รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาและตรวจสอบวิธีการทางเคมีไฟฟ้าเพื่อการประเมินค่าเชิงวิเคราะห์กรดลิโปอิกและสารอนุพันธ์ที่เกี่ยวข้องสำหรับประยุกต์ใช้เพื่อการศึกษาทางด้านคลินิกและอาหาร

โดย ดร. วีณา เสียงเพราะ และคณะ

กรกฎาคม 2553

สัญญาเลขที่…MRG5080278……

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CONTENTS

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บทคัดย่อ

รหัสโครงการ: MRG5080278

ชื่อโครงการ: การพัฒนาและตรวจสอบวิธีการทางเคมีไฟฟ้าเพื่อการประเมินค่าเชิงวิเคราะห์กรดลิโปอิกและสารอนุพันธ์ที่เกี่ยวข้องสำหรับประยุกต์ใช้เพื่อการศึกษาทางด้านคลินิกและอาหาร

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บทคัดย่อ

งานวิจัยนี้มุ่งเน้นความสำคัญไปที่การพัฒนาการใช้ขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์สำหรับหาปริมาณสารที่มีความสำคัญ โดยแบ่งงานวิจัยออกเป็นสองส่วนคือ ส่วนแรก เป็นการพัฒนาตัวตรวจวัดชนิดใหม่ โดยใช้ขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์ร่วมกับระบบไฮเพอร์ฟอร์มานส์ลิควิดโครมาโทกราฟีสำหรับการหาปริมาณของกรดลิโปอิกและสารอนุพันธ์ที่เกี่ยวข้อง ในการศึกษาเบื้องต้นได้ทำการศึกษาการเกิดปฏิกิริยาทางเคมีไฟฟ้าของกรดลิโปอิกและสารอนุพันธ์ที่เกี่ยวข้องที่ขั้วไฟฟ้าฟิล์มบางโบรอโดปไดมอนด์ด้วยไซคลิกโวลแทมเมทรี เปรียบเทียบผลการทดลองที่ได้กับขั้วไฟฟ้ากลาสสิคาร์บอน พบว่าขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์ให้ผลของสัญญาณไซคลิกโวลแทมโมแกรมสำหรับปฏิกิริยาออกซิเดชันของกรดลิโปอิกและสารอนุพันธ์ที่เกี่ยวข้องแบบไม่ผันกลับที่ชัดเจนและให้ค่ากระแสไฟฟ้าที่สูง เมื่อเปรียบเทียบกับขั้วไฟฟ้ากลาสสิคาร์บอน จากนั้นได้นำระบบระบบไฮเพอร์ฟอร์มานส์ลิควิดโครมาโทกราฟีซึ่งต่อกับขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์ไปประยุกต์สำหรับการวิเคราะห์ปริมาณกรดลิโปอิกในสารตัวอย่างอาหารเสริม จากการศึกษาเปอร์เซ็นต์การกลับคืนของสารมาตรฐานที่เติมลงในสารตัวอย่างพบว่าได้ผลการทดลองในช่วง 94.4 ถึง 103.6 เปอร์เซ็นต์ และผลการทดลองที่ได้เป็นที่น่าพอใจมีความสอดคล้องกับค่าที่ได้จากวิธีมาตรฐาน

งานวิจัยส่วนที่สองเป็นการพัฒนาระบบใหม่สำหรับการตรวจวัดปริมาณของเอ็นแอซิทิลแอลซีสเทอีนในตัวอย่างยา วิธีที่พัฒนาขึ้นนี้เป็นวิธีที่ง่าย รวดเร็ว และมีสภาพไวสูงเมื่อเปรียบเทียบกับวิธีมาตรฐาน จากการนำขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์ไปประยุกต์ใช้กับระบบโฟลว์อินเจคชันสำหรับการวิเคราะห์ปริมาณเอ็นแอซิทิลแอลซีสเทอีนในสารมาตรฐานและสารตัวอย่างยาเตรียม จากการศึกษาเปอร์เซ็นต์การกลับคืนของสารมาตรฐานที่เติมลงในสารตัวอย่างพบว่าได้ผลการทดลองในช่วง 97.4 ถึง 103.6 เปอร์เซ็นต์ ผลการทดลองที่ได้เป็นที่น่าพอใจมีความสอดคล้องกับค่าที่ระบุในฉลาก ด้วยค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ต่ำกว่า 3.5 เปอร์เซ็นต์

