions at the two peak maxima. The flow rate was 1.0 mL min^{-1}.

Polydisperse silica and silica gel 60G (5–40 μm) for thin layer chromatography from Merck, (Germany) was also used as a sample. The particles were irregular in shape and samples were prepared with size ranges of <10 μm and 10–20 μm using repeated gravitational settling.

Suggested Gravitational FFF Experiments

GrFFF Fractograms

The GrFFF fractogram is a plot of the detector response versus elution time and is obtained by using a chart recorder or digitized signal using a computer interface. However, it is often advantageous to convert the x axis to elution volume using the volumetric flow rate, as this compensates for changes in flow rate in different GrFFF runs.

$$V_r = \dot{V} t_r \qquad (12)$$

An example of such a fractogram for a mixture of the 5 μm and 10 μm monodisperse silica samples is given in Figure 5. The first peak eluted is a void peak owing to dissolved material or unretained particles that may be either too small to be settled by the gravitational field or so large that retention is negligible. The subsequent retained peaks are due to the sample particles. The mean retention can be characterized by the position of the peak maximum signified by t_r and V_r for elution time and volume measurements, respectively.

GrFFF Retention Order

GrFFF operates in the steric-hyperlayer or lift mode of FFF, where larger particles elute before smaller ones. For the mixture of monodisperse silica samples shown in Figure 5 and Figure 10, the 10 μm beads elute before the 5 μm particles. Note that the elution times are different owing to the different flow rates used in the two different runs. For a polydisperse sample such as those shown in Figure 6 and Figure 7A the particle size will also decrease with increasing V_r.

This elution order can be demonstrated with monodisperse samples by injecting different size particles in individual

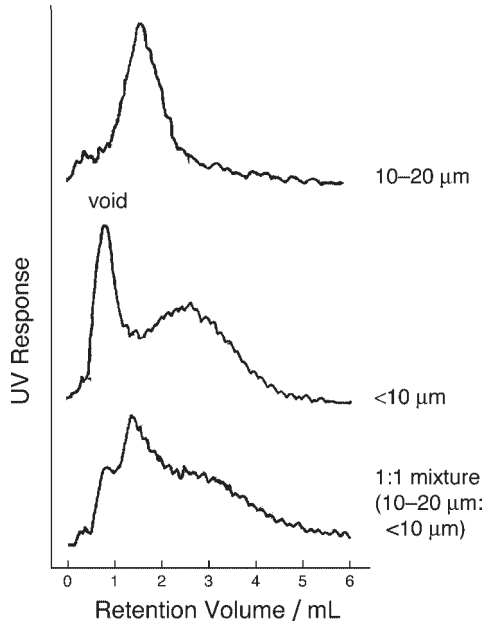

Figure 6. GrFFF fractograms of silica samples with size ranges of 10–20 μm and <10 μm, and a 1:1 mixture of 10–20 μm and <10 μm particles. The carrier was deionized water, flow rate 2.0 mL min^{-1} and relaxation time was 30 s.

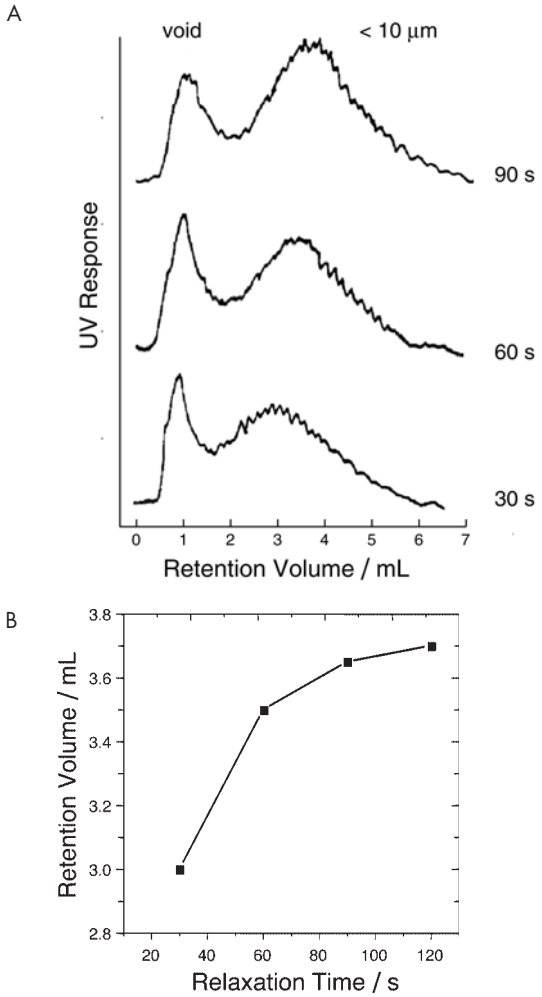

Figure 7. (A) The GrFFF fractograms of silica gel <10 μm with different relaxation times. The carrier was deionized water and the flow rate 1.0 mL min^{-1}. (B) Plot of retention volume versus relaxation time for the <10 μm silica gel.

runs. Alternatively mixtures of several monodisperse samples or broad polydisperse samples can be separated and fractions at appropriate elution times can be collected and the particles observed with an optical microscope as illustrated by the photos in Figure 5.

Effect of Relaxation Time

Runs on the 10 μm monodisperse silica were performed at a flow rate of 1.5 mL min^{-1} with relaxation times of 30, 60, and 90 s. The fractograms are not shown but the relaxation time appeared to have a negligible effect on the fractogram shape or the peak maximum retention volume. Since the calculated relaxation time is only 11 s (see eq 1) effective settling occurs early in the sample migration and the stop-flow relaxation procedure could be safely eliminated with this sample.

The above conclusion may not be valid for smaller particles. This could be investigated by varying the relaxation time for a range of silica samples (<10 μm). Inadequate relaxation is indicated by a shift to lower retention volume and eventually merging of the sample peak with the void peak as the relaxation time is reduced. This effect can be seen in the set of fractograms for the <10 μm silica gel shown in Figure 7A. Obviously 30 s is not enough relaxation time for the smallest particles in the sample causing a loss of resolution from the void and a slight shift in the peak maximum. The plot of peak retention volume versus t_r shown in Figure 7B indicates that a relaxation time of at least 90 s is required for this sample.

Effect of Flow Rate

Figure 8 gives the fractograms of the 10 μm silica at flow rates of 0.20, 0.60, and 1.00 mL min^{-1}. Naturally a higher flow rate results in a lower retention time. More significantly the peak maximum is shifted to a lower retention volume (1.7 mL compared to 2.3 mL for 1.00 and 0.20 mL min^{-1}, respectively). This demonstrates that the lift forces are stronger at higher flow rates, which elevates the particles further away from the accumulation wall and into the higher velocity flow streams.

A series of runs were performed on the 5 μm and 10 μm silica samples. The retention volumes and retention ratios at the peak maximum are plotted as a function of flow rate in Figure 9. Again the peaks shift to lower elution volume and higher retention ratios as the flow rate increases owing to the increased lift force as explained above.

Calculation of Plate Height and Resolution

Separation efficiency in FFF is monitored using the plate height, as in chromatography. The plate height (H) is a mea-

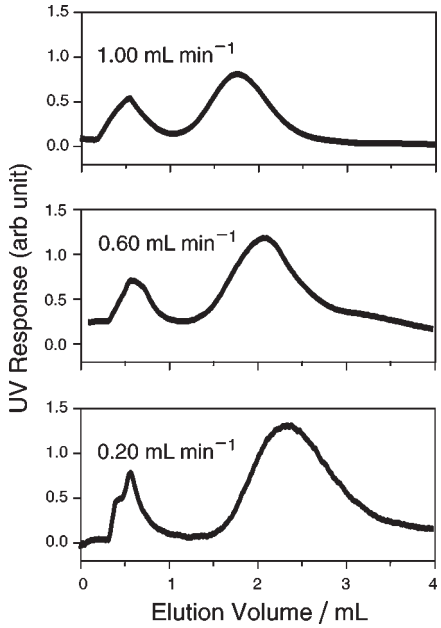

Figure 8. GrFFF fractograms of the 10 μm chromatographic silica particles at different flow rates.

sure of how narrow the peak is maintained during elution and can be calculated by the expression

$$H = \frac{L W^2}{16\, t_r^2} = \frac{L W_{1/2}^2}{5.545\, t_r^2} \qquad (13)$$

where L is the channel length, t_r the peak maximum retention time, and W the width of the peak at the baseline (also estimated as twice the width at half the peak height, $W_{1/2}$).

The plate height is determined by a number of factors as summarized in the van Deemter equation:

$$H = \frac{A}{\langle v \rangle} + B\langle v \rangle + H_I + H_P \qquad (14)$$

The first term results from longitudinal diffusion, which will be negligible for micrometer-size particles. The second term is due to nonequilibrium effects and the third term (H_I) is caused by instrumental peak broadening. The last term (H_P) is related to the sample polydispersity. If a graph of H versus flow rate is plotted and extrapolated to zero flow rate the influence of the latter two quantities can be obtained.

The resolution of two sample peaks is estimated from the expression

$$R_s = \frac{2\left[x_{r(2)} - x_{r(1)}\right]}{W_{(1)} + W_{(2)}} = \frac{1.18\left[x_{r(2)} - x_{r(1)}\right]}{W_{1/2(1)} + W_{1/2(2)}} \qquad (15)$$

where x_r refers to the distance along the fractogram to the peak maximum.

Calculation of Particle Elevation

The particle elevation parameters x_{eq} and δ can be calculated using eqs 11 and 8, respectively. Data (not tabulated) for the 5 and 10 μm silica show that the particles are lifted further away from the accumulation wall by increasing the carrier flow rate. This is obvious from the data in Figure 10, as the retention ratios increase with the flow rate. Negative δ values can result when the viscous drag force causes particles to move slower than anticipated by the simplified theory used here, which does not incorporate a fluid slip function (see discussion in ref *22,* p 85).

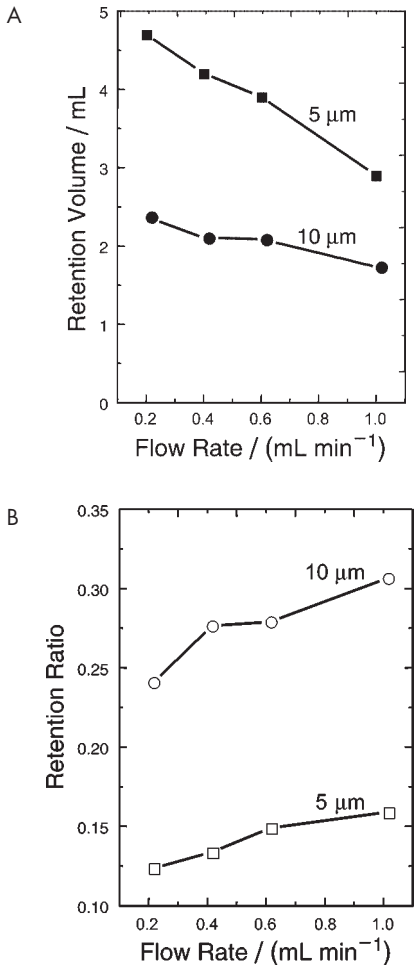

Figure 9. Effect of flow rate on (A) retention volume and (B) retention ratio of 5 μm and 10 μm silica samples.

Effect of Carrier Composition

Experiments to demonstrate the effect of ionic strength of the carrier for the 5 μm silica were performed at a flow rate of 4.0 mL min^{-1} in deionized water as well as in 0.01% and 0.1% sodium dodecyl sulfate (SDS), an anionic surfactant. The retention ratios were 0.69, 0.37, and 0.36, respectively, for these carriers. Clearly very small increases in ionic strength have a large effect on retention. The particle elevation terms x_{eq} and δ were calculated and these parameters clearly demonstrated that the particles move closer to the channel wall as ionic strength increases. This is due to significant charge repulsion between the negatively charged particles and the wall in deionized water, which is reduced by

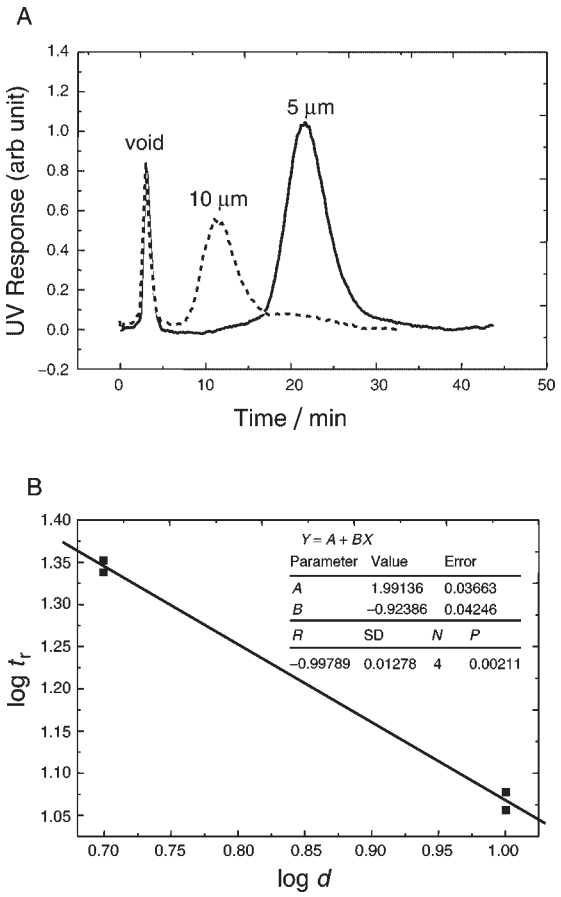

Figure 10. (A) GrFFF fractograms of 5 and 10 µm silica particles and (B) Calibration plot of log t_r versus log d. The flow rate was 0.2 mL min^{-1}.

shielding of the counter ions (Na^+) as the SDS is added. This illustrates that for quantitative use of GrFFF in size determination a small concentration of ions in the carrier is necessary (about 10^{-4} M is usually sufficient) to avoid quite large errors owing to the effect of wall repulsion.

Calculation of Particle-Size Distributions

Conversion from Elution Time to Diameter

By assuming that the silica particles in the monodisperse standards have the same density as the other sample particles used, the conversion of the diameter scale was made employing the empirical formula *(33)*,

$$\log t_{ri} = -S_d \log d_i + \log t_{r1} \qquad (16)$$

where t_r is measured retention time, d is the diameter of the particles, S_d is the size selectivity, and t_{r1} is a constant equal to the extrapolated value of t_r corresponding to particles of unit diameter. Figure 10 shows fractograms of the 5 and 10 µm silica particles and the plot of log t_r versus log d. These plots were used to generate the equation for converting the x axis to a diameter scale as described above. In this case, the calculated selectivity using the data for the 5 µm and 10 µm silica was found to be 0.92 and t_{r1} was 98.0.

It should be noted that there may be a systematic error in this calibration due to the fact that the 5 µm silica standard particles are spherical but the 10 µm silica standard and the silica gel particles are platey in shape. It is know that platey particles experience higher lift forces than spheres of the same volume *(34)*. Thus particles of the same shape should be used for accurate calibration and at least three standards covering the size range of interest would be desirable.

Conversion from UV Detector Signal to Eluted Mass

For micron size particles, the UV detector response, UV_i, at point i along the FFF elution profile is related to the mass concentration of particles of the sample in the eluent ($dm_{p_i}^c/dV_{r_i}$) *(35, 36)*

$$\frac{d\,m_{p_i}^c}{dV_{r_i}} = UV_i\, d_i \qquad (17)$$

where $m_{p_i}^c$ is the mass of sample eluted up to elution volume V_{r_i} and d_i is the particle diameter eluting at V_{r_i}. It should be noted that the superscript c in these quantities signifies, that it is the cumulative amount eluted up to point i on the fractogram. The appropriate y axis for a particle-size distribution, $m_{p_i}^c/(dd_i)$, is given by *(22)*

$$\frac{d\,m_{p_i}^c}{d\,d_i} \approx UV_i\, d_i \frac{\delta V_i}{\delta d_i} \qquad (18)$$

where δd_i is the increment in d_i corresponding to increment δV_{r_i} in V_{r_i} at point i along the fractogram. For such a plot, $m_{p_i}^c/(dd_i)$ versus d_i, the area under the distribution between two size values represents the mass of particles in this size range. Advanced students could be set the assignment to generate such a mass-based size distribution.

Hazards

Normal care and supervision needs to be taken in constructing the channel, assembling the electronic components and conducting the GrFFF experiments. Avoid inhalation of any dry particle samples when making sample suspensions.

Conclusions

Field-flow fractionation is becoming quite widely used separation technique, which warrants inclusion in undergraduate analytical chemistry courses. This article outlines some FFF theory that can be incorporated in lectures and suggests experiments that can be included in laboratory classes. The theory is useful for reinforcing some important aspects of physical chemistry such as settling and fluid flow. The laboratory exercise uses gravitational FFF, which is inexpensive and easy to construct, making it accessible to most institutions. An experiment is given in the Supplemental Material[W] that includes both a student and instructors manual. We have tested this experiment with students in Australia and Thailand and find it an effective way to illustrate FFF and the underlying theory.

Acknowledgments

Thanks are due to the Thailand Research Fund (TRF) including the Royal Golden Jubilee (RGJ) Ph.D. Program for a scholarship for RC and the Postgraduate Education and Research Program in Chemistry (PERCH) for partial support.

[W]Supplemental Material

A tested undergraduate experiment along with an instructor's manual and a glossary of terms are available in this issue of *JCE Online*.

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Microchim Acta (2008) 162: 181–188
DOI 10.1007/s00604-007-0879-8
Printed in The Netherlands

Microchimica Acta

Original Paper

Naphthazarin modified carbon paste electrode for determination of copper(II)

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Received 3 June 2007; Accepted 2 October 2007; Published online 10 December 2007
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Abstract. A carbon paste electrode modified with naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) was fabricated as a copper(II) sensor. Voltammetric and potentiometric detections were investigated. Adsorptive stripping voltammetry offers better sensitivity but poorer repeatability than potentiometry. The applied potential and current passing in voltammetric detection cause irreversible electrode reactions and shortening of the lifetime of the electrode. Potentiometric determination in batch and flow injection using 3% (w/w) of naphthazarin in the paste exhibited linear response with supra-Nernstian slope to copper(II) over a wide concentration range of three orders of magnitude in 0.10 M ammonium acetate medium. The potentiometric detection limits were 1.5×10^{-6} and 3.0×10^{-5} M by batch and flow injection, respectively. The electrode shows a good selectivity for copper(II) over a wide variety of metal ions and was applied to the analysis of alloys. The electrode response time was 50 sec and it showed good reproducibility for at least 60 days.

Keywords: Copper sensor; modified carbon paste electrode; naphthazarin; adsorptive stripping voltammetry; potentiometric detection

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Chemically modified electrodes have gained great interest as chemical sensors due to the electrode surfaces can be tailored to achieve the needed selectivity and sensitivity. Chemical modifiers immobilized on the carbon paste electrodes provide attractive properties such as low cost, ease of modification and no memory effects on the renewable surface [1]. Searching for the selective modifier stills being a challenge task. Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) has capability of forming complex with a number of metal ions [2–4]. It has been successfully used as a chelating agent for spectrophotometric determination of beryllium [5, 6], aluminum [6], thorium and uranium [7], yttrium [8], manganese and zinc [9] and copper [10]. Besides the spectral properties, naphthazarin has also electrochemical properties due to quinone and hydroxyl quinone groups in its molecule. The quinone/hydroquinone couple is well known and widely used as a good model in electrochemical study [11]. Hence, in this work electrochemical detection based on the copper – naphthazarin complex was continuously investigated. Although numerous of chemical modifiers have been reported for incorporation with carbon paste and used as copper sensors. However, detection technique used in each work was either voltammetric [12–20] or potentiometric [21–23] measurement, using the same modifier in carbon paste electrode for both detection techniques has not been reported.

The objectives of this work is to demonstrate the ability of naphthazarin as a modifier immobilized in carbon paste electrode and to use this naphthazarin modified carbon paste electrode (NCPE) as an electrochemical sensor for copper(II) ion. According to the low water solubility of naphthazarin, saturation of naphthazarin in water was reported at the concentration of 1.2×10^{-4} M [24], so this reagent was used as a modifier and the mineral oil in the paste could help holding up naphthazarin on the electrode surface. The characteristics and analytical performance of NCPE as a copper(II) sensor were investigated for voltammetric and potentiometric detections by batch and flow injection modes.

Experimental

Reagents

All chemicals used in this work were of analytical grade. A stock naphthazarin solution (2.00×10^{-3} M) was prepared by dissolving an accurate amount of reagent (98% purity, Fluka) in ethanol. A stock copper solution (0.10 M) was prepared from $Cu(NO_3)_2 \cdot 3H_2O$ in deionized water. More dilute solutions were obtained by dilution with appropriate volumes of 0.10 M ammonium acetate.

Preparation of NCPE and Ag/AgCl electrode

An electrode body was made from a disposable 1 mL polyethylene syringe. For the NCPE, the syringe tip was cut off for the open end of 0.5 cm diameter. The modified paste was prepared as the following: 1 g of pure graphite powder (Aldrich, particle size of 1–2 μm) was mixed with a fixed amount of naphthazarin in ethanol solvent, ultrasonicated for 30 min and evaporated off the ethanol. This receiving powder was transferred to a mortar and mixed well with 0.4 mL mineral oil (Fluka). The modified paste was filled in the electrode body and a copper wire was used for electrode connection. The carbon paste electrode (CPE) was prepared in the same procedure without naphthazarin mixing. The paste electrodes were left with the surface tip up, air dried for 24 h before use in order to reduce oily surface and low response. The new surface of the paste electrode surface was simply renewed by scrapping off about 1–2 mm of the old surface and polished the new surface on a filter paper. One paste electrode can be used for 12–15 renewal surfaces. The home-made Ag/AgCl reference electrode was also prepared [25] in the syringe body for using in the flow system. Its potential was calibrated with the commercial Ag/AgCl electrode (6.0726.100, Metrohm, Switzerland).

Apparatus and setup

An Autolab PGSTAT30 with GPES (General-Purpose Electrochemical System) version 4.9 software (Eco-Chemie, Utrecht, The Netherlands) was used for voltammetric and potentiometric measurements. A six port injector (V-451, Upchurch Scientific) and a peristaltic pump (Masterflex, Cole-Palmer Instrument Co., U.S.A.) were employed.

Voltammetric experiments

Three-electrode system was used for voltammetric experiments. The working electrode was the carbon paste electrode or NCPE. The counter electrode was a platinum wire and the reference was an Ag/AgCl electrode. The 0.10 M ammonium acetate solution was used as a supporting electrolyte medium. Voltammetric records of the quiescent solutions were performed at room temperature ($25 \pm 2\,°C$) after nitrogen flush in the measuring solution for 3 min. Cyclic voltammetric scan rate was 100 mV sec^{-1}. Differential pulse voltammetry conditions were: pulse amplitude 50 mV, pulse period 40 msec and scan rate 10 mV sec^{-1}.

Potentiometric experiments

For the batch method, the potential was recorded from the constant stirred (500 rpm) solution of a copper(II) solution in 0.10 M ammonium acetate medium and potential difference between the indicator electrode (NCPE) and reference electrode (Ag/AgCl electrode) at the steady stage was measured versus time using the Autolab30 instrument. The electrochemical cell used for potential measurements was:

$$\text{NCPE/Cu(II)}, 0.10\,\text{M NH}_4\text{OAc} \,//\, \text{Sat.KCl, AgCl/Ag}$$

The flow injection system was a single flow line connected to a pump. The 0.10 M ammonium acetate was used as the carrier solution. The detector cell body composed of two parts which were cast from the transparent resin and assembled together with screws and knots. The upper part of the detector cell was drilled for fitting the inlet solution tube, indicator and reference electrodes in order to contact to the cell chamber of 210 μL volume. The outlet tube was at the lower part of the cell. An aliquot ($\sim$0.5 mL) of copper(II) in 0.10 M ammonium acetate medium was injected to the injection port and the volume of 280–940 μL was transported to the mixing coil and then to the detector cell. The inlet tube, outlet tube and the mixing coil of 50, 100, 200 or 300 cm length are microbore PTFE tubing of 1.0 mm inner diameter. Potential of the solution was recorded continuously versus time by the Autolab30 instrument.

Sample preparation for alloy sample

An accurate weight of 0.20 g of Nickel Copper Alloy (NBS no. 882) sample was treated with acid digestions as follows. The 6.00 mL of 50% (v/v) HNO_3 was added to the sample and the mixture was boiled till clear before the volume was adjusted to 100 mL. Then a further 50-fold dilution was made by the solution of 0.10 M ammonium acetate.

Results and discussion

Voltammetric detection

Figures 1 and 2 represent cyclic voltammograms obtained by employing the CPE and the NCPE. Electrochemical reactions of naphthazarin on the working electrode are due to the quinone/hydroquinone couple concerning protonation and deprotonation of the quinone groups. There are several possible and complicated pathways of electrode reactions of a quinone. The possible pathway of quinone reduction in aqueous solvent depends strongly on the pH of the solution and

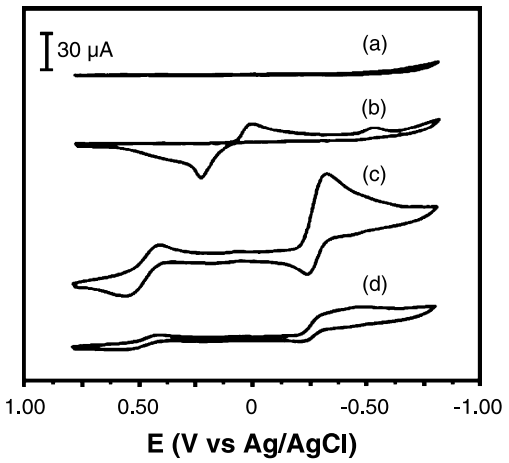

Fig. 1. Cyclic voltammograms on CPE of (*a*) supporting electrolyte: 0.1 M NH_4OAc, (*b*) 1×10^{-4} M Cu(II) ion, (*c*) 2×10^{-4} M naphthazarin, (*d*) mixture of 1×10^{-4} M Cu(II) ion and 2×10^{-4} M naphthazarin. Scan rate = 100 mV sec^{-1}

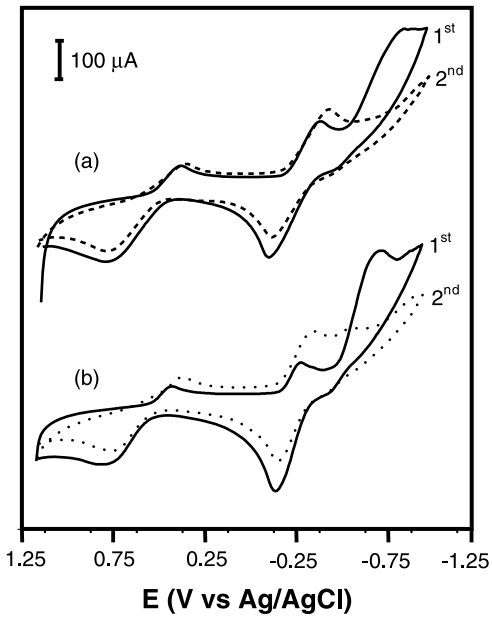

Fig. 2. Cyclic voltammograms taken for two cycles on 3% (w/w) NCPE of (*a*) supporting electrolyte: 0.1 M NH_4OAc, (*b*) 1×10^{-4} M Cu(II) ion in supporting electrolyte. Scan rate = 100 mV sec^{-1}

the pK_a values of the acid-base functions of the quinone, semiquinone radicals and the hydroquinone [24, 26]. Mechanism on the reactions involving should be investigated (as discussion on possibility is given in ESI).

In order to get the high sensitivity of detection, adsorptive stripping voltammetric technique was used. The NCPE was immersed in a stirred copper(II) solution in 0.10 M NH_4OAc medium for a fixed period. At this step copper ion was preconcentrated by complex-

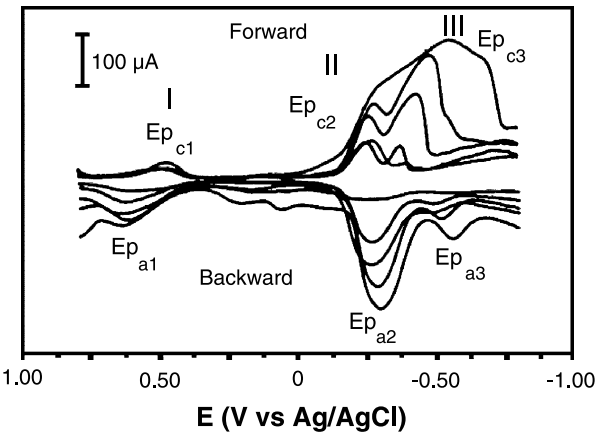

Fig. 3. Differential pulse stripping voltammograms from a 3% (w/w) NCPE in 0.1 M NH_4OAc after 5 min preconcentration in different concentrations of Cu(II): 1, 5, 10 and 100 μM

ation with naphthazarin and deposited on the electrode surface. The electrode was then rinsed with water, stirred 1 min in 0.10 M NH_4OAc medium and transferred to the new deaerated electrolyte medium. The response signals were recorded by taking differential pulse voltammograms in the cathodic scan and anodic scan right after the cathodic scan as in Fig. 3. Higher peak currents are obtained from the preconcentration step with higher concentrations of copper(II) solutions. In spite of the peak potential was shifted at higher concentration of copper(II), the cathodic peak current at vicinity III ($Ep_{c3} \sim -0.37$ V) shows good linear relationship with copper ion concentration up to 5 μM so that this reduction peak is interesting for further quantitative determination of Cu(II) after the open-circuit preconcentration in copper(II) solution.

Effect of supporting electrolytes

Various supporting electrolytes (KNO_3, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, KCl, NaOAc and $NaClO_4$) were studied to be used in the measuring step. In sodium perchlorate electrolyte, the reduction peak was at −0.54 V and it was good separated from the nearby characteristic peak of pure nahthazarin at −0.33 V and the peak current at −0.54 V was increased with increasing the concentration of copper ion in the preconcentration step as shown in Fig. 4.

Effect of naphthazarin content in the carbon paste

Five different naphthazarin contents (1, 3, 5, 8, 10 and 15% w/w) in the carbon paste were tested for the

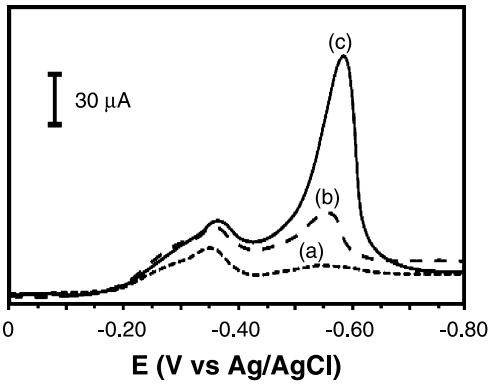

Fig. 4. Differential pulse voltammograms on the 1% (w/w) NCPE in 0.10 M $NaClO_4$ electrolyte after 20 min preconcentration in (*a*) 0.10 M NH_4OAc (*b*) 1 μM copper(II) in 0.10 M NH_4OAc and (*c*) 5 μM copper(II) in 0.10 M NH_4OAc. Pulse amplitude 50 mV and scan rate 10 mV sec^{-1}

response signals. The NCPE of 1% and 3% (w/w) naphthazarin in the paste did not show significant different response but the higher naphthazarin percentages revealed higher residual currents and broadening peaks so that 1% (w/w) NCPE was chosen.

Working concentration range on the NPCE

Sensitivity of copper detection depends on the preconcentration step. Longer deposition time resulted in higher peak current. Linear relationship of the peak current at −0.54 V and the copper ion concentration was obtained from 1.0–5.0 μM (0.06–0.32 ppm) using 20 min deposition time on the 1% (w/w) NCPE. The concentration range could be extended to 10 μM if shorter deposition time as 5 min was used due to the limited active surface for copper ion accumulation.

Surface electrode renewal

Homogeneity of naphthazarin in the carbon paste and surface regeneration were crucial for accuracy and precision result. We found that reversing potential scan after each differential voltammogram taken could help better precision because naphthazarin in carbon paste could return back to the former form by the reverse potential scan as in cyclic voltammetry. Different methods of electrode surface regenerations were studied. The 1.0 M acetic acid and 0.01 M EDTA solutions were used to wash the NCPE surface after each measurement but the precision of measurements were poorer than using the new scrapped off and polished surface. With the reverse potential scan, responses of a 1% (w/w) NCPE to 5 μM copper ion using 5 min deposition time gave% RSDs ($n = 5$) of the measuring current signals as: 13.3, 12.8, 12.4 and 7.4% upon using an electrode with: old surface, acetic acid washing surface, EDTA washing surface and new surface, respectively. Without reversing the potential scan, an electrode with new surface resulted in bad reproducible measuring signal as % RSD ($n = 5$) was high as 23.9%. The applied potential and current passing in voltammetric detection cause irreversible electrode reaction and shortening the life time of electrode so reversing of the potential scan is recommended as a part of surface electrode renewal after each measurement.

Effect of foreign ions

Effect of foreign cations and anions present in the analyte solution is represented in Table 1. Among the studied cations and anions, interference from Al(III) and EDTA were the most pronounced due to Al(III) can also form complex with naphthazarin and EDTA can form complex with Cu(II) better than naphthazarin.

The disadvantages of the adsorptive stripping voltammetric technique are complicated and time consuming steps (preconcentration, voltammetric record and electrode surface regeneration) involved. One measurement was about 15–30 min depended on deposition time. The applied potential and current passing can cause easy electrode fouling and shortening the life time of electrode. Benefit of the technique is selective adsorption and less interference as the medi-

Table 1. Tolerance limits for other ions interfere the voltammetric response of 3 μM Cu(II) using 1% (w/w) NCPE and deposition time 5 min. The mole ratios shown gave relative deviation within ±10% of that obtained from only Cu(II)

Cation	Mole ratio Cu(II): cation	Anion	Mole ratio Cu(II): anion
Ba(II)	1:10	F^-	1:9600
Ca(II)	1:70	Cl^-	1:7500*
Ni(II)	1:4	Br^-	1:3000
Co(II)	1:1*	I^-	1:3000*
Cd(II)	1:100	CN^-	1:3000
Pb(II)	1:5	SCN^-	1:100
Zn(II)	1:5	PO_4^{3-}	1:100
Mn(II)	1:10*	EDTA	1:0.05
Al(III)	1:0.5	citrate	1:10
Cr(III)	1:1	oxalate	1:1
Fe(III)	1:4	tartrate	1:70*

* Increasing signal.

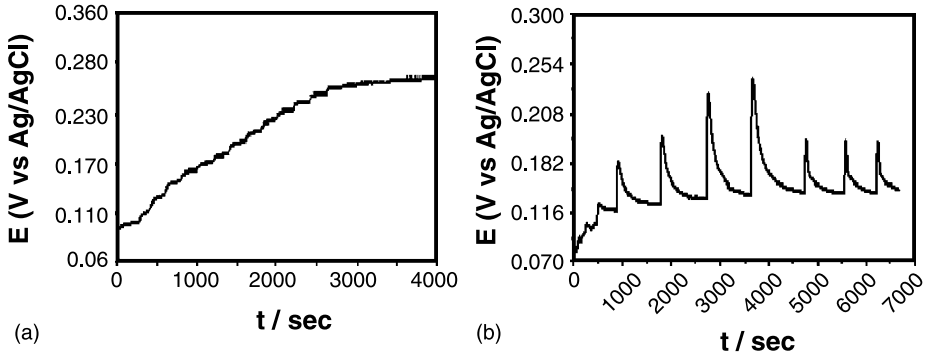

Fig. 5. Potentiometric responses of the 3% (w/w) NCPE indicator electrode to copper(II) ion at various concentrations in 0.1 M NH_4OAc medium by (a) batch and (b) flow injection techniques

um was changed between the preconcentration and measurement steps.

Potentiometric detection

Potentiometric detection at zero current is a simple technique for using the modified electrode both in batch and flow injection. The same electrode surface was used for a series of consecutive measurements and suitable for flow injection with continuous operation. Deviation of the surface-to-surface was avoided and reproducibility was improved. The copper(II) ions are extracted from solution and deposited upon the electrode surface through complexation with the immobilized naphthazarin reagent, resulted in the changing potential response by different amounts of copper ions in the solutions. This could lead to supra-Nernstian slope. In batch method, the potential response of the NCPE reached steady state within 50 sec and remained constant afterward (Fig. 5a). Potentiometric response in the flow system has the peak form with pronounce tailing due to convection predominates in dispersion control (Fig. 5b). The peak potentials give linear relationship with log activities of copper(II) over wider concentration range than the voltammetric technique but the sensitivity of the technique is lower.

Effect of naphthazarin content in the carbon paste electrode

For this purpose, eight electrodes were prepared. The amounts of carbon powder and mineral oil were constant at the ratio of 1 g carbon powder: 0.4 mL oil for each electrode. The proportions of naphthazarin in these eight electrodes were 0.5, 1.0, 3.0, 5.0, 10.0, 15.0, 20.0 and 25.0% (w/w). Calibration graphs for batch method over copper(II) concentration range of 2.0×10^{-6}–2.9×10^{-3} M were examined. The resulting supra-Nernstian slopes and correlation coefficients are 0.0454 (0.9909), 0.0455 (0.9894), 0.0478 (0.9969), 0.0477 (0.9947), 0.0494 (0.9806), 0.0600 (0.9133), 0.0600 (0.9133) and 0.0671 (0.9820) V per decade concentration. These results showed that the slopes increased by increasing the percentages of naphthazarin modifier. An electrode with naphthazarin in the carbon paste of more than 15.0% gave nonlinear electrode response. The electrode with 3.0% of modifier was chosen for further potentiometric study.

Batch potentiometric response

The potentiometric responses of 3.0% NCPE to copper(II) together with some cations from batch method were investigated. The calibration graph of copper(II) concentration range 2.0×10^{-6}–2.9×10^{-3} M (0.13–184 ppm) was constructed by sequential adding known amount of standard copper(II) solution into a 100 mL of 0.1 M NH_4OAc solution. Plot of potential response against log [activity of copper ion] gave linear regression line with correlation coefficient of 0.9969 and supra-Nernstian slope of 0.0478 ± 0.0021 V. Detection limit was determined from the intersection of the two extrapolated segments of the calibration graph [28] and was found to be 1.5×10^{-6} M (0.10 ppm). The NCPE showed no selectivity to Cr(III), Ni(II), Co(II), Mn(II) and Zn(II) but the potential response was linear increased with Pb(II) concentration from 5.0×10^{-6}–2.8×10^{-3} M with sub-Nernstian slope = 0.025 and correlation coefficient = 0.988. The Fe(III) ion showed obviously increase of the NCPE response when its concentration was more than 5.6×10^{-4} M. Also presence of Al(III) is prone to interfere with copper(II) response. Selectivity coefficient values of 3.0% NCPE respected to these three foreign ions, determined by the Matched Potential

Method [28] were found to be: $K_{Cu,Pb} = 0.0014$, $K_{Cu,Al} = 0.0095$ and $K_{Cu,Fe} = 0.058$.

Optimization of FI variables

Effect of mixing coil lengths (11, 45, 90, 110, 130, 180, 280 and 380 cm) was studied with the copper concentration range 5×10^{-5}–1×10^{-2} M. A shorter coil length, the signal to noise ratio was higher than that at the longer coil length, the longer time was needed to get the signals so that the mixing coil length of 90 cm was chosen.

Effect of flow rates (0.5, 1.0, 1.5, 2.0 and 3.0 mL min^{-1}) was performed with the copper concentration range of 5.0×10^{-5}–1.0×10^{-2} M. A lower flow rate led to a longer period in peak returning to baseline but a higher flow rate of more than 1.5 ml min^{-1} resulted in bubble in flow tube and noisy signals. A flow rate of 1.0 mL min^{-1} was chosen due to the best fit of the linear calibration graph.

Effect of sample injection volume at the injection port was varied by using different tubing lengths to give the volumes of 280, 534, 816 and 940 μL. No significant differences in slopes and R^2 of the calibration graphs obtained at the same concentration range. The smallest sample injection volume of 280 μL was chosen.

Higher copper concentration injected into the system gave longer return times, base line drift and hence, a decrease in sample throughput. Surface renewal of the NCPE was also required. The virgin NCPE surfaces were somewhat less efficient for copper ion detection. The potential drift was found obviously at the initial injection of copper ion solution. However, after a few injections, the reproducible signals were obtained. The similar behaviors were found in other work [13] which copper(I) was preconcentrated on 2,9-dimethyl-1,10-phenanthroline modified carbon paste electrode. It was hypothesized that the early depositions of copper ion might assist the ligand molecules to orient in the carbon paste and attaining higher degree of ordering on the electrode surface that facilitates complexation.

Calibration graph in FI

Using optimized condition: mixing coil length of 90 cm, flow rate at 1.0 mL min^{-1} and sample injection volume of 280 μL, calibration graph was studied in the concentration range of 5.0×10^{-5}–1.0×10^{-2} M (0.32–635 ppm). Although the baseline drift was found, plot of the peak height signal (obtained from the difference between the potential at peak and base point) against log copper concentration gave the linear regression line as $y = (0.0444 \pm 0.0010)x + (0.2208 \pm 0.0057)$ with correlation coefficient 0.9951 (precision from five 3% (w/w) NCPEs). Reproducibility of the slope and R^2 of calibration curve was checked and it was found that the electrode could be used for more than 60 days as data in Table 2. The time for peak returning to baseline was about 10–20 min depending on the copper(II) concentration in the injection solution. Detection limit was determined from the calibration graph to be 3.0×10^{-5} M (1.9 ppm). The wider working concentration range and higher detection limit were obtained comparing to the batch method. This could be due to that amount of copper ion reached the electrode surface was diluted in the flow tube.