คำหลัก : ขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์ กรดลิโปอิก เอ็นแอซิทิลแอลซีสเทอีน ระบบของไหลระบบไฮเพอร์ฟอร์มานส์ลิควิดโครมาโทกราฟี

Abstract

Project Code : MRG5080278

Project Title : The development and validation of electrochemical techniques for the evaluation of α-lipoic acid and related compounds: Application in clinical and food samples

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Project Period : **2 years**

Abstract:

This research focused on the development of using boron-doped diamond (BDD) thin film electrode as a new detector for the determination of important analytes. It was separated into two parts. The first part was focused on the use of the boron-doped diamond (BDD) thin film electrode coupled with high performance liquid chromatography for the determination of lipoic acid and related compounds. The electrooxidation of lipoic acid and related compounds at the BDD electrode using cyclic voltammetry was studied first. The results obtained were compared with those of the glassy carbon (GC) electrode. At the BDD electrode, lipoic acid and related compounds provided a highly and well-defined irreversible cyclic voltammograms. In addition, this proposed method was applied to determine lipoic acid in food supplements. According to the method based on the standard addition technique, recoveries of spiked standard solutions were determined. The recoveries of analytes in the sample were 94.4-103.6%. Compared to standard method, results obtained are satisfactory.

The second part, a new methodology for the determination of N-acetyl-L-cysteine in drug samples was developed. The proposed methodology offers a simple, fast and sensitive assay through which N-acetyl-L-cysteine could be quantified down to the nanomolar level. In addition, the BDD electrode was useful as detector in flow injection system for the quality control and analysis of N-acetyl-L-cysteine in both standard and drug samples. The recoveries of analytes in the sample were 957.4-103.6%. The results agree reasonably well with the label and also showed very good precision in an acceptable range of less than 3.5 %.

Keywords : Boron-doped diamond thin film electrode, lipoic acid, N-acetyl-L-cysteine, flow based system, high performance liquid chromatography

บทสรุปโครงการ

Executive Summary

วัตถุประสงค์

1. เพื่อตรวจสอบสารต้านอนุมูลอิสระต่าง ๆ เช่น กรดลิโปอิก โดยใช้ขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์และเทคนิคไซคลิกโวลแทมเมทรี
2. เพื่อเปรียบเทียบสมบัติทางเคมีไฟฟ้าของสารต้านอนุมูลอิสระเมื่อใช้ขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์และขั้วไฟฟ้ากลาสสิคาร์บอนโดยใช้เทคนิคไซคลิกโวลแทมเมทรี
3. ตรวจสอบอุปกรณ์ตรวจวัดที่ประกอบขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์เข้ากับระบบโฟลว์อินเจคชัน โดยนำไปทดลองใช้หาปริมาณของสารต้านอนุมูลอิสระได้
4. หาภาวะที่เหมาะสมในการวิเคราะห์สารต้านอนุมูลอิสระเพื่อปรับปรุงวิธีการวิเคราะห์ให้สามารถวิเคราะห์สารต้านอนุมูลอิสระที่ความเข้มข้นต่ำลงได้
5. นำอุปกรณ์ตรวจวัดที่ประกอบขั้วไฟฟ้าฟิล์มบางโบรอนโดปไดมอนด์เข้ากับระบบไฮดปอร์ฟอร์มานส์ลิควิดโครมาโทกราฟีและหาภาวะที่เหมาะสมสำหรับการวิเคราะห์สารต้านอนุมูลอิสระในสารตัวอย่าง เช่น อาหาร ได้
6. ได้วิธีการวิเคราะห์ที่เหมาะสมเพื่อแยกและวิเคราะห์ปริมาณสารต้านอนุมูลอิสระในตัวอย่างต่าง ๆ เช่น อาหาร