Table 2. Long-term reproducibility study of a 3% (w/w) NCPE by FI-potentiometric measurement

Day*	Calibration**	Slope	R^2
1	$y = 0.0394x + 0.1830$	0.0394	0.9637
3	$y = 0.0452x + 0.2114$	0.0452	0.9933
6	$y = 0.0435x + 0.2283$	0.0435	0.9961
13	$y = 0.0472x + 0.2374$	0.0472	0.9956
19	$y = 0.0437x + 0.2274$	0.0437	0.9936
35	$y = 0.0536x + 0.2577$	0.0536	0.9939
48	$y = 0.0440x + 0.2154$	0.0440	0.9960
51	$y = 0.0491x + 0.2432$	0.0491	0.9996
60	$y = 0.0459x + 0.2074$	0.0459	0.9905
67	$y = 0.0430x + 0.1959$	0.0430	0.9889

* After fabrication.
** In the concentration range of 5.0×10^{-5}–1.0×10^{-2} M.

Interference study in FI

Each cation or anion at various concentration was mixed with 1 mM Cu(II) ion in 0.10 M NH_4OAc me-

Table 3. Tolerance limits for other ions interfere the potentiometric response of 1 mM Cu(II) using a 3% (w/w) NCPE in the flow system with sample injection volume of 280 μL, 90 cm mixing coil and flow rate at 1.0 mL min^{-1}. The mole ratios shown gave the potentiometric signals deviation within ±5% of that obtained from only Cu(II)

Cation	Mole ratio Cu(II): cation	Anion	Mole ratio Cu(II): anion
Ni(II)	1:45	F^-	1:100
Zn(II)	1:55	Cl^-	1:60
Mn(II)	1:18	Br^-	1:40
Al(III)	1:14	SCN^-	1:0.4
Cr(III)	1:1	PO_4^{3-}	1:0.2
Fe(III)	1:7	EDTA	1:0.2
		citrate	1:0.1
		oxalate	1:0.1

Table 4. Comparison of analytical characteristics using the 3% (w/w) NCPE for potentiometric measurement of copper(II) in batch and flow injection

Parameter	Batch	Flow injection*
Response time	2–5 min	31–35 sec
Working range (correlation coefficient, R^2)	2.0×10^{-6}–2.9×10^{-3} M (0.997)	5.0×10^{-5}–1.0×10^{-2} M (0.999)
Sensitivity (as linear slope)	47.8 ± 0.2 mV/decade	38 ± 9.7 mV/decade
Sample volume required	20–100 mL	0.5–1 mL
Detection limit	1.5×10^{-6} M (0.10 ppm)	3.0×10^{-5} M (1.9 ppm)
Measurement precision in potential		
of 1.0×10^{-4} M Cu(II)	$E = 0.208 \pm 0.022$ V %RSD = 10.4 ($n = 5$)	$E = 0.022 \pm 0.003$ V %RSD = 12.1 ($n = 5$)
1.0×10^{-3} M Cu(II)	$E = 0.256 \pm 0.016$ V %RSD = 6.3 ($n = 5$)	$E = 0.059 \pm 0.003$ V %RSD = 5.6 ($n = 5$)
Cu(II) determination in NBS Ni–Cu–Al Alloy no. 882 (Cu = 31.02 ± 0.04% w/w other ions: Ni = 65.25, Al = 2.85, Ti = 0.57, C = 0.006, Mn = 0.0007, S = 0.0014, Si = 0.006, Fe = 0.009)	$32.48 \pm 1.05\%$ ($n = 4$)	$31.54 \pm 0.76\%$ ($n = 4$)

* Using 280 μL injection volume, 90 cm mixing coil and flow rate of 1.0 mL min^{-1}.

dium. The optimum condition of the FI system was used for construction the calibration graph. Tolerance limit of several ions defined as the mole ratio of the copper ion: other ion that changed the 3 μM Cu(II) signal ~5% are shown in Table 3. The tolerance limits in FI of the most cations studied are higher than those found in adsorptive voltammetric technique except Cr(II) while the tolerance limits of the anions are lower. In adsorptive voltammetric technique the reaction of interfering ions upon the electrode surface is the main cause but in potentiometric detection, the surface reaction and ionic strength of the solution resulted in more effect on the potential response.

Comparison between batch and FI

Analytical characteristics using the 3% (w/w) NCPE for potentiometric detection of copper(II) in batch and FI and analysis results of an reference alloy sample are shown in Table 4. No significant difference of the mean values from the certified value were obtained from both batch and FI with potentiometric detection (at P = 0.05).

Acknowledgements. The Thailand Research Fund is acknowledged for support.

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Fast and simultaneous detection of heavy metals using a simple and reliable microchip-electrochemistry route: An alternative approach to food analysis

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Received 11 April 2007; received in revised form 25 June 2007; accepted 26 June 2007
Available online 1 July 2007

Abstract

This paper reports, for the first, the fast and simultaneous detection of prominent heavy metals, including: lead, cadmium and copper using microchip CE with electrochemical detection. The direct amperometric detection mode for microchip CE was successfully applied to these heavy metal ions. The influences of separation voltage, detection potential, as well as the concentration and pH value of the running buffer on the response of the detector were carefully assayed and optimized. The results clearly show that reliable analysis for lead, cadmium, and copper by the degree of electrophoretic separation occurs in less than 3 min using a MES buffer (pH 7.0, 25 mM) and L-histidine, with 1.2 kV separation voltage and −0.8 V detection potential. The detection limits for Pb^{2+}, Cd^{2+}, and Cu^{2+} were 1.74, 0.73 and 0.13 μM (S/N = 3). The %R.S.D. of each peak current was <6% and migration times <2% for prolonged operation. To demonstrate the potential and future role of microchip CE, analytical possibilities and a new route in the raw sample analysis were presented. The results obtained allow the proposed microchip CE-ED acts as an alternative approach for metal analysis in foods.
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Keywords: Microchip CE; Electrochemical detection; Heavy metals; Microanalysis

1. Introduction

The traditional format of capillary electrophoresis is quickly being replaced by microdevice platforms in modern analytical chemistry, offering improvements in cost, resolution, speed, quantitation and automation. This aspect of miniaturization is well matched with the ultimate goal of rapid screening and simultaneous detection of a large number of samples [1,2]. The miniaturization of an integrated system improves the advantages inherent to capillary electrophoresis.

Heavy metals are particularly worrisome contaminants in foods and the environment. In general, they are not biodegradable and they have long biological half-lives. Heavy metals have potential for accumulation in humans from various plants and other natural sources, posing serious health hazards for conditions such as renal failure, symptoms of chronic toxicity, and liver damage. According to the World Health Organization (World Health Organization, 1995), lead, cadmium, chromium, and other heavy metals must be controlled in food sources in order to assure public safety.

Lead and cadmium are among the most abundant heavy metals on earth, and are particularly toxic. Excessive concentrations of these metals in food is associated with the etiology of a number of diseases, especially with cardiovascular, renal, neurological, and bone diseases [3,4]. In addition, they are also implicated in causing carcinogenesis, mutagenesis, and teratogenesis [5]. Copper, another important metal, is an essential trace element for the human body and contributes to important intracellular metabolic events [6]. A copper imbalance can result in a severe human ailment, from either an excess or deficiency of this key element [7]. A major reason to monitor levels of toxic metals in foods follows from the fact that contamination of the general environment has increased. The sources of this environmental pollution are quite varied, ranging from industrial

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0039-9140/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2007.06.034

and traffic emissions to the use of purification mud and agricultural expedients. In order to study the toxicity of heavy metals in food, a simple, sensitive and accurate detection method is required.

Many analytical methods have been developed for determination of individual metals in food products, including: titration [8], colorimetric analysis [9,10], UV–vis spectroscopy [11–13], and either flame or graphite furnace atomic absorption spectrometry (AAS) [14–17]. In addition to these techniques, methods for multi-elemental determination have been developed; these are ion chromatography (IC) [18–20] and inductively coupled plasma combined with either atomic emission spectrometry (ICP–AES) [21,22] or mass spectrometry (ICP–MS) [23–25]. Although these methodologies are rapid and sensitive for the determination of trace amounts of metals, they require complicated instrumentation with high capital and operational costs. Furthermore, these methods are not easily implemented into fully portable analytical tools for screening, detecting, identifying, and quantifying metal ions.

Capillary electrophoresis (CE) has been widely applied to the separation and determination of different metal species, and has proved to be a fast, high-resolution separation technique [26–31]. The detection methods typically used in combination with CE separation technique are ultraviolet light (UV), fluorescence or laser-induced fluorescence (LIF) detection. The lack of a strong chromophore from metal ions has certainly been one of the major limitations in the analysis of most metals by these methods. In addition, these detection modes suffer from lack of sensitivity when a miniaturized device is used. Electrochemical detection (ED) offers high sensitivity and selectivity for metals that are readily oxidized or reduced. This technique is readily suitable and compatible with microfabrication technology that has been successfully employed in microchip CE.

Separation of metal ions by microchip CE has attracted significant attention, offering a number of advantages including speed of analysis, portability, flexibility, and compatibility with integrated analytical systems, allowing development of "micro-total-analysis systems (μTAS)" [1,32–38]. There are a few published examples of metal ion separation performed on a microchip CE. The detection methods are largely focused on the utilization of complexing agents, such as fluorescent metal complexation [39]. Although excellent separation of metal ions was obtained, the designs of microchip sensors were not compact or portable, but tedious and expensive. Unlike other researchers, Li et al. [40] reported the use of microchip CE with indirect amperometric detection of heavy metals. Their results demonstrated good separation and detection, unfortunately, the configuration of microchip and detector was still complicated. Alternatively, we have focused on the simple, direct quantitation of metals by electrochemical reaction, which will enable the incorporation of a portable, simple, and inexpensive detector.

This work addresses the need for developing an inexpensive and field portable analytical device for metal ions which would permit the rapid screening and detection of contaminated food or waste materials on site. The detection of metal ions was accomplished using a microchip CE/amperometric detection system, in which screen-printed carbon electrode is placed at the end of channel. This approach simplifies the fabrication of the working electrode and also provides a convenient and sensitive method for the determination of metal ions by amperometry. Furthermore, by simply changing the electrode, we can easily remove or clean the detection electrode, which is prone to contamination. In this manner, we propose an attractive portable device for screening and analyzing a complex system containing different metal species, such as food. The optimization, characterization, and attractive performance characteristics of a microchip CE, and its successful application to complex samples (such as vegetable juices) are reported in the following sections.

2. Experimental

2.1. Chemicals

Lead(II) nitrate was obtained from Aldrich (Germany). Cadmium(II) sulfate was obtained from Baker Analyzed (USA). Copper(II) sulphate was obtained from BDH (England). Standard stock solutions of all analytes were freshly prepared each day. The running buffer solution (BGE) for separation was prepared from 2-morpholinoethanesulfonic acid (MES), (s)-2-amiono-3-(4-imidazyl) propionic acid (L-histidine) which were obtained from Fluka (USA). Sodium hydroxide and Hydrochloric acid were purchased from Merck (Germany), and were used for pH adjustment of the electrolytic buffer solution. All reagents were used without further purification. Pure deionized water (Millipore, USA) was used to prepare all aqueous solutions. The sample solutions were prepared by diluting corresponding stock solutions in running buffer. All solutions for electrophoresis experiments were filtered through a 0.45 μm membrane filter (Altech) before use.

2.2. Apparatus

Borofloat glass chips with simple-cross single-separation channels (16 mm × 95 mm × 2.2 mm) were obtained from Micralyne (model MC-BF4-001, Canada). The microchip has a four-way injection cross that are connected to the three reservoirs and the channel. The original waste reservoir was cut off, leaving the channel outlet at the end of the chip, facilitating the end-column electrochemical detection. The chip had a 90 mm long separation channel (from injection cross to the channel outlet) and a 10 mm long injection channel (between the sample and buffer reservoir). All channels were etched to a depth of 20 μm and a width at the top of the channel of 50 μm.

The integrated CE-ED microchip system was described previously [36]. The CE microchip was placed in a laboratory-built Plexiglas holder for housing the separation chip and electrochemical detector, allowing convenient replacement. The holder consisted of a sample, running buffer, and unused reservoirs. Short pipette tips were cut and inserted into the fluidic ports of the various reservoirs on the glass chip for providing solution contact between the channel on the chip and the corresponding reservoir on the chip holder. Platinum wires were inserted into the compartments to provide the electrical contacts to a high-

voltage power supply. A home-made high voltage power supply, with an adjustable voltage range between 0 and +4000 V, was used for controlling the injection and separation. The amperometric detector, placed in the waste reservoir, at the separation channel outlet, consisted of an Ag/AgCl wire reference electrode, a platinum wire counter electrode, and a screen-printed carbon working electrode.

2.3. End-column amperometric detection

Amperometric detection was performed with a computer controlled electrochemical analyzer (Autolab potentiostat, PG-30, Methrom) using the "amperometric *i–t* curve" mode. The electropherograms were recorded at a fixed detection potential, −0.8 V versus Ag/AgCl wire. The screen-printed carbon working electrode was placed opposite the outlet of the separation channel through a plastic screw. The distance between the electrode surface and the channel outlet was controlled by a plastic screw and a thin-layer spacer. The electrochemical detection compartment was filled with 25 mM MES/L-histidine buffer solution (pH 7.0). All experiments were done at room temperature, sample injection were performed after the baseline current had stabilized.

2.4. Electrophoresis procedures

Before electrophoresis, the channels of each glass chip were treated by rinsing with deionized water, 0.1 M NaOH, and again with deionized water for 10 min each. The reservoirs were cleaned and the reservoir for sample solution was filled with sample solution, while all other reservoirs were filled with running buffer. Each of the corresponding pipette tips on the micro-channel chip was filled with their respective solution. Injection was carried out by applying the desired potential, 1200 V, between the sample reservoir and the grounded detection reservoir for 3 s, while all other reservoirs were allowed to float. Separation was performed by switching the high-voltage contacts and applying the corresponding separation voltages to the running buffer reservoir with the detection reservoir grounded. As soon as the voltage was switched to perform electrophoresis separation, the electrochemical analyzer was actuated to record signals.

2.5. Sample preparation

Sample solutions were blended for 300 s with a homogenizer. Two millilitre of sample solution was placed in capped centrifuge tubes and then centrifuged for 10 min at 3500 rpm. 0.5 mL of supernatant was then transferred to a centrifuge tube fitted with a filter membrane (0.45 μm Nylon membranes) and centrifuged for 5 min. The filtrate was placed in capped tubes. 0.5 mL of filtrate-sample solution was spiked with 50–100 μL of 2 mM mixed metal ion stock solution (lead(II), cadmium(II), and copper(II) ions) and further diluted with 25 mM MES and L-histidine buffer (pH 7.0) to give final concentrations of 100, 200, 400, 600, 800, and 1000 μM, respectively. Finally, the solutions were analyzed by microchip CE-ED.

3. Results and discussion

In general, the first step in using microchip CE for the determination of metal ions is the selection of the background electrolyte co-ion and subsequently, the optimization of the resolution and detection sensitivity of system. In this work, since electrochemical detection is used; the background electrolyte has a large effect on the signal sensitivity. To obtain a high signal-to-noise ratio, the conductivity of the background electrolyte co-ion, which is directly related to the electrophoretic mobility, should differ from those of analytes as much as possible. It is well known that carrier electrolytes with low-mobility co-ions are preferred for the analysis of small ions. 2-(*N*-Morpholino)ethanesulfonic acid (MES) + histidine is a typical carrier electrolyte with a low-mobility co-ion used for the separation of small ions because both compounds have relatively low mobilities. Moreover, their pK_a values are almost identical, which make them excellent components of buffer system. In the following parts, the influence of some experimental parameters such as the buffer pH, buffer concentration, the separation voltage and the detection potential on the separation efficiency and detection sensitivity are reported in detail.

3.1. Influence of the buffer pH

The running buffer pH is the first important parameter for optimization in microchip CE, the same as in conventional CE. Running buffer pH has effect on EOF rate, degree of ionization, mobility, and separation efficiency of analytes. Thus, the pH values of running buffer were examined in the pH range 6.0–8.5. All buffers contained 20 mM MES and 20 mM L-histidine. Table 1 shows the currents obtained and resolution of three metal ions (Cd(II), Pb(II), and Cu(II)). It can be seen that as buffer pH changed from 6.5 to 7.5, the three metal ions could be well separated. The highest current signal was obtained at buffer pH 7.0. Since the background noise increased relative to the signal intensity and took longer to stabilize between samples, a buffer pH of 7.0 was selected as the optimal pH for all subsequent work.

3.2. Influence of the buffer concentration

Buffer concentration is another important parameter that affects the separation efficiency and detection sensitivity. Electropherograms of 1.0 mM lead, cadmium and copper were performed in varying concentrations of pH 7.0 running buffer.

Table 1
Current and Resolution of three metal ions standards

pH of BGE	Current (nA)			Resolution	
	Pb(II)	Cd(II)	Cu(II)	$R_{Pb,Cd}$	$R_{Cd,Cu}$
6	7.35	7.35	1.87	–	7.75
6.5	1.98	14.81	7.88	2.58	2.51
7	13.49	25.71	17.09	1.91	3.33
7.5	13.25	18.48	10.99	2.18	2.31
8	14.69	20.42	13.69	1.45	1.18
8.5	11.38	11.89	10.35	1.79	1.05

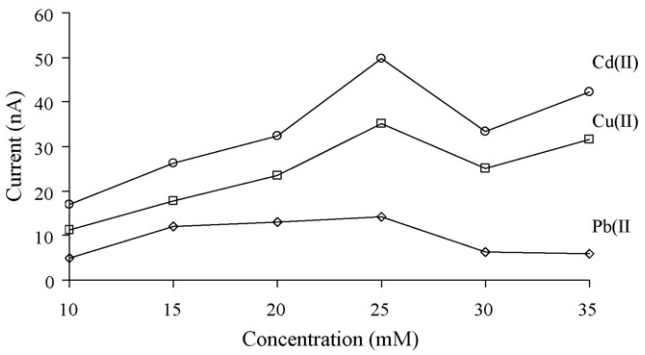

Fig. 1. The effects of the buffer concentration on peak currents. Experimental parameters: sample, 1.0 mM lead, cadmium and copper cations; running buffer, MES and L-His pH 7.0; separation voltage, 1000 V; detection potential, −0.8 V; sampling time, 3 s; working electrode, screen-printed carbon electrode.

We found that the peak current of the three cations increased with increasing buffer concentrations from 10 to 25 mM, and then decreased with further increase of the buffer concentration as shown in Fig. 1. This phenomenon can be attributed to two reasons: (1) higher concentrations of running buffer result in high ionic strength, which will decrease the difference of conductivity between the sample zone and running buffer zone and result in low signal, and (2), higher concentrations of the running buffer makes for electrostacking, which results in relatively higher concentration of the sample zone and then a relatively high signal is obtained. A running buffer concentration of 25 mM was chosen for optimal detection sensitivity.

3.3. Influence of detection potential

Since the detection potential strongly affects the sensitivity and detection limits of microchip CE with an electrochemical system, the optimal detection potential was determined by investigating a hydrodynamic voltammogram. Fig. 2 shows the results which must draw a balance between low applied potentials and enough sensitivity for all the metal ions involved at screen-printed carbon electrode. We observed that the amperometric signal of heavy metals ions increased with the increase of detection potential from −0.70 to −0.85 V. However, the baseline current and the corresponding noise level become large at higher reduction potential. The sensitivity of the signal for the three ions with the change of detection potential was also different. In order to perform the simultaneous determination of lead, cadmium, and copper, the detection potential which most influences the measurement process was optimized. From the results, it can be seen that cadmium and copper have a similar change rate of the signal, while the relative peak current of lead has a lower change rate with changing the detection potential. This suppression of the lead signal could be explained by intermetallic effect of cupper [41]. There is the formation of intermetallic/solid between Cu–Pb. The phenomenon may result in low signal of Pb^{2+}, also resulting in possible calibration problem. To compromise between the sensitivity and signal-to-noise characteristics of three metals, especially Pb^{2+}, a detection potential of −0.8 V was chosen since it offered the most favorable results.

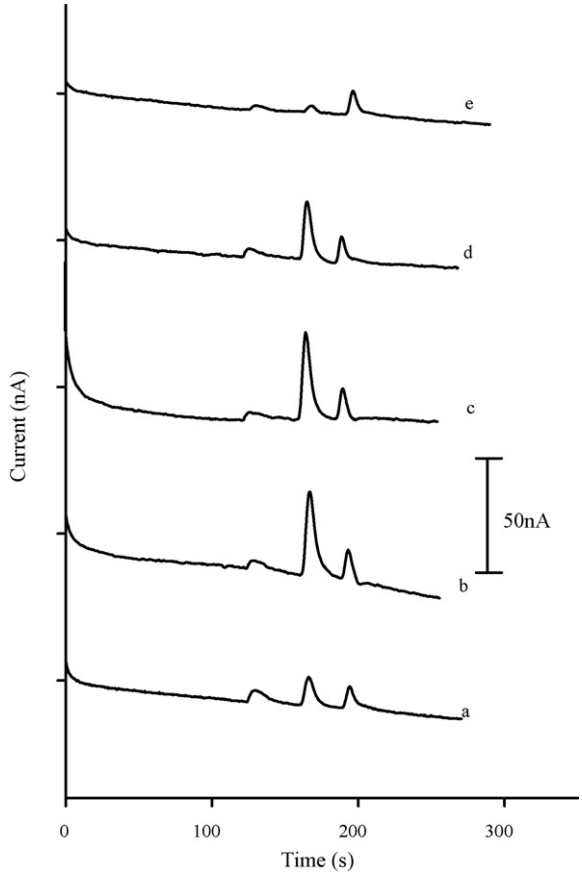

Fig. 2. Electropherograms of 1.0 mM lead, cadmium and copper cations at different detection potentials, −0.90 V (a), −0.85 V (b), −0.80 V (c), −0.75 V (d), −0.70 V (e). Experimental parameters: running buffer, 25 mM MES and L-His pH 7.0; separation voltage, 1000 V; sampling time, 3 s; working electrode, screen-printed carbon electrode.

3.4. Influence of separation voltage

The separation voltage affects the electric field strength, which in turn affects the EOF and the migration velocity of charged particles, which determine the migration times of the analytes. Moreover, higher separation voltages may result in higher Joule heating. The effect of separation voltage on the migration time of the analytes is shown in Fig. 3A. As expected, increasing the voltage gives shorter migration times but also increases the background noise, resulting in a higher detection limit. The migration times were dramatically decreased for all three metal ions, from 120 to 90 s for lead, 150 to 110 s for cadmium, and 175 to 140 s for copper. Although the resolution of analytes can be improved to some extent, too low a separation voltage will increase the analytical time considerably, which in turn causes severe peak broadening (Fig. 3B). Based on experiments, 1200 V was chosen as the optimum voltage to strike a good compromise.

3.5. Effect of oxygen

The effect of oxygen using batch system by screen-printed carbon electrode compared to that by glassy carbon electrode

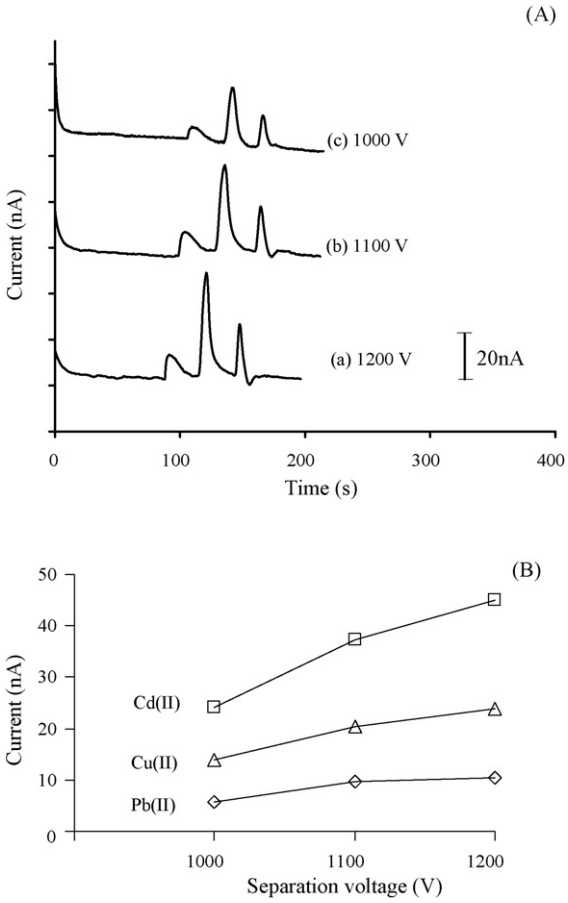

Fig. 3. Electropherograms of 1.0 mM lead, cadmium and copper cations at different separation voltages. The relationship between the peak currents and the separation voltage (A), 1200 V (a), 1100 V (b), 1000 V (c). The relationship between the peak current and the separation time (B). Other parameters same as in Fig. 2.

was studied. It was found that there was a little effect of oxygen on the background signal using screen-printed carbon electrode whereas the high effect of oxygen on the background signal using glassy carbon was obtained. It indicates that screen-printed carbon electrode is less sensitive to oxygen. We also investigated the effect of oxygen of three metals using normal and degas solutions. It was found that the peak current decreased around 11% for $Cd^{2+}$and 21% for Pb^{2+}, respectively. For Cu $^{2+}$, very small change of the peak current was obtained (data not shown). For the microchip separation system, the experiment was performed in a very small system. Therefore, the effect of oxygen on the determination of these metals can be negligible. From this point, it should be an advantage of this proposed method that it does not need to perform the experiment under the oxygen-free condition.

3.6. Linear range and detection limits

According to the previous studies on pH value and concentration of the running buffer, as well as the separation voltage and the detection potential; optimized conditions of 1200 V separation voltage, −0.8 V detection potential, with 25 mM (pH 7.0) of MES + L-histidine as running buffer were obtained. Under

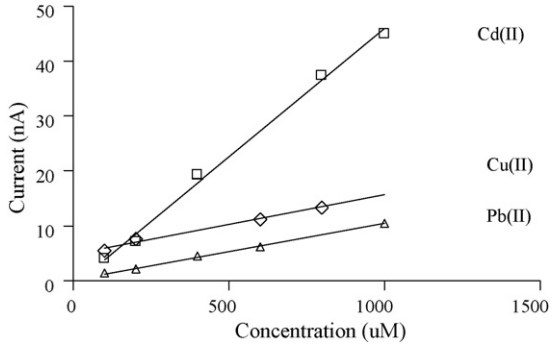

Fig. 4. The relationship between the peak currents and the concentrations. Experimental parameters: running buffer, 25 mM MES and L-His pH 7.0; separation voltage, 1200 V; detection potential, −0.8 V; sampling time, 3 s; working electrode, screen-printed carbon electrode.

the selected conditions, a series of the standard mixture solutions of lead, cadmium, and copper with a concentration range of 100–1000 μM was tested to determine the linearity for all analytes at the screen-printed carbon electrode in this system. The data are shown in Fig. 4. The results of regression analysis on calibration curves are 0.9977, 0.9902, and 0.9958 for lead, cadmium, and copper, respectively. The detection limits were evaluated on the basis of a minimum signal-to-noise ratio of 3. The calibration curves exhibit excellent linear behavior over the concentration range of about micromolar orders of magnitude, with detection limits ranging from 0.13 to 1.74 μM for all the metal ions (data shown in Table 2).

A standard mixture solution of lead, cadmium, and copper (1 mM each) was analyzed ten times to determine the reproducibility of the peak current and migration time for all analytes under the optimal conditions in this experiment. The relative standard deviations (R.S.D.s) of peak current and migration time are 4.20% and 1.47% for lead, 5.27% and 1.40% for cadmium, 3.92% and 1.20% for copper, respectively. It is indicated that the proposed system exhibited an excellent performance on both separation and detection for prolonged operation.

3.7. Sample analysis

The analytical potential of screen-printed carbon electrodes in constant EC detection coupled with microchip CE for the separation of lead, cadmium, and copper in juice samples were investigated. By applying the optimum conditions described above, the injection of three metal ions were analyzed by standard addition method. We studied the metal ions of three different juice samples obtained from a local market. The results are

Table 2
The reproducibility of peak current, retention time and limit of detection of metals

Metal ions	%R.S.D. of peak current	%R.S.D. of retention time	LOD (μM)
Cu(II)	3.92	1.20	0.13
Cd(II)	5.27	1.40	0.73
Pb(II)	4.20	1.47	1.74

Table 3
Determination of Pb(II), Cd(II), and Cu(II) in different samples by means of microchip CE-ED using screen-printed carbon electrode

Sample	Amount added (μM)			Amount found (μM)			%Recovery ($n = 3$)			%R.S.D. ($n = 3$)		
	Pb	Cd	Cu	Pb	Cd	Cu	Pb	Cd	Cu	Pb	Cd	Cu
Green vegetable juice	1000	750	750	1032	791.85	784.35	103.20	105.58	104.58	2.93	2.10	2.11
	750	500	500	757.95	477.15	459.65	101.06	95.43	91.93	2.37	2.39	2.67
	500	250	250	486.95	269.75	250.08	97.39	107.90	100.03	3.40	2.13	3.75
Tomato juice	1000	800	800	1012.8	775.44	781.84	101.28	96.93	97.73	2.65	0.90	2.68
	800	600	400	791.12	603.06	389.80	98.89	100.51	97.45	4.25	1.63	4.76
	400	200	200	399.92	200.50	191.18	99.98	100.25	95.59	6.48	6.07	6.23
Pine apple juice	800	800	800	819.12	803.84	810.96	102.39	100.48	101.37	3.56	2.87	2.28
	600	600	600	586.26	639.66	621.66	97.71	106.61	103.61	1.57	2.71	2.25
	400	200	500	396.56	214.62	490.05	99.14	107.31	98.01	2.13	1.87	2.57

shown in Table 3. Recoveries ranged from 91.93 to 107.90 with ten replicate each, giving %R.S.D.s of 0.90–6.48. The results show that the proposed methods can be efficiently used for the determination of metal ions in practical samples. Such the high speed of analysis and efficiency of proposed method, it should be possible to separate and detect other metals in different kinds of food by simply varying the detection potential.

4. Conclusions

This work presents the first application of microchip CE-ED in direct mode for the simultaneous determination of lead, cadmium, and copper in vegetable juices. It has been demonstrated that microchip CE-ED is characterized by its simplicity, quickness, higher resolution and sensitivity, excellent reproducibility, low expense of operation and minor amounts of samples and reagent. The reproducibility of quantitative analysis by this method is satisfactory. Electrochemical detection coupled with microchip CE enables selective and sensitive detection of the electroactive constituents and simplification of the electropherograms. We conclude that microchip CE-ED is a powerful technique for the study of heavy metal ions and has become an alternate, competitive and supplementary method for HPLC, because of its special attributes. In particular, microchip CE-ED provides an attractive approach for a portable analytical device for rapid screening and the analysis of complex systems simultaneously containing metals and heavy metals. Such a microchip CE-ED system may have wide applications, particularly in food analysis.

Acknowledgements

The authors would like to acknowledge the support from the National Research Council of Thailand (NRCT) through the improvement of food safety and food quality for new world economy project, the Thailand Research Fund (Research Team Promotion Grant), and Graduate Thesis Grant, Chulalongkorn University.

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ภาคผนวก ข

ภาคผนวก ข1 รางวัลและเกียรติยศ

ภาคผนวก ข2 ข่าวผลงานด้านการวิจัย

ภาคผนวก ข3 การเขียนหนังสือเพื่อเผยแพร่ประโยชน์ด้านการวิจัย

ภาคผนวก ข1

ข่าวรางวัลและเกียรติยศ

ข่าวรางวัลและเกียรติยศ

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

รับรางวัล “บุคคลดีเด่นของชาติ”

สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์) ประจำปี 2547

จากคณะกรรมการเอกลักษณ์ของชาติ สำนักนายกรัฐมนตรี

ภาคผนวก ข2

ข่าวผลงานด้านการวิจัย

ข่าวผลงานด้านการวิจัย

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

“นักเคมีสร้างอุปกรณ์หาสารรั่วระดับนาโน”

บทสัมภาษณ์ จากหนังสือพิมพ์กรุงเทพธุรกิจ หน้า 9

เมื่อวันศุกร์ที่ 10 มิถุนายน 2548

สำนักงานกองทุนสนับสนุนการวิจัย
THE THAILAND RESEARCH FUND

เมธีวิจัยอาวุโส สกว. ๒๕๔๗-๒๕๔๘
TRF Senior Research Scholar 2004-2005

สาขาเคมีวิเคราะ

ศาสตราจารย์ ดร.เกตุ กรุดพัน
Professor Dr. Kate Grudpa

ประวัติส่วนตัว

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์ เกิดเมื่อวันที่ 2 ตุลาคม พ.ศ. 2496 ที่ อำเภอบางกรวย จังหวัดนนทบุรี เป็นบุตรนายศักดิ์ และนางอุบล กรุดพันธ์ สมรสกับ นางธนกร กรุดพันธ์ มีธิดา 1 คน (นางสาวศุภรา กรุดพันธ์) เริ่มการศึกษาที่ จังหวัดนนทบุรี โดยเริ่มระดับประถมศึกษาที่โรงเรียนวัดศรีประวัติ(ประชานิยม) ระดับมัธยมศึกษาตอนต้นที่โรงเรียนบางกรวย และระดับมัธยมศึกษาตอนปลาย ที่โรงเรียนเขมาภิรตาราม สำเร็จการศึกษาระดับปริญญาตรี สาขาวิชาเคมี จากมหาวิทยาลัยเชียงใหม่ ในปี พ.ศ. 2517 และระดับปริญญาเอก สาขาเคมีวิเคราะห์ จาก Liverpool John Moores University ในปี พ.ศ. 2524 ด้วยทุน Research assistantship ของ Liverpool Education Authority ได้มีประสบการณ์หลังปริญญาเอกหลายแห่ง รวมถึง Alexander von Humboldt Fellow ที่ Karl rule Research Center เยอรมนี (พ.ศ.2534-2535) และ The Royal Society Visiting Fellow ที่ Liverpool John Moores University (พ.ศ.2541)

ประวัติการทำงาน

เริ่มรับราชการที่ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ ในตำแห่ง อาจารย์ พ.ศ.2517 ดำรงตำแหน่ง ศาสตราจารย์ (โดยวิธีพิเศษ) ใน พ.ศ.2534 ดำรงตำแหน่งศาสตราจารย์ (โดยเสนอผลงานวิจัยล้วนๆ) ใน พ.ศ.2547 เคยเป็นสมาชิก อาจารย์ มหาวิทยาลัยเชียงใหม่ และทำงานบริหารในระดับต่างๆ เช่น ผู้ช่วยคณบดี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ เป็นต้น ได้ริเริ่มโครงการปริญญาเอกร่วมสถาบันในต่างประเทศ ในลักษณะ Sandwich Program ตั้งแต่ปี พ.ศ.2534 เป็นกรรมการสภาวิจัยแห่งชาติ สาขาวิทยาศาสตร์เคมีและเภสัช (พ.ศ.2545-ปัจจุบัน) ได้รับเกียรติจากทบวงการพลังงานปรมาณูระหว่างประเทศ (International Atomic Energy : IAEA) เพื่อทำงานด้านต่าง ๆ ได้แก่ เป็น Course Director ของการจัด Regional Training Course for South Asia in Water Monitoring ที่ภาควิชาเคมี มหาวิทยาลัยเชียงใหม่ (3-21 พฤศจิกายน พ.ศ.2540) เป็นผู้เขียนรายงาน ให้คำแนะนำด้าน Nuclear and Radiochemistry ที่ประเทศพม่า (Yangon University และ Mawlamyai University) (7-22 ตุลาคม พ.ศ.2538) และเป็นผู้จัดการฝึกอบรมระดับชาติ ทาง Nuclear and Radiochemistry โดยการสนับสนุนของ IAEA และรัฐบาลสหพันธ์

สาธารณรัฐเยอรมันซึ่งจัดขึ้นที่มหาวิทยาลัยเชียงใหม่จำนวน ครั้ง ได้ฝึกอบรมให้นักวิทยาศาสตร์ชาวพม่าอีก 6 คน ในระยะเวลามากกว่า 5 ปี ได้รับเชิญเป็นผู้ร่วมจัดการประชุมนานาชาติในหลายโอกาส เช่น ICAS 2001 (International Union of Pure and Applied Chemistry Congress on Analytical Science 2001) สิงหาคม พ.ศ. 2544 กรุงโตเกียว ประเทศญี่ปุ่น ได้รับเกียรติเป็น Co-organizer ในการจัดประชุมวิชาการนานาชาติ FACIFICHEM 2005 (19-20 December 2005) ใน Symposium#16 เป็นกรรมการใน International Advisory Board ของการจัดประชุมวิชาการนานาชาติ เช่น International Flow Analysis IX และ X ที่เมือง Geelong ประเทศออสเตรเลีย (February 2003) และโปรตุเกส (September 2006) เป็นสมาชิก ใน Streering Committee ในการจัด 12th และ 13th International Conference on Flow Injection Analysis, ที่เมือง Marida, Venezuela: (December 2003) และที่เมือง Las Vegas ประเทศสหรัฐอเมริกา (April 2005) เป็นผู้ประสานงานฝ่ายไทยในการจัด Interfaces of Analytical Sciences : Workshop of US and Thai Analytical Scientists ในเดือนมกราคม 2549 ที่เชียงใหม่โดยการสนับสนุนของ National Science Foundation (NSF) และหน่วยงานของไทยรวมถึงสำนักงานกองทุนสนับสนุนการวิจัยด้วย นอกจากนี้ยังได้รับเชิญเป็นวิทยากรบรรยายนำ และเป็นประธานในการประชุมวิชาการในระดับชาติและนานาชาติหลายครั้ง เช่น The 9th International Conference on Flow Injection Analysis ที่เมือง Seattle, USA, ในเดือนสิงหาคม พ.ศ.2541 ได้รับรางวัลนักวิจัยดีเด่นแห่งชาติ สาขาวิทยาศาสตร์เคมี-เภสัช ปี พ.ศ.2542 (สำนักงานคณะกรรมการวิจัยแห่งชาติ) นักศึกษาเก่าดีเด่น ปี พ.ศ.2542 (มหาวิทยาลัยเชียงใหม่) "Science & Technology Research Grant" ปี พ.ศ.2543 (Thailand Toray Science Foundation) เมธีวิจัยอาวุโส สกว. ครั้งที่ 1 ปี พ.ศ.2544-2546 ครั้งที่ 2 ปี พ.ศ.2547-2550 ได้รับรางวัลนักวิทยาศาสตร์ ดีเด่น ปี พ.ศ. 2544 (มูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์) และรางวัลเกียรติยศแก่นักศึกษาเก่ามหาวิทยาลัยเชียงใหม่ที่ประสบความสำเร็จในชีวิต จากสมาคมนักศึกษาเก่ามหาวิทยาลัยเชียงใหม่ รับรางวัล "JAFIA Scientific Award" จาก The Japanese Association for Flow Injection Analysis (JAFIA) (คนเอเชียคนแรกนอกญี่ปุ่น) บุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์) ประจำปี พ.ศ. 2547 จากสำนักงานเสริมสร้างเอกลักษณ์ของชาติ

นอกจากนี้ยังได้รับทุน/รางวัล/เกียรติยศ ต่าง ๆ เช่น ทุนพัฒนานักวิจัยรุ่นที่ 1 (2537-2540) และทุนองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา ของสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ทุนวิจัยคณะกรรมการวิจัยแห่งชาติร่วมมือกับประเทศอังกฤษ (พ.ศ. 2541-2542) Editorial Advisory Board ของวารสาร Talanta (Elsevier) (พ.ศ. 2543-ปัจจุบัน) และของวารสาร Journal of Flow Injection Analysis (Japanese Association for Flow Injection Analysis) (พ.ศ.2546-ปัจจุบัน) Associate Editor ของวารสาร Water Research (Elsevier) (พ.ศ. 2546- ปัจจุบัน) Editorial board ของวารสาร Laboratory Robotics and Automation (LRA) (Wiley) (พ.ศ.2540-2543) Guest Editor ของ special issue "The 11th International Conference on Flow Injection Analysis (ICFIA 2001) 11 Talanta, vol. 58 No. 6 Guest Editor ของ special issue "FIA in the Pacific Rims" ของ Laboratory Robotics and Automation, vol.12, No.3, 2000

ผลงานวิจัยโดยสรุป

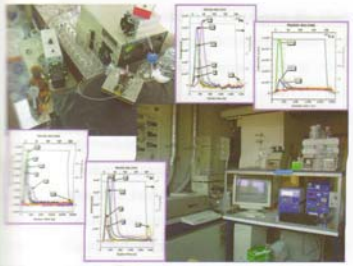
ได้สนใจในการวิจัยศึกษาและพัฒนาการวิเคราะห์ทางเคมีแผนใหม่โดยเฉพาะเทคนิคที่เกี่ยวข้องกับการไหล (Flow - based Analysis) โดยเน้นการพัฒนาฐานความรู้ของไทยเพื่อแก้ปัญหาของไทยและได้องค์ความรู้ใหม่เกิดนวัตกรรมใหม่ที่ได้รับการยอมรับในระดับสากลด้วย ผลงานหลายชิ้นเป็นเรื่องใหม่ในสาขาที่ทำการวิจัยที่เป็นรากฐานของการนำไปใช้ประโยชน์ในวงการต่าง ๆ เช่น อุตสาหกรรมเกษตร สิ่งแวดล้อม การแพทย์และการศึกษา

ในระดับอุดมศึกษา ได้มีความคิดริเริ่มในการทำงานอย่างจริงจังและต่อเนื่องในการค้นคว้าศึกษาวิจัย และพัฒนาทางด้านนี้ประมาณ 20 ปีจนเกิดประสิทธิผลของงานเป็นรูปธรรม โดยเริ่มแรกได้สนใจพัฒนาระบบการวิเคราะห์ทางเคมีในเทคนิค Flow Injection Analysis (FIA) ทั้งในส่วนของการพัฒนาอุปกรณ์ เครื่องมือที่มีราคาถูก การพัฒนาเทคนิคต่างๆ และปฏิกิริยาเคมีที่เกี่ยวข้องเพื่อให้ได้วิธีการวิเคราะห์แบบใหม่ที่มีความถูกต้อง แม่นยำสูง ประหยัดค่าใช้จ่ายและเวลาคน เป็นที่รู้จักโดยทั่วไปว่า "Low Cost Man"

การที่ได้สนใจเริ่มการทำวิจัยเพื่อศึกษาและพัฒนาการวิเคราะห์โดยเทคนิค Flow Injection Analysis (FIA) เนื่องจากเป็นการท้าทายในความสามารถที่จะทำวิจัยในความจำกัดในหลายๆ ด้าน โดยเฉพาะงบประมาณแต่ต้องการผลลัพธ์เพื่อแก้ไขปัญหาในประเทศและเกิดองค์ความรู้ใหม่ๆ ทางวิชาการเป็นที่ยอมรับ ในระดับสากลด้วยเมื่อพิจารณาถึงการควบคุมการผลิตในอุตสาหกรรม หรือการวิจัยในวิทยาศาสตร์สาขาต่างๆ สิ่งสำคัญที่ต้องคำนึงถึงเกี่ยวกับข้อมูลผลการวิเคราะห์เหล่านี้โดยทั่วไป ได้แก่ ความถูกต้อง ความแม่นยำ และความน่าเชื่อถือของข้อมูล เพราะการได้ข้อมูลที่ผิดพลาดจะทำให้การสรุปผลหรือการตัดสินใจผิดพลาด ก่อให้เกิดผลเสียได้ นอกจากนี้ต้องพิจารณาถึงสิ่งที่ต้องใช้ไปในการให้ได้มาซึ่งผลการวิเคราะห์ที่ต้องการ เช่น ค่าใช้จ่าย เวลา แรงงาน ซึ่งนอกจากจะนำมาใช้ในการแก้ปัญหาทางการวิเคราะห์ที่กำลังเผชิญอยู่ในปัจจุบันแล้ว ยังเป็นพื้นฐานหรือเครื่องมือในการวิจัยพัฒนาสิ่งใหม่ๆ ให้เกิดขึ้นด้วย สำหรับประเทศไทยยังมีการวิจัยพัฒนาทางด้านเคมีวิเคราะห์น้อยมาก ทำให้ต้องสูญเสียเงินตราในกิจกรรมทางการวิเคราะห์เคมีแก่ต่างประเทศเป็นจำนวนเงินที่สูงมาก เช่น การซื้อเครื่องมือวิเคราะห์ต่างๆ ทั้งที่เป็นแบบง่ายหรือชนิดที่ซับซ้อน รวมถึงอุปกรณ์และชิ้นส่วนของเครื่องมือวิเคราะห์ เป็นต้น จึงเป็นผลให้ขาดความรู้พื้นฐานวิทยาศาสตร์ในการพัฒนาเทคโนโลยีขึ้นเอง กลุ่มวิจัยได้มองเห็นปัญหาข้างต้นและเล็งเห็นถึงศักยภาพของเทคนิค Flow Injection Analysis (FIA) ในการพัฒนาวิธีวิเคราะห์ที่อาศัยเครื่องมือที่ไม่ซับซ้อน ราคาถูก มีค่าใช้จ่ายในการวิเคราะห์ต่ำแต่มีประสิทธิภาพสูง จึงได้มุ่งความสนใจพัฒนาระบบการวิเคราะห์ทางเคมีโดยเทคนิค Flow Injection Analysis และเทคนิคที่เกี่ยวข้อง เช่น Sequential Injection Analysis หรือ Bead Injection Analysis โดยเกิดนวัตกรรมในการพัฒนาเครื่องมือ-อุปกรณ์ เช่น การพัฒนาระบบ Flow Injection Systems (เช่น On-line dialysis, in-value column device) ใช้งานร่วมกับ Ion Chromatograph เกิดนวัตกรรมเกี่ยวกับเทคนิค เช่น On- line solvent extraction, การศึกษา Size-based speciation, Flow-injection with bead injection systems เป็นต้น การศึกษาเกี่ยวกับปฏิกิริยาเคมี โดยเฉพาะศึกษาปฏิกิริยาที่ใช้รีเอเจนต์ที่ไม่เสถียร แต่ราคาไม่แพง เช่น ด่างทับทิม ($KMnO_4$) หรือสารในไบฝรั่ง เป็นต้น การพัฒนาการวิเคราะห์ดังกล่าวนำไปสู่การใช้ประโยชน์ในกิจการในสาขาต่างๆ เช่น การศึกษาทางสิ่งแวดล้อม การควบคุมคุณภาพในอุตสาหกรรม การวิเคราะห์ทางเกษตรกรรม และการวิเคราะห์ในทางการแพทย์ เป็นต้น

งานวิจัยในอนาคต

จากการเริ่มด้วยการใช้วัสดุอุปกรณ์ราคาถูกเพื่อทำการวิจัยเทคนิค Flow Injection Analysis (FIA) ซึ่งเกี่ยวกับการไหลอย่างต่อเนื่อง (Continuous Flow) ทั้งนี้จะขยายการวิจัยเพื่อครอบคลุมเทคนิคการวิเคราะห์เกี่ยวกับการไหล (Flow-based Analysis) โดยพัฒนาเทคนิคที่เกิดขึ้นมาภายหลัง ได้แก่ Sequential Injection Analysis (SIA) ซึ่งจะไม่ไหลไปทางเดียวแต่จะใช้การไหลกลับไปกลับมา อาจบังคับให้ไหลๆ หยุดๆ ได้โดยการควบคุมด้วยคอมพิวเตอร์ นอกจากนี้จะเกี่ยวข้องกับการวิจัยเพื่อศึกษาเทคนิค Bead Injection Analysis (BIA) ที่ให้สารแขวนลอยของบีดไหลไปในท่อเล็กๆ แล้วกักไว้ที่ส่วนของการตรวจวัดซึ่งทำให้สามารถใช้ประโยชน์ของการเกิดปฏิกิริยาบนพื้นผิวของบีด แล้วไล่บีดออกทิ้งไป ใช้บีด



ชุดใหม่สำหรับการวิเคราะห์ในครั้งใหม่ จึงเกิดประโยชน์ขึ้นหลายประการรวมถึงการลดการรบกวน (Inference) ได้ด้วย ทั้งนี้สนใจที่จะวิจัยเพื่อพัฒนาระบบและเทคนิคการวิเคราะห์แบบใหม่ที่รวมถึง Lab-on-Value (LOV) และ Lab-at-Value (LAV) (เป็นเทคนิคที่นำเสนอโดยกลุ่มวิจัยฯ) รวมถึงเทคนิค Stopped-Flow based Analysis เพื่อประสิทธิภาพในกระบวนการวิเคราะห์ด้วยเทคนิคต่างๆ ที่เกี่ยวข้องดังกล่าวใช้พื้นฐานทาง Microfluidics ซึ่งจะเป็นประโยชน์ในการวิจัยเพื่อพัฒนาเพื่อลดขนาดลง (down-scaling หรือ miniaturization) ซึ่งจะเกี่ยวข้องกับ Lab-on-Chip (LOC) ทั้งนี้ สนใจที่จะพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยใช้เทคนิคที่อิงการไหล โดยพัฒนาระบบเครื่องมือ (Instrumentation) การวิจัยเพื่อให้ได้แนวทางใหม่ ๆ (Novel approaches) เพื่อใช้ในการไหล การพัฒนาใช้สารใหม่ (New materials) เพื่อการวิเคราะห์ทางเคมี และการประยุกต์เทคนิคการไหลเพื่องานต่างๆ รวมถึงการประยุกต์ทางการแพทย์ เช่น การคัดกรองผู้ป่วยโรคมะเร็ง โรคตับ โรคกระดูก โรคธาลัสซีเมีย งานวิจัยทางด้านนี้น่าจะเสริมกับแนวทางการพัฒนาแพทย์ทางไกล (Telehealth) แนวทางศึกษาการตอบสนองของกลุ่มเนื้อเยื่อ/ เซลล์ต่อการกระตุ้นของยา การคัดกรองอย่างรวดเร็วในการศึกษาทางยา (High sample through-put screening) เป็นต้น การประยุกต์เพื่อการวิเคราะห์ทางเคมีเพื่อการเกษตรและอุตสาหกรรมเกษตร การวิเคราะห์ในการศึกษาทางสิ่งแวดล้อม

การนำผลงานวิจัยไปใช้ประโยชน์

ประโยชน์ที่เกิดขึ้นจากงานวิจัยจะมีผลกระทบทั้งในแวดวงวิชาการเองแล้วน่าจะมีผลต่อสังคมด้วย ทั้งนี้ โดยการผ่านความร่วมมือของบุคลากรจากหลายหลายสถาบันในลักษณะเครือข่าย (Networking) และหลากหลายสาขาวิชา (Multidisplinary) จะทำให้เกิดการพัฒนางานวิจัยในลักษณะบูรณาการ ซึ่งจะเป็นประโยชน์และมีผลกระทบต่อองค์การวิชาการและสังคมด้วย จึงสนใจที่พยายามมองถึงการพัฒนาฐานความรู้ของไทย ใช้ทรัพยากรเพื่อแก้ปัญหาของประเทศ เพื่อให้ได้ความรู้ใหม่ที่ได้รับการยอมรับในระดับสากล ทั้งนี้สามารถนำองค์ความรู้และผลงานไปใช้ประโยชน์ในด้านการแพทย์ เกษตรกรรม อุตสาหกรรม และสิ่งแวดล้อม โดยคำนึงถึงการพัฒนาทรัพยากรมนุษย์ ไปพร้อมกันด้วยโดยจะพยายามผลักดันให้มีความร่วมมือในระหว่างบุคลากรของสถาบันต่าง ๆ ทั้งในประเทศและต่างประเทศ เช่น กลุ่มวิจัยต่างๆ ในประเทศญี่ปุ่น มีความร่วมมือกับ Japanese Association for Flow Injection Analysis และกลุ่มวิจัยต่าง ๆ ในสหรัฐอเมริกา โดยจัดประชุมเชิงปฏิบัติการ Interfaces of Analytical Sciences : Workshop of US and Thai Analytical Scientists พยายามทำงานเป็นทีมโดยดึงศักยภาพที่เด่นของสมาชิกแต่ละคนออกมาใช้อย่างมีประสิทธิภาพ เพื่อวางแผนเป็นระบบวิเคราะห์และสังเคราะห์ความรู้อย่างเป็นระบบ

Biography and Research Summary

Professer Dr. Kate Grudpan received his B.S. (Chemistry) from Chiang Mai University in 1974, and Ph.D. degree in analytical chemistry from Liverpool John Moores University, UK in 1981. He became a lecturer in the Department of Chemistry, Faculty of Science, Chiang Mai University in 1974, where is now a professor. Professor Dr.Grudpan has received many awards and honors during his career due to his extensive research work. He received the "JAFIA scientific Award" (2002 by the Japanese Association for Flow Injection Analysis, JAFIA, Outstanding Person of Thailand in Science and Technology (Analytical Chemistry) (2004 by the Office of the National Uniqueness of Thailand), Outstanding Scientist (2001 by the Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King), Outstanding

Researcher in Chemical and Pharmaceutical Sciences (1999 by the National Research Council of Thailand), and TRF Senior Research Scholar I (2001, by the Thailand Research Fund). He has served on various committees including of the National Research Council of Thailand in chemical and pharmaceutical sciences (2002-present), editorial advisory boards: Talanta (Elsevier) (2000-present) and Journal of Flow Injection Analysis (Japanese Association for Flow Injection Analysis) (2003-present), Associate Editor: Water Research (Elsevier) (2003-present), Editorial board: Laboratory Robotics and Automation (LRA) (Wiley) (1997-2000), and Guest editor for special issues of the above journals. Professer Dr. Grudpan has been invited to be a course director, an organizer, and a co-organizer for national and international training courses, workshops and Conferences including International Atomic Energy Agency (IAEA) courses, PACIFICHEM 2005, International Conference on Flow Injection Analysis (ICFIA), Flow Analysis Conference, and the Interfaces of Analytical Sciences: Workshop of U.S. and Thai Analytical Scientists.

Professor Dr.Grudpan's research concerns development in various aspects of analytical sciences with an emphasis on flow-based techniques with cost-effectiveness and miniaturization. Following the experiences in microfluidics with flow injection (FI), further development would involve sequential injection (SI), bead injection (BI), Lab-on-Valve (LOV), Lab-at-Valve (LAV), and Lab-on-Chip (LOC) approaches.

Consideration of downscaling/miniaturization and micro-total analysis (TAS) will be of interest.

Developments of micro-and nano-scale analysis will be made. They involve: development of instrumentation, novel approaches for flow-based analysis, new materials employed in chemical analysis and applications of flow-based analysis, which would aim for agricultural, environmental, process control and clinical/medical analysis. For the last, fast screening for diseases using some biomarkers will also be investigated as well as high sample through-put screening in the drug discovery study. The fast screening for diseases should be useful in telehealth.

The research should have impact on communities and society.

ผลงานวิชาการระหว่างปี ค.ศ.2000-ปัจจุบัน

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มหาวิทยาลัยเชียงใหม่
CHIANG MAI UNIVERSITY

รางวัลมหาวิทยาลัยเชียงใหม่ ประจำปี ๒๕๕๐

"ช้างทองคำ" และ "โล่ประกาศเกียรติคุณ" และ ประกาศเกียรติคุณ "ศาสตราจารย์"

นักวิจัยดีเด่น สาขาวิทยาศาสตร์และเทคโนโลยี

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

ตำแหน่ง ศาสตราจารย์ ระดับ 10

สังกัด ภาควิชาเคมี คณะวิทยาศาสตร์

เริ่มรับราชการในตำแหน่งอาจารย์ เมื่อวันที่ 15 พฤษภาคม 2517

ได้รับแต่งตั้งให้ดำรงตำแหน่งรองศาสตราจารย์ โดยวิธีพิเศษ เมื่อวันที่ 11 กันยายน 2534

ได้รับพระบรมราชโองการโปรดเกล้าฯ แต่งตั้งให้ดำรงตำแหน่งศาสตราจารย์ ระดับ 10 เมื่อวันที่ 22 กันยายน 2547

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์ ได้บุกเบิกงานวิจัยทางเคมีวิเคราะห์แผนใหม่โดยอาศัยหลักการไหล (flow-based analytical techniques) หลากหลายเทคนิคมาอย่างต่อเนื่องเป็นเวลามากกว่า 25 ปี ซึ่งในปัจจุบันสามารถลดขนาดของการวิเคราะห์ลงมาใช้สารเคมีในปริมาณที่น้อยในระดับไมโครลิตร เป็นระบบอัตโนมัติที่พัฒนาขึ้นมาจากบุคลากรวิจัยของมหาวิทยาลัยเชียงใหม่ โดยเน้นการประยุกต์สำหรับการแก้ไขปัญหาเคมีวิเคราะห์ในประเทศไทย ที่ประหยัดทั้งเวลาและค่าใช้จ่าย พร้อมทั้งเกิดนวัตกรรมในระดับสากล เป็นรากฐานในการศึกษาวิจัยในแขนงต่างๆ ทั้งทางด้านสิ่งแวดล้อม การวิเคราะห์ทางยา การวิเคราะห์สารบ่งชี้ทางชีวภาพเพื่อคัดกรองในการวินิจฉัยโรคต่างๆ และทางเกษตรกรรม โดย การวิจัยพัฒนาที่อาศัยอาศัยหลักการไหล ดังกล่าวทำให้ได้ระบบและวิธีการวิเคราะห์แบบใหม่ที่มีความถูกต้อง แม่นยำ ประหยัดค่าใช้จ่ายและเวลา เป็นที่ยอมรับในวงวิชาการ โดยได้เผยแพร่ผลงานวิจัยในวารสารวิชาการนานาชาติมากกว่า 100 เรื่อง ทั้งนี้ **ศาสตราจารย์ ดร.เกตุ กรุดพันธ์** มีความมุ่งมั่นในการวิจัยเพื่อการพัฒนาที่ยั่งยืนตามแนวพระราชดำริในแนวทางเศรษฐกิจพอเพียง เพื่อความเป็นอยู่ที่ดีขึ้นสำหรับคนทุกระดับ

มหาวิทยาลัยเชียงใหม่
CHIANG MAI UNIVERSITY

รางวัลมหาวิทยาลัยเชียงใหม่ ประจำปี ๒๕๕๐

"ช้างทองคำ" และ "โล่ประกาศเกียรติคุณ" และ ประกาศเกียรติคุณ "ศาสตราจารย์"

นักวิจัยดีเด่น สาขาวิทยาศาสตร์และเทคโนโลยี

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

ตำแหน่ง ศาสตราจารย์ ระดับ 10

สังกัด ภาควิชาเคมี คณะวิทยาศาสตร์

เริ่มรับราชการในตำแหน่งอาจารย์ เมื่อวันที่ 15 พฤษภาคม 2517

ได้รับแต่งตั้งให้ดำรงตำแหน่งรองศาสตราจารย์ โดยวิธีพิเศษ เมื่อวันที่ 11 กันยายน 2534

ได้รับพระบรมราชโองการโปรดเกล้าฯ แต่งตั้งให้ดำรงตำแหน่งศาสตราจารย์ ระดับ 10 เมื่อวันที่ 22 กันยายน 2547

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์ ได้บุกเบิกงานวิจัยทางเคมีวิเคราะห์แผนใหม่โดยอาศัยหลักการไหล (flow-based analytical techniques) หลากหลายเทคนิคมาอย่างต่อเนื่องเป็นเวลามากกว่า 25 ปี ซึ่งในปัจจุบันสามารถลดขนาดของการวิเคราะห์ลงมาใช้สารเคมีในปริมาณที่น้อยในระดับไมโครลิตร เป็นระบบอัตโนมัติที่พัฒนาขึ้นมาจากบุคลากรวิจัยของมหาวิทยาลัยเชียงใหม่ โดยเน้นการประยุกต์สำหรับการแก้ไขปัญหาเคมีวิเคราะห์ในประเทศไทย ที่ประหยัดทั้งเวลาและค่าใช้จ่าย พร้อมทั้งเกิดนวัตกรรมในระดับสากล เป็นรากฐานในการศึกษาวิจัยในแขนงต่างๆ ทั้งทางด้านสิ่งแวดล้อม การวิเคราะห์ทางยา การวิเคราะห์สารบ่งชี้ทางชีวภาพเพื่อคัดกรองในการวินิจฉัยโรคต่างๆ และทางเกษตรกรรม โดย การวิจัยพัฒนาที่อาศัยอาศัยหลักการไหล ดังกล่าวทำให้ได้ระบบและวิธีการวิเคราะห์แบบใหม่ที่มีความถูกต้อง แม่นยำ ประหยัดค่าใช้จ่ายและเวลา เป็นที่ยอมรับในวงวิชาการ โดยได้เผยแพร่ผลงานวิจัยในวารสารวิชาการนานาชาติมากกว่า 100 เรื่อง ทั้งนี้ **ศาสตราจารย์ ดร.เกตุ กรุดพันธ์** มีความมุ่งมั่นในการวิจัยเพื่อการพัฒนาที่ยั่งยืนตามแนวพระราชดำริในแนวทางเศรษฐกิจพอเพียง เพื่อความเป็นอยู่ที่ดีขึ้นสำหรับคนทุกระดับ

รองศาสตราจารย์เกตุ กรุดพันธ์

บุคคลดีเด่นของชาติ

สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์)

ประจำพุทธศักราช ๒๕๔๗

นักวิจัย มช.

รับรางวัล "บุคคลดีเด่นของชาติ"

รองศาสตราจารย์ ดร.เกตุ กรุดพันธ์ อาจารย์นักวิจัย ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ รับเชิดชูเกียรติบุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี ด้านเคมีวิเคราะห์ ประจำปี 2547

คณะอนุกรรมการคัดเลือกและแผยแพร่ผลงานดีเด่นของชาติ โดยคณะกรรมการเอกลักษณ์ของชาติ ได้สรรหาคัดเลือกผลงานดีเด่นของบุคคล หน่วยงาน และโครงการดีเด่นของชาติ เพื่อเป็นการส่งเสริมและให้กำลังใจบุคคลและหน่วยงานที่ทำคุณประโยชน์ต่อประเทศชาติ โดยในปีนี้ได้พิจารณาคัดเลือกเป็นที่เรียบร้อย ผลปรากฏว่า รองศาสตราจารย์ ดร.เกตุ กรุดพันธ์ รองศาสตราจารย์ระดับ 9 ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ ได้รับการประกาศเกียรติคุณ เป็นบุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์) ประจำปี 2547 ซึ่งคณะอนุกรรมการฯ จะได้นำผู้ได้รับการประกาศเกียรติคุณ เข้าเฝ้าทูลละอองธุลีพระบาทพระบาทสมเด็จพระเจ้าอยู่หัว เพื่อรับพระราชทานรางวัลเกียรติคุณบุคคลและหน่วยงานดีเด่นของชาติในโอกาสต่อไป

รองศาสตราจารย์ ดร.เกตุ กรุดพันธ์ เป็นผู้ได้รับการยกย่องเชิดชูเกียรติมาแล้วหลายรางวัล อาทิ เมธีวิจัยอาวุโส สำนักงาน คณะกรรมการวิจัยแห่งชาติ สาขาเคมี ประจำปี 2544 - 2546 นักวิทยาศาสตร์ดีเด่น สาขาเคมีวิเคราะห์ ปี 2544 จากมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยี ในพระบรมราชูปถัมภ์ นักวิจัยดีเด่นแห่งชาติ สาขาวิทยาศาสตร์เคมี-เภสัช ปี 2542 ของสภาวิจัยฯ และ นักศึกษาเก่าดีเด่นมหาวิทยาลัยเชียงใหม่ ประจำปี 2542 เป็นต้น

คณะกรรมการเอกลักษณ์ของชาติ
สำนักนายกรัฐมนตรี

ขอมอบเกียรติบัตรนี้ให้แก่

รองศาสตราจารย์เกตุ กรุดพันธ์
บุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์)

ประจำปีพุทธศักราช ๒๕๔๗

จึงขอมอบเกียรติบัตรนี้เพื่อเป็นเกียรติ และศักดิ์ศรีสืบไป

(พลเอกวิษณุ เครืองาม)
รองนายกรัฐมนตรี
ประธานกรรมการเอกลักษณ์ของชาติ

(พลเอกพิจิต ศิริสัมพันธ์)
ประธานอนุกรรมการคัดเลือกและเผยแพร่ผลงานดีเด่นของชาติ

สำนักงานกองทุนสนับสนุนการวิจัย
THE THAILAND RESEARCH FUND

เมธีวิจัยอาวุโส สกว. ๒๕๔๗-๒๕๔๘
TRF Senior Research Scholar 2004-2005

สาขาเคมีวิเคราะ

ศาสตราจารย์ ดร.เกตุ กรุดพัน
Professor Dr. Kate Grudpa

ประวัติส่วนตัว

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์ เกิดเมื่อวันที่ 2 ตุลาคม พ.ศ. 2496 ที่ อำเภอบางกรวย จังหวัดนนทบุรี เป็นบุตรนายศักดิ์ และนางอุบล กรุดพันธ์ สมรสกับ นางธนกร กรุดพันธ์ มีธิดา 1 คน (นางสาวศุภรา กรุดพันธ์) เริ่มการศึกษาที่ จังหวัดนนทบุรี โดยเริ่มระดับประถมศึกษาที่โรงเรียนวัดศรีประวัติ(ประชานิยม) ระดับมัธยมศึกษาตอนต้นที่โรงเรียนบางกรวย และระดับมัธยมศึกษาตอนปลาย ที่โรงเรียนเขมาภิรตาราม สำเร็จการศึกษาระดับปริญญาตรี สาขาวิชาเคมี จากมหาวิทยาลัยเชียงใหม่ ในปี พ.ศ. 2517 และระดับปริญญาเอก สาขาเคมีวิเคราะห์ จาก Liverpool John Moores University ในปี พ.ศ. 2524 ด้วยทุน Research assistantship ของ Liverpool Education Authority ได้มีประสบการณ์หลังปริญญาเอกหลายแห่ง รวมถึง Alexander von Humboldt Fellow ที่ Karl rule Research Center เยอรมนี (พ.ศ.2534-2535) และ The Royal Society Visiting Fellow ที่ Liverpool John Moores University (พ.ศ.2541)

ประวัติการทำงาน

เริ่มรับราชการที่ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ ในตำแห่ง อาจารย์ พ.ศ.2517 ดำรงตำแหน่ง ศาสตราจารย์ (โดยวิธีพิเศษ) ใน พ.ศ.2534 ดำรงตำแหน่งศาสตราจารย์ (โดยเสนอผลงานวิจัยล้วนๆ) ใน พ.ศ.2547 เคยเป็นสมาชิก อาจารย์ มหาวิทยาลัยเชียงใหม่ และทำงานบริหารในระดับต่างๆ เช่น ผู้ช่วยคณบดี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ เป็นต้น ได้ริเริ่มโครงการปริญญาเอกร่วมสถาบันในต่างประเทศ ในลักษณะ Sandwich Program ตั้งแต่ปี พ.ศ.2534 เป็นกรรมการสภาวิจัยแห่งชาติ สาขาวิทยาศาสตร์เคมีและเภสัช (พ.ศ.2545-ปัจจุบัน) ได้รับเกียรติจากทบวงการพลังงานปรมาณูระหว่างประเทศ (International Atomic Energy : IAEA) เพื่อทำงานด้านต่าง ๆ ได้แก่ เป็น Course Director ของการจัด Regional Training Course for South Asia in Water Monitoring ที่ภาควิชาเคมี มหาวิทยาลัยเชียงใหม่ (3-21 พฤศจิกายน พ.ศ.2540) เป็นผู้เขียนรายงาน ให้คำแนะนำด้าน Nuclear and Radiochemistry ที่ประเทศพม่า (Yangon University และ Mawlamyai University) (7-22 ตุลาคม พ.ศ.2538) และเป็นผู้จัดการฝึกอบรมระดับชาติ ทาง Nuclear and Radiochemistry โดยการสนับสนุนของ IAEA และรัฐบาลสหพันธ์

สาธารณรัฐเยอรมันซึ่งจัดขึ้นที่มหาวิทยาลัยเชียงใหม่จำนวน ครั้ง ได้ฝึกอบรมให้นักวิทยาศาสตร์ชาวพม่าอีก 6 คน ในระยะเวลามากกว่า 5 ปี ได้รับเชิญเป็นผู้ร่วมจัดการประชุมนานาชาติในหลายโอกาส เช่น ICAS 2001 (International Union of Pure and Applied Chemistry Congress on Analytical Science 2001) สิงหาคม พ.ศ. 2544 กรุงโตเกียว ประเทศญี่ปุ่น ได้รับเกียรติเป็น Co-organizer ในการจัดประชุมวิชาการนานาชาติ FACIFICHEM 2005 (19-20 December 2005) ใน Symposium#16 เป็นกรรมการใน International Advisory Board ของการจัดประชุมวิชาการนานาชาติ เช่น International Flow Analysis IX และ X ที่เมือง Geelong ประเทศออสเตรเลีย (February 2003) และโปรตุเกส (September 2006) เป็นสมาชิก ใน Streering Committee ในการจัด 12th และ 13th International Conference on Flow Injection Analysis, ที่เมือง Marida, Venezuela: (December 2003) และที่เมือง Las Vegas ประเทศสหรัฐอเมริกา (April 2005) เป็นผู้ประสานงานฝ่ายไทยในการจัด Interfaces of Analytical Sciences : Workshop of US and Thai Analytical Scientists ในเดือนมกราคม 2549 ที่เชียงใหม่โดยการสนับสนุนของ National Science Foundation (NSF) และหน่วยงานของไทยรวมถึงสำนักงานกองทุนสนับสนุนการวิจัยด้วย นอกจากนี้ยังได้รับเชิญเป็นวิทยากรบรรยายนำ และเป็นประธานในการประชุมวิชาการในระดับชาติและนานาชาติหลายครั้ง เช่น The 9th International Conference on Flow Injection Analysis ที่เมือง Seattle, USA, ในเดือนสิงหาคม พ.ศ.2541 ได้รับรางวัลนักวิจัยดีเด่นแห่งชาติ สาขาวิทยาศาสตร์เคมี-เภสัช ปี พ.ศ.2542 (สำนักงานคณะกรรมการวิจัยแห่งชาติ) นักศึกษาเก่าดีเด่น ปี พ.ศ.2542 (มหาวิทยาลัยเชียงใหม่) "Science & Technology Research Grant" ปี พ.ศ.2543 (Thailand Toray Science Foundation) เมธีวิจัยอาวุโส สกว. ครั้งที่ 1 ปี พ.ศ.2544-2546 ครั้งที่ 2 ปี พ.ศ.2547-2550 ได้รับรางวัลนักวิทยาศาสตร์ ดีเด่น ปี พ.ศ. 2544 (มูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์) และรางวัลเกียรติยศแก่นักศึกษาเก่ามหาวิทยาลัยเชียงใหม่ที่ประสบความสำเร็จในชีวิต จากสมาคมนักศึกษาเก่ามหาวิทยาลัยเชียงใหม่ รับรางวัล "JAFIA Scientific Award" จาก The Japanese Association for Flow Injection Analysis (JAFIA) (คนเอเชียคนแรกนอกญี่ปุ่น) บุคคลดีเด่นของชาติ สาขาวิทยาศาสตร์และเทคโนโลยี (ด้านเคมีวิเคราะห์) ประจำปี พ.ศ. 2547 จากสำนักงานเสริมสร้างเอกลักษณ์ของชาติ

นอกจากนี้ยังได้รับทุน/รางวัล/เกียรติยศ ต่าง ๆ เช่น ทุนพัฒนานักวิจัยรุ่นที่ 1 (2537-2540) และทุนองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา ของสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ทุนวิจัยคณะกรรมการวิจัยแห่งชาติร่วมมือกับประเทศอังกฤษ (พ.ศ. 2541-2542) Editorial Advisory Board ของวารสาร Talanta (Elsevier) (พ.ศ. 2543-ปัจจุบัน) และของวารสาร Journal of Flow Injection Analysis (Japanese Association for Flow Injection Analysis) (พ.ศ.2546-ปัจจุบัน) Associate Editor ของวารสาร Water Research (Elsevier) (พ.ศ. 2546- ปัจจุบัน) Editorial board ของวารสาร Laboratory Robotics and Automation (LRA) (Wiley) (พ.ศ.2540-2543) Guest Editor ของ special issue "The 11th International Conference on Flow Injection Analysis (ICFIA 2001) 11 Talanta, vol. 58 No. 6 Guest Editor ของ special issue "FIA in the Pacific Rims" ของ Laboratory Robotics and Automation, vol.12, No.3, 2000

ผลงานวิจัยโดยสรุป

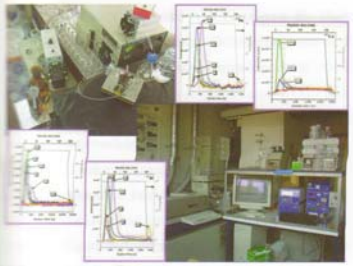
ได้สนใจในการวิจัยศึกษาและพัฒนาการวิเคราะห์ทางเคมีแผนใหม่โดยเฉพาะเทคนิคที่เกี่ยวข้องกับการไหล (Flow - based Analysis) โดยเน้นการพัฒนาฐานความรู้ของไทยเพื่อแก้ปัญหาของไทยและได้องค์ความรู้ใหม่เกิดนวัตกรรมใหม่ที่ได้รับการยอมรับในระดับสากลด้วย ผลงานหลายชิ้นเป็นเรื่องใหม่ในสาขาที่ทำการวิจัยที่เป็นรากฐานของการนำไปใช้ประโยชน์ในวงการต่าง ๆ เช่น อุตสาหกรรมเกษตร สิ่งแวดล้อม การแพทย์และการศึกษา

ในระดับอุดมศึกษา ได้มีความคิดริเริ่มในการทำงานอย่างจริงจังและต่อเนื่องในการค้นคว้าศึกษาวิจัย และพัฒนาทางด้านนี้ประมาณ 20 ปีจนเกิดประสิทธิผลของงานเป็นรูปธรรม โดยเริ่มแรกได้สนใจพัฒนาระบบการวิเคราะห์ทางเคมีในเทคนิค Flow Injection Analysis (FIA) ทั้งในส่วนของการพัฒนาอุปกรณ์ เครื่องมือที่มีราคาถูก การพัฒนาเทคนิคต่างๆ และปฏิกิริยาเคมีที่เกี่ยวข้องเพื่อให้ได้วิธีการวิเคราะห์แบบใหม่ที่มีความถูกต้อง แม่นยำสูง ประหยัดค่าใช้จ่ายและเวลาคน เป็นที่รู้จักโดยทั่วไปว่า "Low Cost Man"

การที่ได้สนใจเริ่มการทำวิจัยเพื่อศึกษาและพัฒนาการวิเคราะห์โดยเทคนิค Flow Injection Analysis (FIA) เนื่องจากเป็นการท้าทายในความสามารถที่จะทำวิจัยในความจำกัดในหลายๆ ด้าน โดยเฉพาะงบประมาณแต่ต้องการผลลัพธ์เพื่อแก้ไขปัญหาในประเทศและเกิดองค์ความรู้ใหม่ๆ ทางวิชาการเป็นที่ยอมรับ ในระดับสากลด้วยเมื่อพิจารณาถึงการควบคุมการผลิตในอุตสาหกรรม หรือการวิจัยในวิทยาศาสตร์สาขาต่างๆ สิ่งสำคัญที่ต้องคำนึงถึงเกี่ยวกับข้อมูลผลการวิเคราะห์เหล่านี้โดยทั่วไป ได้แก่ ความถูกต้อง ความแม่นยำ และความน่าเชื่อถือของข้อมูล เพราะการได้ข้อมูลที่ผิดพลาดจะทำให้การสรุปผลหรือการตัดสินใจผิดพลาด ก่อให้เกิดผลเสียได้ นอกจากนี้ต้องพิจารณาถึงสิ่งที่ต้องใช้ไปในการให้ได้มาซึ่งผลการวิเคราะห์ที่ต้องการ เช่น ค่าใช้จ่าย เวลา แรงงาน ซึ่งนอกจากจะนำมาใช้ในการแก้ปัญหาทางการวิเคราะห์ที่กำลังเผชิญอยู่ในปัจจุบันแล้ว ยังเป็นพื้นฐานหรือเครื่องมือในการวิจัยพัฒนาสิ่งใหม่ๆ ให้เกิดขึ้นด้วย สำหรับประเทศไทยยังมีการวิจัยพัฒนาทางด้านเคมีวิเคราะห์น้อยมาก ทำให้ต้องสูญเสียเงินตราในกิจกรรมทางการวิเคราะห์เคมีแก่ต่างประเทศเป็นจำนวนเงินที่สูงมาก เช่น การซื้อเครื่องมือวิเคราะห์ต่างๆ ทั้งที่เป็นแบบง่ายหรือชนิดที่ซับซ้อน รวมถึงอุปกรณ์และชิ้นส่วนของเครื่องมือวิเคราะห์ เป็นต้น จึงเป็นผลให้ขาดความรู้พื้นฐานวิทยาศาสตร์ในการพัฒนาเทคโนโลยีขึ้นเอง กลุ่มวิจัยได้มองเห็นปัญหาข้างต้นและเล็งเห็นถึงศักยภาพของเทคนิค Flow Injection Analysis (FIA) ในการพัฒนาวิธีวิเคราะห์ที่อาศัยเครื่องมือที่ไม่ซับซ้อน ราคาถูก มีค่าใช้จ่ายในการวิเคราะห์ต่ำแต่มีประสิทธิภาพสูง จึงได้มุ่งความสนใจพัฒนาระบบการวิเคราะห์ทางเคมีโดยเทคนิค Flow Injection Analysis และเทคนิคที่เกี่ยวข้อง เช่น Sequential Injection Analysis หรือ Bead Injection Analysis โดยเกิดนวัตกรรมในการพัฒนาเครื่องมือ-อุปกรณ์ เช่น การพัฒนาระบบ Flow Injection Systems (เช่น On-line dialysis, in-value column device) ใช้งานร่วมกับ Ion Chromatograph เกิดนวัตกรรมเกี่ยวกับเทคนิค เช่น On- line solvent extraction, การศึกษา Size-based speciation, Flow-injection with bead injection systems เป็นต้น การศึกษาเกี่ยวกับปฏิกิริยาเคมี โดยเฉพาะศึกษาปฏิกิริยาที่ใช้รีเอเจนต์ที่ไม่เสถียร แต่ราคาไม่แพง เช่น ด่างทับทิม ($KMnO_4$) หรือสารในไบฝรั่ง เป็นต้น การพัฒนาการวิเคราะห์ดังกล่าวนำไปสู่การใช้ประโยชน์ในกิจการในสาขาต่างๆ เช่น การศึกษาทางสิ่งแวดล้อม การควบคุมคุณภาพในอุตสาหกรรม การวิเคราะห์ทางเกษตรกรรม และการวิเคราะห์ในทางการแพทย์ เป็นต้น

งานวิจัยในอนาคต

จากการเริ่มด้วยการใช้วัสดุอุปกรณ์ราคาถูกเพื่อทำการวิจัยเทคนิค Flow Injection Analysis (FIA) ซึ่งเกี่ยวกับการไหลอย่างต่อเนื่อง (Continuous Flow) ทั้งนี้จะขยายการวิจัยเพื่อครอบคลุมเทคนิคการวิเคราะห์เกี่ยวกับการไหล (Flow-based Analysis) โดยพัฒนาเทคนิคที่เกิดขึ้นมาภายหลัง ได้แก่ Sequential Injection Analysis (SIA) ซึ่งจะไม่ไหลไปทางเดียวแต่จะใช้การไหลกลับไปกลับมา อาจบังคับให้ไหลๆ หยุดๆ ได้โดยการควบคุมด้วยคอมพิวเตอร์ นอกจากนี้จะเกี่ยวข้องกับการวิจัยเพื่อศึกษาเทคนิค Bead Injection Analysis (BIA) ที่ให้สารแขวนลอยของบีดไหลไปในท่อเล็กๆ แล้วกักไว้ที่ส่วนของการตรวจวัดซึ่งทำให้สามารถใช้ประโยชน์ของการเกิดปฏิกิริยาบนพื้นผิวของบีด แล้วไล่บีดออกทิ้งไป ใช้บีด



ชุดใหม่สำหรับการวิเคราะห์ในครั้งใหม่ จึงเกิดประโยชน์ขึ้นหลายประการรวมถึงการลดการรบกวน (Inference) ได้ด้วย ทั้งนี้สนใจที่จะวิจัยเพื่อพัฒนาระบบและเทคนิคการวิเคราะห์แบบใหม่ที่รวมถึง Lab-on-Value (LOV) และ Lab-at-Value (LAV) (เป็นเทคนิคที่นำเสนอโดยกลุ่มวิจัยฯ) รวมถึงเทคนิค Stopped-Flow based Analysis เพื่อประสิทธิภาพในกระบวนการวิเคราะห์ด้วยเทคนิคต่างๆ ที่เกี่ยวข้องดังกล่าวใช้พื้นฐานทาง Microfluidics ซึ่งจะเป็นประโยชน์ในการวิจัยเพื่อพัฒนาเพื่อลดขนาดลง (down-scaling หรือ miniaturization) ซึ่งจะเกี่ยวข้องกับ Lab-on-Chip (LOC) ทั้งนี้ สนใจที่จะพัฒนาการวิเคราะห์ระดับไมโครและนาโนโดยใช้เทคนิคที่อิงการไหล โดยพัฒนาระบบเครื่องมือ (Instrumentation) การวิจัยเพื่อให้ได้แนวทางใหม่ ๆ (Novel approaches) เพื่อใช้ในการไหล การพัฒนาใช้สารใหม่ (New materials) เพื่อการวิเคราะห์ทางเคมี และการประยุกต์เทคนิคการไหลเพื่องานต่างๆ รวมถึงการประยุกต์ทางการแพทย์ เช่น การคัดกรองผู้ป่วยโรคมะเร็ง โรคตับ โรคกระดูก โรคธาลัสซีเมีย งานวิจัยทางด้านนี้น่าจะเสริมกับแนวทางการพัฒนาแพทย์ทางไกล (Telehealth) แนวทางศึกษาการตอบสนองของกลุ่มเนื้อเยื่อ/ เซลล์ต่อการกระตุ้นของยา การคัดกรองอย่างรวดเร็วในการศึกษาทางยา (High sample through-put screening) เป็นต้น การประยุกต์เพื่อการวิเคราะห์ทางเคมีเพื่อการเกษตรและอุตสาหกรรมเกษตร การวิเคราะห์ในการศึกษาทางสิ่งแวดล้อม

การนำผลงานวิจัยไปใช้ประโยชน์

ประโยชน์ที่เกิดขึ้นจากงานวิจัยจะมีผลกระทบทั้งในแวดวงวิชาการเองแล้วน่าจะมีผลต่อสังคมด้วย ทั้งนี้ โดยการผ่านความร่วมมือของบุคลากรจากหลายหลายสถาบันในลักษณะเครือข่าย (Networking) และหลากหลายสาขาวิชา (Multidisplinary) จะทำให้เกิดการพัฒนางานวิจัยในลักษณะบูรณาการ ซึ่งจะเป็นประโยชน์และมีผลกระทบต่อองค์การวิชาการและสังคมด้วย จึงสนใจที่พยายามมองถึงการพัฒนาฐานความรู้ของไทย ใช้ทรัพยากรเพื่อแก้ปัญหาของประเทศ เพื่อให้ได้ความรู้ใหม่ที่ได้รับการยอมรับในระดับสากล ทั้งนี้สามารถนำองค์ความรู้และผลงานไปใช้ประโยชน์ในด้านการแพทย์ เกษตรกรรม อุตสาหกรรม และสิ่งแวดล้อม โดยคำนึงถึงการพัฒนาทรัพยากรมนุษย์ ไปพร้อมกันด้วยโดยจะพยายามผลักดันให้มีความร่วมมือในระหว่างบุคลากรของสถาบันต่าง ๆ ทั้งในประเทศและต่างประเทศ เช่น กลุ่มวิจัยต่างๆ ในประเทศญี่ปุ่น มีความร่วมมือกับ Japanese Association for Flow Injection Analysis และกลุ่มวิจัยต่าง ๆ ในสหรัฐอเมริกา โดยจัดประชุมเชิงปฏิบัติการ Interfaces of Analytical Sciences : Workshop of US and Thai Analytical Scientists พยายามทำงานเป็นทีมโดยดึงศักยภาพที่เด่นของสมาชิกแต่ละคนออกมาใช้อย่างมีประสิทธิภาพ เพื่อวางแผนเป็นระบบวิเคราะห์และสังเคราะห์ความรู้อย่างเป็นระบบ

Biography and Research Summary

Professer Dr. Kate Grudpan received his B.S. (Chemistry) from Chiang Mai University in 1974, and Ph.D. degree in analytical chemistry from Liverpool John Moores University, UK in 1981. He became a lecturer in the Department of Chemistry, Faculty of Science, Chiang Mai University in 1974, where is now a professor. Professor Dr.Grudpan has received many awards and honors during his career due to his extensive research work. He received the "JAFIA scientific Award" (2002 by the Japanese Association for Flow Injection Analysis, JAFIA, Outstanding Person of Thailand in Science and Technology (Analytical Chemistry) (2004 by the Office of the National Uniqueness of Thailand), Outstanding Scientist (2001 by the Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King), Outstanding

Researcher in Chemical and Pharmaceutical Sciences (1999 by the National Research Council of Thailand), and TRF Senior Research Scholar I (2001, by the Thailand Research Fund). He has served on various committees including of the National Research Council of Thailand in chemical and pharmaceutical sciences (2002-present), editorial advisory boards: Talanta (Elsevier) (2000-present) and Journal of Flow Injection Analysis (Japanese Association for Flow Injection Analysis) (2003-present), Associate Editor: Water Research (Elsevier) (2003-present), Editorial board: Laboratory Robotics and Automation (LRA) (Wiley) (1997-2000), and Guest editor for special issues of the above journals. Professer Dr. Grudpan has been invited to be a course director, an organizer, and a co-organizer for national and international training courses, workshops and Conferences including International Atomic Energy Agency (IAEA) courses, PACIFICHEM 2005, International Conference on Flow Injection Analysis (ICFIA), Flow Analysis Conference, and the Interfaces of Analytical Sciences: Workshop of U.S. and Thai Analytical Scientists.

Professor Dr.Grudpan's research concerns development in various aspects of analytical sciences with an emphasis on flow-based techniques with cost-effectiveness and miniaturization. Following the experiences in microfluidics with flow injection (FI), further development would involve sequential injection (SI), bead injection (BI), Lab-on-Valve (LOV), Lab-at-Valve (LAV), and Lab-on-Chip (LOC) approaches.

Consideration of downscaling/miniaturization and micro-total analysis (TAS) will be of interest.

Developments of micro-and nano-scale analysis will be made. They involve: development of instrumentation, novel approaches for flow-based analysis, new materials employed in chemical analysis and applications of flow-based analysis, which would aim for agricultural, environmental, process control and clinical/medical analysis. For the last, fast screening for diseases using some biomarkers will also be investigated as well as high sample through-put screening in the drug discovery study. The fast screening for diseases should be useful in telehealth.

The research should have impact on communities and society.

ผลงานวิชาการระหว่างปี ค.ศ.2000-ปัจจุบัน

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มหาวิทยาลัยเชียงใหม่
CHIANG MAI UNIVERSITY

รางวัลมหาวิทยาลัยเชียงใหม่ ประจำปี ๒๕๕๐

"ช้างทองคำ" และ "โล่ประกาศเกียรติคุณ" และ ประกาศเกียรติคุณ "ศาสตราจารย์"

นักวิจัยดีเด่น สาขาวิทยาศาสตร์และเทคโนโลยี

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์

ตำแหน่ง ศาสตราจารย์ ระดับ 10

สังกัด ภาควิชาเคมี คณะวิทยาศาสตร์

เริ่มรับราชการในตำแหน่งอาจารย์ เมื่อวันที่ 15 พฤษภาคม 2517

ได้รับแต่งตั้งให้ดำรงตำแหน่งรองศาสตราจารย์ โดยวิธีพิเศษ เมื่อวันที่ 11 กันยายน 2534

ได้รับพระบรมราชโองการโปรดเกล้าฯ แต่งตั้งให้ดำรงตำแหน่งศาสตราจารย์ ระดับ 10 เมื่อวันที่ 22 กันยายน 2547

ศาสตราจารย์ ดร.เกตุ กรุดพันธ์ ได้บุกเบิกงานวิจัยทางเคมีวิเคราะห์แผนใหม่โดยอาศัยหลักการไหล (flow-based analytical techniques) หลากหลายเทคนิคมาอย่างต่อเนื่องเป็นเวลามากกว่า 25 ปี ซึ่งในปัจจุบันสามารถลดขนาดของการวิเคราะห์ลงมาใช้สารเคมีในปริมาณที่น้อยในระดับไมโครลิตร เป็นระบบอัตโนมัติที่พัฒนาขึ้นมาจากบุคลากรวิจัยของมหาวิทยาลัยเชียงใหม่ โดยเน้นการประยุกต์สำหรับการแก้ไขปัญหาเคมีวิเคราะห์ในประเทศไทย ที่ประหยัดทั้งเวลาและค่าใช้จ่าย พร้อมทั้งเกิดนวัตกรรมในระดับสากล เป็นรากฐานในการศึกษาวิจัยในแขนงต่างๆ ทั้งทางด้านสิ่งแวดล้อม การวิเคราะห์ทางยา การวิเคราะห์สารบ่งชี้ทางชีวภาพเพื่อคัดกรองในการวินิจฉัยโรคต่างๆ และทางเกษตรกรรม โดย การวิจัยพัฒนาที่อาศัยอาศัยหลักการไหล ดังกล่าวทำให้ได้ระบบและวิธีการวิเคราะห์แบบใหม่ที่มีความถูกต้อง แม่นยำ ประหยัดค่าใช้จ่ายและเวลา เป็นที่ยอมรับในวงวิชาการ โดยได้เผยแพร่ผลงานวิจัยในวารสารวิชาการนานาชาติมากกว่า 100 เรื่อง ทั้งนี้ **ศาสตราจารย์ ดร.เกตุ กรุดพันธ์** มีความมุ่งมั่นในการวิจัยเพื่อการพัฒนาที่ยั่งยืนตามแนวพระราชดำริในแนวทางเศรษฐกิจพอเพียง เพื่อความเป็นอยู่ที่ดีขึ้นสำหรับคนทุกระดับ

INNOVATION INVENTION

นักเคมีสร้างอุปกรณ์หาสารรั่วระดับนาโน

นักวิจัยเชียงใหม่คิดค้นเทคนิคใหม่ วิเคราะห์สารเคมีตกค้าง หรือตรวจหาสารเคมีรั่วไหลได้อัตโนมัติ เผยสามารถประยุกต์ใช้วิเคราะห์ทางการแพทย์และสาธารณสุข ขณะที่นักลงทุนไทยและต่างชาติสนใจต่อยอดงานวิจัยสู่พาณิชย์

รศ.ดร.เกตุ กรุตพันธ์ นักวิจัยจากห้องปฏิบัติการสู่ความเป็นเลิศทางวิชาการ ด้านการพัฒนาเพื่อการลดขนาดในการวิเคราะห์โดยการไหล ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ เปิดเผยว่า ทีมวิจัยได้พัฒนาเทคนิคการวิเคราะห์ทางเคมีแบบใหม่ โดยใช้เทคนิคการไหลของสาร (Flowbased Analysis) สามารถช่วยโรงงานอุตสาหกรรมตรวจวิเคราะห์สารเคมีตกค้างในผลิตภัณฑ์ต่างๆ อาทิ อาหาร กุ้งหรือไก่สดส่งออก รวมทั้งตรวจหาสารเคมีรั่วไหลในกระบวนการผลิต

ทีมวิจัยได้พัฒนาเพื่อเพิ่มระบบและวิธีการวิเคราะห์ที่มีอยู่ ให้มีประสิทธิภาพและรวดเร็วขึ้น โดยระบบที่คิดค้นขึ้นนี้สามารถตรวจสอบเคมีได้ตั้งแต่ 60-360 ตัวอย่างต่อชั่วโมง และตรวจวิเคราะห์ได้แม้กระทั่งสารเคมีปนเปื้อนหรือตกค้างในระดับต่ำเพียง 1 ใน 1000 ล้านส่วน รวมทั้งตัวเครื่องประกอบขึ้นเองภายในประเทศ ส่งผลให้การวิเคราะห์มีราคาถูก

"เครื่องมือวิเคราะห์ที่สร้างขึ้นนี้ได้รับการยอมรับในวงการวิชาการระดับสากล และยังได้รับความร่วมมือจากสถาบันการศึกษาทั้งในและต่างประเทศมาร่วมทำงานวิจัย รวมถึงภาคเอกชนเองได้เข้ามามีส่วนร่วมในการพัฒนางานวิจัยให้เป็นประโยชน์ในเชิงพาณิชย์ โดยสำนักงานนวัตกรรมแห่งชาติ (สนช.) สนับสนุนทุนวิจัย" รศ.ดร.เกตุ กล่าว

นอกจากนี้ เทคนิคการไหลที่พัฒนาขึ้นยังสามารถประยุกต์ใช้กับการตรวจวิเคราะห์ด้านต่างๆ อาทิ การวิเคราะห์ทางสิ่งแวดล้อม ซึ่งจากความรู้ที่ได้นำไปสู่การสร้างเครื่องมือแบบอัตโนมัติเพื่อตรวจหาโลหะหนักที่ปนเปื้อนในน้ำได้อย่างรวดเร็ว เช่น ตะกั่ว แคดเมียม ทองแดง สังกะสี โดยได้รับความร่วมมือระหว่างกลุ่มวิจัยกับบริษัทเอกชนในสวิตเซอร์แลนด์ อีกทั้งสามารถวิเคราะห์น้ำเสียจากโรงงาน

ทั้งนี้ ทีมวิจัยยังได้ร่วมมือกับกลุ่มวิจัยจากญี่ปุ่น พัฒนาเครื่องมือวิเคราะห์คลอไรด์และเครื่องวิเคราะห์สารเคมีในระดับนาโน โดยอาศัยพื้นฐานความรู้จากเทคนิคการไหล ซึ่งขณะนี้อยู่ในขั้นตอนการพิจารณาต้นแบบเชิงพาณิชย์ และยังมีความร่วมมือกับนักวิจัยสวีเดนคิดค้นวิธีการวิเคราะห์อะไมโลสในแป้งและข้าว

ในส่วนการใช้ประโยชน์ทางการแพทย์ สามารถนำไปสร้างเครื่องมือตรวจคัดกรองผู้ป่วยมะเร็งและโรคข้อ เพื่อช่วยในการวินิจฉัยโรคให้ถูกต้อง รวดเร็วและราคาถูก โดยเครื่องดังกล่าวกำลังอยู่ในขั้นตอนของการพัฒนาเช่นกัน จากนั้นจึงจะนำไปใช้ประโยชน์จริงในอนาคต

ภาคผนวก ค

การจัดประชุมวิชาการประจำปี

ภาคผนวก ค1	The 4th Annual Symposium on TRF Senior Research Scholar On "Development of Micro-and Nano-scale Analysis by Flow-based Techniques I"
ภาคผนวก ค2	The 5th Annual Symposium on TRF Senior Research Scholar on "Development of Micro-and Nano-scale Analysis by Flow-based Techniques II"
ภาคผนวก ค3	The 6th Annual Symposium: TRF Senior Research Scholar and Research group on Innovation on Analytical Instrumentation CHE: "Development of Flow-based Analysis for Better Life Quality"
ภาคผนวก ค4	Interfaces of Analytical Sciences: Workshop of U.S. and Thai Analytical Scientists

ภาคผนวก ค1

การจัดประชุมวิชาการประจำปี

The 4th Annual Symposium on TRF Senior Research Scholar On

"Development of Micro-and Nano-scale Analysis by Flow-based Techniques I"

The 4th Annual Symposium on TRF Senior Research Scholar on "Development of Micro-and Nano-scale Analysis by Flow-based Techniques I"

Program & Abstracts

19th September 2005

Chiang Mai University

Chiang Mai, THAILAND

The 4th Annual Symposium on TRF Senior Research Scholar on
"Development of Micro-and Nano-scale Analysis by Flow-based Techniques I"
At Room 1 ScB 2
Faculty of Science, Chiang Mai University
19th September 2004

8.15 - 8.30	Registration	
8.30 - 8.45	**Opening session** - "Symposium Objectives" and "Progress on the Development of Micro-and Nano-scale Analysis by Flow-based Techniques: the phase II on the TRF Senior Scholar Project" by Prof. Dr. Kate Grudpan, TRF Senior Research Scholar - Opening address by the Director, Academic Division, TRF	
8.45 - 9.10	**Plenary Lecture I:** Prof. Dr. Tadao Sakai, Aichi Institute of Technology, Japan.	
9.10 - 9.35	**Plenary Lecture II:** Prof. Dr. Shoji Motomizu, Department of Chemistry, Faculty of Science, Okayama University, Japan.	
9.35 - 10.00	*Coffee break*	
10.00 - 10.15	**Invited Lecture I:** Prof. Dr. H. Itabashi, Gunma University, Japan.	
10.15 - 10.30	**Invited Lecture II:** Assoc. Prof. Dr. N. Teshima, Aichi Institute of Technology, Japan.	
10.30 - 10.45	**Invited Lecture III:** Dr. K. Higuchi, Ogawa Co. Ltd., Japan.	
10.45 - 10.55	*Oral presentation 1	(for P-01, Dr. D. Nacapricha)
10.55 - 11.05	*Oral presentation 2	(for P-02-03, W. Siangproh)
11.05 - 11.15	*Oral presentation 3	(for P-08, Dr. R. Burakham)
11.15 - 11.25	*Oral presentation 4	(for P-09, W. Siriangkhawut)
11.25 - 11.35	*Oral presentation 5	(for P-10, N. Miyoshi)
11.35 - 11.45	*Oral presentation 6	(for P-11, T. Suekane)
11.45 - 11.55	*Oral presentation 7	(for P-12, S. Somnam)
11.55 - 12.05	*Oral presentation 8	(for P-13, K. Watla-iad)
12.05 - 12.15	*Oral presentation 9	(for P-14, D. Somprayoon)
12.15 - 13.30	*Lunch*	
13.30 - 14.30	Discussion at Posters	
14.30 - 16.00	Discussion in Overall	

* Note (1) Some from the all posters presented
(2) Should be with in 3 slides

List of Presentations

No.	Title
PL-01	Multi Element Detection Systems using Flow-based Techniques
PL-02	Ultratrace Analytical Chemistry Coupled with Flow-Based Techniques: Its Great Contribution to Now-Going Manufacturing
ILP-01	All Injection Analysis for Determination of Chromium(VI)
ILP-02	Flow Analysis of Gaseous Samples: Nitrogen Oxides in Air and Acetone in Breath
ILP-03	Simple and Mild On-Line Photo-Chemical Decomposition / Analytical Method for Organic Phosphorus Compounds
ILP-04	Non-Poisonous Analytical Method for Nitrate Based on Photo-Chemical Reduction
P-01	Application of Boron-doped Diamond Thin Film Electrode for Ion Chromatography
P-02	Microchip Capillary Electrophoresis with Screen-printed Carbon Electrode for the Analysis of Plastic Explosives
P-03	Determination of Hydrazine Compounds Using Microchip Capillary Electrophoresis and a Cobalt Phthalocyanine Modified Electrochemical Detector
P-04	Electrochemical Oxidation of Malachite Green and Leucomalachite Green at Boron-Doped Diamond Thin Film Electrode Applied to Flow Injection System
P-05	Electrochemical Analysis Sulfonamides by FIA and HPLC Coupled with Amperometric Detection in Eggs Using Boron-doped Diamond Thin Film Electrode
P-06	Analysis of Carboxylic Acids using Capillary Zone Electrophoresis with Pre-Capillary Derivatization
P-07	Anion Exchange Chromatography for Determination of Carboxylic Acids in Cane Juice and Raw Sugar

P-08	Sequential Injection-Lab-at-Valve for On-line Micro Extraction
P-09	Sequential Injection-Monosegmented Flow for On-line Standard Addition Voltammetric Determination of Some Heavy Metals
P-10	Determination of Vanadium by Using Chelating Disk for Collection / Concentration
P-11	Simple FIA System for Monitoring Ammonia in Air: Development for a Study in Thailand
P-12	Hydrodynamic Flow System for the Determination of Iron
P-13	Sequential Injection Analysis for Bradford Protein in Milk
P-14	Online Determination of Bone Alkaline Phosphatase Using Flow Injection-bead Injection System
P-15	SIA-Capillary Immunoassay System for Determination of Hyaruronic Acid
P-16	Novel Approach for Micro-titration Using SIA-LOV
P-17	Flow Injection Determination of Iron Using Guava Leaf Extract as an Alternative Natural Reagent
P-18	Reinvestigation of the Electrochemical Reactions in the Phosphomolybdate Formation
P-19	Spectrophotometric Determination of Dissolved Inorganic Carbon (DIC) and Dissolved Organic Carbon (DOC) in Natural Water by Using Sequential Injection Analysis with On-line UV Photo-Oxidation
P-20	Lab-on-a-Chip in Thailand
P-21	A Simple Sequential Injection Analysis Method for Determination of Iron
P-22	Spectrophotometric Determination of Zinc in Pharmaceutical Preparations by Use of a Sequential Injection Analysis System with 1-(2-Pyridylazo)-2-Naphthol and Non-Ionic Surfactant

P-23	Optical Sensor for Determination of Copper in Micro Flow System
P-24	Colorimetric Flow-Injection Analysis Assay of Tetracycline Antibiotics using A Dual Light–Emitting Diode based Detector
P-25	Online Sulfide Removal for Phosphate Determination
P-26	Optimization of Capillary Electrophoresis Technique for Separation of Organophosphopous
P-27	Calibration Method for Simultaneous Determination of Chlorate and Chlorite from Flow Injection-Differential Pulse Voltammogram in Agrochemical Samples
P-28	Program for Fast Identification of Gasoline Trademarks by their Dynamic Interfacial Pressure

A Report on the 4th Annual Symposium on TRF Senior Research Scholar on Flow Based Analysis in Thailand*

Tadao Sakai

Department of Applied Chemistry, Aichi Institute of Technology, 1247 Yachigusa, Yakusa-cho, Toyota 470-0392, Japan

*Translated from original Japanese, published in Journal of Flow Injection Analysis, Vol. 22, No. 2, pp. 138-139, to this English version by Associate Professor Norio Teshima, Aichi Institute of Technology, Japan.

The 4th Annual symposium on TRF senior research scholar on "Development of micro- and nano-scale analysis by flow-based techniques I" was held in Chiang Mai University on Sep. 19th, 2005. The TRF has been supported by Thai government for 6 years. This symposium was organized by Prof. Kate Grudpan of Chiang Mai University who has a strong relationship to Japanese Association for Flow Injection Analysis (JAFIA) members. Kate was promoted to professor on November, 2004. I am very pleased to hear that he is the first professor of analytical chemistry field in Thailand and also the first professor in Department of Chemistry, Chiang Mai University. I believe that all JAFIA members must also feel happy to hear the good news. Kate organized the 11th International Conference on Flow Injection Analysis held on December, 2001 which was a very impressive conference. He was a winner of FIA Award for Science in 2003 when we celebrated the twenty fifth anniversary of the founding of JAFIA. Also, he has been to Japan many times, and I think that he has enjoyed friendship with JAFIA members for the last decade. The symposium has been dependent on his great effort for these days. Prof. Shoji Motomizu of Okayama University as a JAFIA member attended the 1st TRF symposium. I have also attended the TRF symposium since the 2nd. Last year Prof. Motomizu, Prof. Hiroyuki Ukeda of Kochi University, Prof. Hideyuki Itabashi of Gunma Univesity, Dr. Keiro Higuchi of F・I・A instruments (his current affiliation: Ogawa & Co.) and I attended the 3rd symposium, and Dr. Norio Teshima joined Japanese participants from this symposium.

Recently, the Thai government has been taking positive attitude to deal with education for young generation and the international cultural and academic exchanges. The symposium has been developed by Thailand Research Fund (TRF) and Postgraduate Education and Research Program in Chemistry. The project is evaluated every three years, and the Phase II project has just begun since this year. Kate told me that the primary aim of this project was to develop miniaturization and micro-total-analysis system (μTAS) such as lab-at-valve (LAV), micro-titration, SIA with in-line standard addition and stopped-flow techniques. The researches on flow-based analytical methods of analysis have been expanding from Chiang Mai University to other universities such as Mahidol University, Chulalongkorn University, Khon Kean University and so on. Also, the symposium gives good opportunities to young researchers such as posdocs, Ph.D. and/or M.S. students, so that they can internationalize themselves and build up their international relationships. Prof. Gary D. Chiristian of University of Washington (USA), Prof. Jaromir Ruzicka of University of Washington, Prof. Ian D. McKelvie of Monash University

(Australia), Elo H. Hansen of Technical University of Denmark (Denmark) and Prof. Purnendu K. Dasgupta of Texas Tech University (USA) have contributed their help to the project.

An opening session started at 8:30 am, and Prof. Kate gave us his opening talk which included the aim and successful results of the symposium, followed by an address given by the secretariat of the TRF. There were two plenary lectures (PL I, PL II) and three invited lectures (IL I, IL II, IL III) as follows.

PL I: "Multi element detection systems using flow-based techniques" by Tadao Sakai (Aichi Institute of Technology).

PL II: "Ultratrace analytical chemistry coupled with flow-based techniques: its great contribution to now-going manufacturing" by Shoji Motomizu (Okayama University)

IL I: "All injection analysis for determination of chromium(VI) by Hideyuki Itabashi (Gunma University).

IL II: "Flow analysis of gaseous samples: nitrogen oxides in air and acetone in breath" by Norio Teshima (Aichi Institute of Technology).

IL III: "Simple and mild on-line photo-chemical decomposition/analytical method for organic phosphorus compounds" by Keiro Higuchi (Ogawa & Co.).

Thereafter, there were nine oral presentations by Ph.D. or M.S. students. In this section, Miss Miyoshi and Mr. Suekane who are Japanese exchange students from Prof. Motomizu's group to Prof. Kate's group (four months) gave their talks about their works at Chiang Mai University as follows.

N. Miyoshi "Determination of vanadium by using chelating disk for collection/concentration".

T. Suekane "Simple FIA system for monitoring ammonia in air: development for a study in Thailand".

I admired their good talks. In Japan, a lot of foreign students are invited, however, M.S. course students are not sent to abroad very often. I believe that they must have valuable and wonderful experience. Some students in Kate's group have helped them every weekend out of pure friendship. I think that they are exchanging academic things and their culture as well. This may be "real international exchanges". On the other hand, many Thai Ph.D. students are sent to abroad such as USA, Australia, UK, Japan and so on for one year under a student supporting program by Thai government before getting their Ph.D. degree. I think that Japan should adopt such education system.

After lunch, there were thirty two poster presentations. In the poster session, Thai students were explaining their work eagerly to the Japanese attendances and asking some comment from us. When I was seeing their behavior, I felt great responsibility.

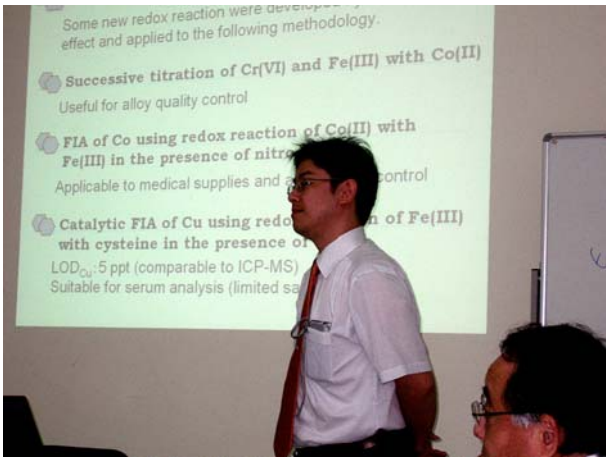
A discussion concerning expectation of developments in FIA and SIA was presided over by Kate in a closing session. The discussion covered "the spread of FIA instruments for practical use", "automation of FIA", "computer science", "zero-emission type FIA", "on-site analysis system", etc.

After the TRF symposium, Dr. Duangjai Nacapricha of Mahidol University and Dr. Orawon Chailapakul of Chulalongkorn University invited me and Teshima to their Universities. In Mahidol University and Chulalongkorn University, I gave lectures entitled "Two phase titration using ion association reaction with hydrophobic indicators" and "Ion association reactions for trace metals and onium compounds analyses using monoprotic acid dyes", respectively. Also, Teshima gave lectures entitled "New heater device and catalytic reaction available for flow-based analysis" (at Mahidol) and "Effect of ligand on redox reaction of metal ions and its application to analytical chemistry" (at Chulalongkorn). Drs. Nacapricha and Chailapukul guided us to each faculty. We also enjoyed talking with them and their students in coffee break with sweets. I appreciate their all kindness.

Prof. Motomizu visited Khon Kean University and Mahasarakham University to give lectures. Prof. Itabashi and Dr. Higuchi talked with Kate about collaboration with Chiang Mai University. We all Japanese members got back to Japan safely.

This exchange has given great contribution to Thai/Japanese researchers and students. I have warm and friendly feeling for Kate' group members. Therefore it is possible to develop various flow-based methods of analysis under collaboration with each other.

I hope that other JAFIA members would join us and I would take my students to the next symposium.



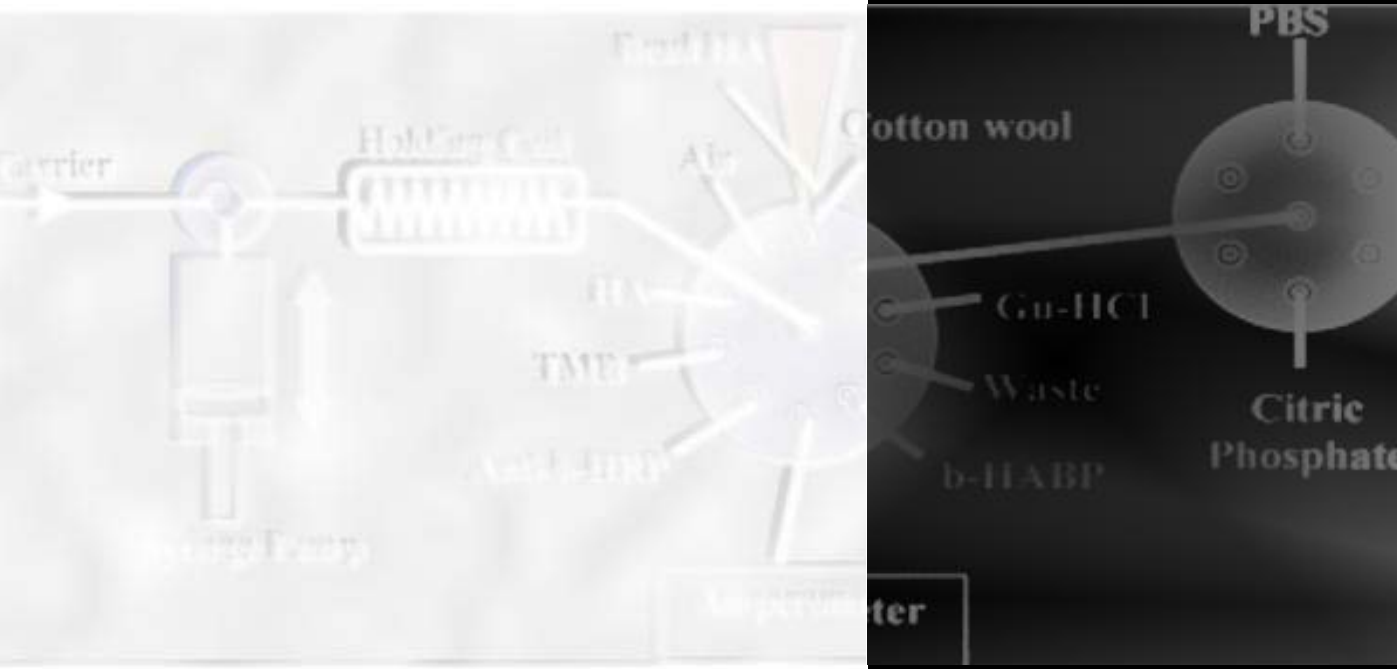
ภาคผนวก ค2

การจัดประชุมวิชาการประจำปี

The 5th Annual Symposium on TRF Senior Research Scholar on "Development of Micro-and Nano-scale Analysis by Flow-based Techniques II"

The 5th Annual Symposium on
TRF Senior Research Scholar

Development of Micro-and Nano-scaleAnalysis Flow-based Techniques II



Program & Abstracts

12 August 2006

Chiang Mai University
Chiang Mai, THAILAND

The 5th Annual Symposium on TRF Senior Research Scholar on
"Development of Micro-and Nano-scale Analysis by Flow-based Techniques "
Seminar Room, Floor 2, SCB2
Faculty of Science, Chiang Mai University
12 August 2006

8.15 - 8.30	Registration	
8.30 – 9.00	**Opening session** - "Symposium Objectives" and "Progress on the Development of Micro- and Nano-scale Analysis by Flow-based Techniques: the phase II on the TRF Senior Scholar Project" by Prof. Dr. Kate Grudpan, TRF Senior Research Scholar - Opening address by the Director, Academic Division, TRF (Prof. Dr. Vichai Boonsaeng)	
9.00 - 9.30	**Plenary Lecture:** *"Developments of Flow-Based Methods for Automated Chemical Analysis Using Computer-Controlled Liquid Flow Devices":* Prof. Dr. Shoji Motomizu	
9.30 - 9.50	**Invited Lecture:** *"Spectrophotometric Flow Analyzer with Automatic Valve Switching System for Trace Phenols"* Assoc. Prof. Dr. Norio Teshima	
9.50 - 10.00	*Oral presentation 1	(Asst. Prof. Dr. Supaporn K. Hartwell)
10.00 - 10.20	*Coffee break*	
10.20 - 10.30	*Oral presentation 2	(Asst. Prof. Dr. Daungjai Nacapricha)
10.30 - 10.40	*Oral presentation 3	(Ms. Weena Siangproh)
10.40 - 10.50	*Oral presentation 4	(Dr. Rodjana Burakham)
10.50 - 11.00	*Oral presentation 5	(Ms. Wanida wonsawat)
11.00 - 11.10	*Oral presentation 6	(Mr. Tinakorn Kanyanee)
11.10 - 11.20	*Oral presentation 7	(Ms. Saiphon Chanpaka)
11.20 - 11.30	*Oral presentation 8	(Mr. Lucksagoon Ganranoo)
11.30 - 11.40	*Oral presentation 9	(Ms. Watsaka Siriangkhawut)
11.40 - 11.50	*Oral presentation 10	(Mr. Sila Kittiwachana)
11.50 - 12.00	*Oral presentation 11	(Dr. Sukon Prasitwattanaseree)
12.00 - 13.00	*Lunch*	
13.00 - 14.30	Discussion at Posters	
14.30 - 16.30	*Coffee Break and* Discussion in Overall	

* Note (1) Some from the all posters presented
(2) Should be within 3 slides

A Report on the 5th Annual Symposium on TRF Senior Research Scholar on Flow Based Analysis in Thailand

Norio Teshima
Department of Applied Chemistry, Aichi Institute of Technology, Japan

The 5th annual symposium on TRF senior research scholar on "Development of micro- and nano-scale analysis by flow-based techniques II" was held in Chiang Mai University (CMU) on Aug. 12th, 2006. This symposium under the auspices of the Thailand Research Fund (TRF) has been organized by Prof. Kate Grudpan of CMU since 2002. I had stayed at Chiang Mai for about 3 weeks by his kind invitation for our collaboration. During my stay, this symposium was held, so that I could take an opportunity of seeing his all group members' efforts to arrange the symposium successfully in advance. On that day they were wearing uniform and establishing their good teamwork.

The research project has active collaboration with oversea institutions: University of Washington, USA (Profs. Gary D. Christian, Jaromir Ruzicka, Robert E. Synovec), Monash University, Australia (Dr. Ron Beckett, Assoc. Prof. Ian D. Mckelvie), Karlsruhe Research Centre, Germany (Prof. Thomas Fanghenel, Horst Geckeis), University of Plymouth, England (Prof. Paul J. Worsfold), Okayama University, Japan (Prof. Shoji Motomizu), Aichi Institute of Technology, Japan (Prof. Tadao Sakai), Texas Tech University, USA (Prof. Pernendu K. Dasgupta), Gunma University, Japan (Prof. Hideyuki Itabashi), Kochi University , Japan (Prof. Hiroyuki Ukeda) and Ogawa & Co., Japan (Dr. Keiro Higuchi). The first Workshop between U.S. and Thai Analytical Scientists was held in Thailand on January 4th – 8th, 2006 in order to facilitate their collaboration.

An opening session was opened with Kate's address about the objectives and progress of the project. After that, there were two welcome opening addresses by the Dean of Faculty of Science (Assist. Prof. Mongkon Rayanakorn) and the Director of Academic Research Division of the TRF (Prof. Vichai Boonsaeng). The Director gave the silver plates to Profs. Shoji Motomizu (Okayama University, Japan) and Tadao Sakai (Aichi Institute of Technology, Japan) and to me, although Tadao Sakai could not attend the symposium. I would like to congratulate Tadao Sakai on his 2006 Japan Society for Analytical Chemistry Award. Also, we praised Thai lady researchers for their awards. Bunches of flowers were given to them: Assist. Prof. Duangjai Nacapricha (Mahidol University, Thailand), Assoc. Prof. Orawon Chailapakul (Chulalongkorn University, Thailand) and Dr. Supaporn K. Hartwell (CMU, Thailand).

There were plenary (PL-01) and invited (IL-01) lectures:

PL-01 "Development of Flow-Based Methods for Automated Chemical Analysis Using Computer-Controlled Liquid Flow Devices" by Shoji Motomizu, Narong Lenghor and Sarawut Somnam (Okayama University). In this paper S. Motomizu presented studies on FIA and SIA systems assembled with solenoid pumps and valves and new concept of SIMA (simultaneous injection-effective mixing analytical method).

IL-01 "Spectrophotometric Flow Analyzer with Automatic Valve Switching System for Trace Phenols" by Norio Teshima and Tadao Sakai (Aichi Institute of Technology).

Thereafter, 11 oral presentations were on the morning session.

S. K. Hartwell presented study on immunochromatographic assay for specific proteoglycans which are a potential biomarker for ovarian cancer.

D. Nacapricha used a boron-doped diamond thin film as sensor for flow analysis of three β-agonists: salbutamol, terbutaline and clenbuterol.

W. Siangproh reported on a FIA of Sudan I, II, III and IV in food using glassy carbon modified with carbon nanotube-ionic liquid gel, and also W. Wonsawat determined the four Sudan dyes in non-aqueous system by a FIA with amperometric detection.

R. Burakham developed an on-line sample preparation for liquid chromatography and capillary electrophoresis.

T. Kanyanee performed an elegant sampling and analysis system for trace SO_2 determination using soap bubble.

S. Chanpaka used a sequential injection analysis (SIA) system with lab-at-valve (LAV) format for on-line micro-solvent extraction of tetracycline.

L. Ganranoo adapted a SI-LAV stopped flow system to a kinetic study on the reaction of iodide and persulfate as a model reaction.

W. Siriangkhawut measured some heavy metals such as Zn, Cd and Pb using Bi film flow-through electrode with anodic stripping voltammetric detection.

S. Prasitwattanaseree demonstrated chemometrics techniques for micro- and nano-scale analysis: classification of gasoline trademarks and simultaneous determination of chlorate and chlorite in agrochemical samples.

S. Kittiwachana described principal component regression (PCR) and partial least square (PLS) procedures for the determination of protein in cow milk, although he was absent because he was in a temple as a new trainee monk (I learned this kind of Thai traditional culture).

I believe that their oral presentations made themselves (especially for Ph.D. students) internationalized.

There were thirty eight poster presentations in the afternoon session. Many Thai students were discussing their successful results lively.

Kate presided at a closing session. He mentioned that studies on analytical chemistry have impacts to academic communities in Thailand by considering the data from the science citation index (Web of Science). Also, he addressed a joint symposium between Thailand and Japan on 2007. Some participants were requested to address their remarks. Ron Beckett gave his comments in supporting that future collaboration would lead to good research, and Shoji Motomizu commented on the expectation of developments in flow-based techniques. I mentioned that I hoped *Journal of Flow Injection Analysis* would be much more recognized as a valuable international journal.

After the symposium, we enjoyed a wonderful dinner at a good restaurant near the campus. Fortunately, a sudden shower waited for us till we all got into the restaurant.

I had continued my stay at Chaing Mai after the symposium, and I could take an opportunity to give a lecture to undergraduate students in Department of Chemistry of CMU. The title was “Flow-Based Methods of Water and Clinical Analyses”.

I observed that the Symposium activated the young generations (especially students) in experiencing to contribution in organizing the Symposium apart from academic experiences.

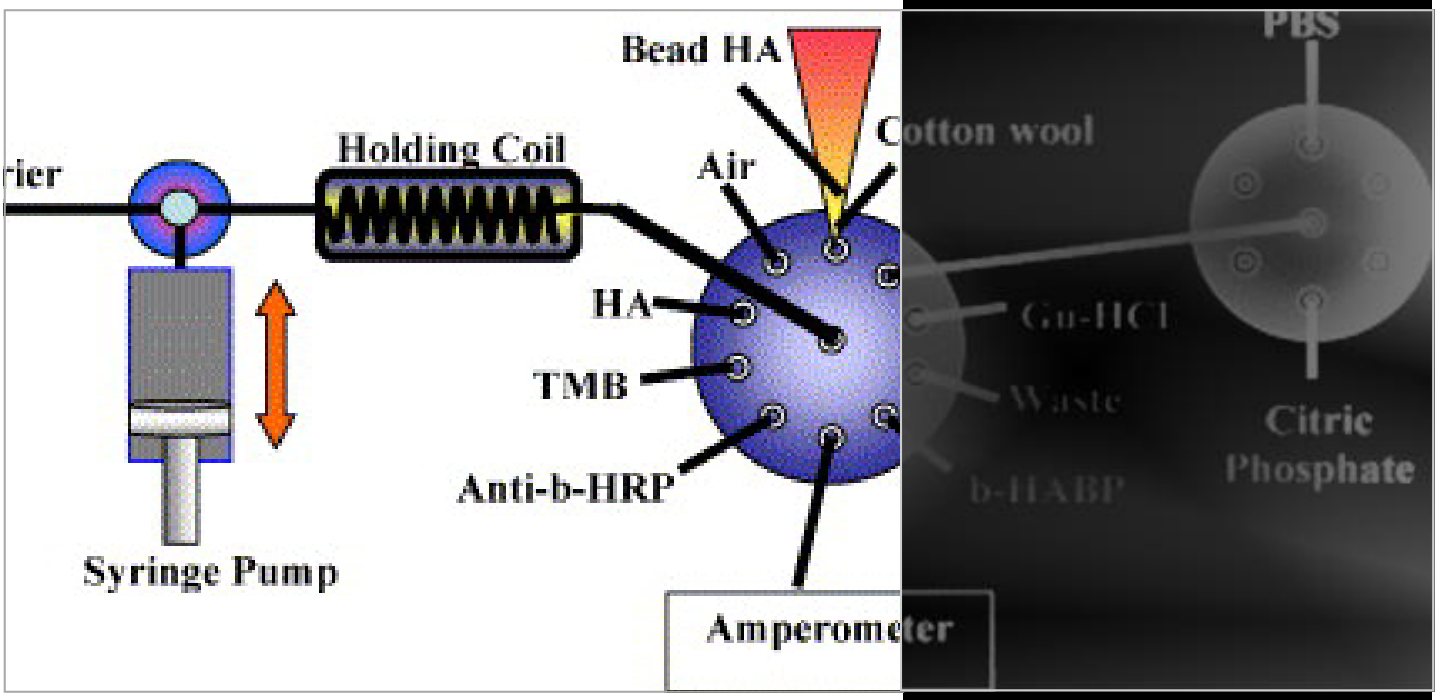
ภาคผนวก ค3

การจัดประชุมวิชาการประจำปี

The 6th Annual Symposium: TRF Senior Research Scholar and Research group on Innovation on Analytical Instrumentation CHE: "Development of Flow-based Analysis for better life quality"

The 6th Annual Symposium on
TRF Senior Research Scholar and
Research Group on Innovation on Analytical Instrumentation CHE

Development of Flow-based Analysis for Better Life Quality



Program & Abstracts

16 August 2007

Chiang Mai University
Chiang Mai, THAILAND

The 6th Annual Symposium: TRF Senior Research Scholar and Research Group on Innovation on Analytical Instrumentation CHE: "Development of Flow-based Analysis for better life quality"

Seminar Room, Floor 2, SCB2
Faculty of Science, Chiang Mai University
16 August 2007

8.15 – 8.30	Registration
8.30 – 9.00	**Opening session** - "Symposium Objectives" and "Progress of the research work" by Prof. Dr. Kate Grudpan - "Opening address by the Director, Academic Division, TRF (Prof. Dr. Vichai Boonsaeng)
9.00 – 9.25	**Keynote Lecture**: "Computer-Assisted Fully Automated Flow Analysis System (CAFCA)": Prof. Dr. Shoji Motomizu
9.25 – 9.50	**Keynote Lecture**: "Development of Field-Flow Fractionation (Interaction of Theory and Experiment)": Dr. Ron Beckett
9.50- 10.05	*Coffee break*
10.05 – 10.10	*Oral presentation 1 (Asst. Prof. Dr. Duangjai Nacapricha)
10.10 – 10.15	*Oral presentation 2 (Ms. Kanokporn Boonsong)
10.15 – 10.20	*Oral presentation 3 (Ms. Rie Fukuda)
10.20 – 10.25	*Oral presentation 4 (Mr. Kazuki Matsumura)
10.25 – 10.30	*Oral presentation 5 (Ms. Kanchana Watla-iad)
10.30 – 11.35	*Oral presentation 6 (Mr. Sarawut Somnam)
11.35 – 11.40	*Oral presentation 7 (Mr. Lucksagoon Ganranoo)
10.40 – 10.45	*Oral presentation 8 (Mr. Tinakorn Kanyanee)
10.45 – 10.50	*Oral presentation 9 (Dr. Vannajan Sanghiran Lee)
10.50 – 10.55	*Oral presentation 10 (Ms. Supada Khonyoung)
10.55 – 12.00	Discussion at Poster Session I
12.00 – 13.00	*Lunch*
13.00 – 14.30	Discussion at Poster Session II
14.30 – 16.30	*Coffee Break and* Discussion in Overall

*Note: Some from all posters presented; each presenter will brief the highlight of poster(s) with 3-5 slides