การดำเนินงานวิจัย และผลงานวิจัยที่ได้รับอย่างย่อ ๆ

1. ค้นคว้าข้อมูลและเอกสารที่เกี่ยวข้อง
2. ได้ผลการตรวจสอบสมบัติทางเคมีไฟฟ้าของกรดลิโปอิกและสารอนุพันธ์ลิโปเอไมด์ เมื่อใช้ขั้วไฟฟ้าโบรอนโดปไดมอนด์และขั้วไฟฟ้ากลาสสิคาร์บอน ด้วยเทคนิคไซคลิกโวลแทมเมทรี โดยที่ทำการแสกนช่วงศักย์ไฟฟ้าตั้งแต่ 0 โวลต์ ถึง 1.1 โวลต์ สำหรับกรดลิโปอิก และช่วงศักย์ไฟฟ้าตั้งแต่ 0 โวลต์ ถึง 1.2 โวลต์ สำหรับลิโปเอไมด์
3. ได้ภาวะที่เหมาะสมในการวิเคราะห์กรดลิโปอิกและสารอนุพันธ์ เพื่อปรับปรุงวิธีการวิเคราะห์ให้สามารถวิเคราะห์ได้ที่ความเข้มข้นต่ำ และเปรียบเทียบสมบัติทางเคมีไฟฟ้าของกรดลิโปอิกและสารอนุพันธ์ลิโปเอไมด์ เมื่อใช้ขั้วไฟฟ้าโบรอนโดปไดมอนด์และขั้วไฟฟ้ากลาสิคาร์บอนโดยใช้เทคนิคไซคลิกโวลแทมเมทรี จากการทดลองพบว่า กรดลิโปอิกและสารอนุพันธ์ลิโปเอไมด์ สามารถเกิดปฏิกิริยาแบบผันกลับไม่ได้ที่ขั้วไฟฟ้าทั้งสองชนิด แต่ขั้วไฟฟ้าโบรอนโดปไดมอนด์ จะให้ค่าสัญญาณของ

กระแสต่อพื้นหลังที่ต่ำกว่าขั้วไฟฟ้ากลาสิคาร์บอน จึงทำให้ค่าสัญญาณกระแสไฟฟ้าที่ตรวจวัดได้ด้วยขั้วไฟฟ้าโบรอนโดปไดมอนด์มีค่าที่สูงกว่าการใช้ขั้วไฟฟ้ากลาสสิคาร์บอน

4. จากการศึกษาผลของอัตราการสแกนในช่วง 0.01 โวลต์ต่อวินาที ถึง 0.3 โวลต์ต่อวินาที และนำผลที่ได้มาสร้างกราฟระหว่างค่ากระแสไฟฟ้าที่ตรวจวัดได้และรากที่สองของอัตาการแสกน พบว่า ได้ความสัมพันธ์เป็นเส้นตรง ทั้งกรณีของกรดลิโปอิกและสารลิโปเอไมด์ แสดงว่า กระแสที่วัดได้จากปฏิกิริยาออกเดชันของสารทั้งสามชนิดถูกควบคุมด้วยกระบวนการแพร่ และเกิดการดูดซับของสารที่ผิวหน้าขั้วไฟฟ้าเพียงเล็กน้อย
5. จากการศึกษาผลความเข้มข้นของกรดลิโปอิกและลิโปเอไมด์ด้วยเทคนิคไซคลิกโวลแทมเมทรี พบว่าขั้วไฟฟ้าโบรอนโดปไดมอนด์สามารถตรวจวัดสารได้ในระดับความเข้มข้นต่ำถึงไมโครโมลาร์ แสดงให้เห็นว่าขั้วไฟฟ้าโบรอนโดปไดมอนด์มีสภาพไวต่อการตรวจวัดสารที่สูง
6. ได้ภาวะที่เหมาะสมโดยใช้เทคนิคแอมเพอโรเมทรี ร่วมกับการตรวจวัดด้วยระบบโฟลว์อินเจคชันโดยใช้ขั้วไฟฟ้าโบรอนโดปไดมอนด์เป็นขั้วไฟฟ้าทำงาน จากผลการทดลองพบว่าศักย์ไฟฟ้าที่เหมาะสมในการตรวจวัด กรดลิโปอิกและสารอนุพันธ์ ได้แก่ สารลิโปเอไมด์ คือ 1.05 โวลต์
7. ได้ภาวะที่เหมาะสมสำหรับการแยกและวิเคราะห์กรดลิโปอิกและสารลิโปเอไมด์ คือ สารมารถแยกสารทั้งสองด้วยคอลัมน์ C18 และใช้เฟสเคลื่อนที่คือสารผสมของสารละลายฟอสเฟตพีเอช 2.5 ต่ออะซิโตไนไตรด์ ในอัตราส่วน 1:1 โดยสามารถทำการแยกสารได้ภายในเวลา 5 นาที ได้ช่วงความสัมพันธ์เชิงเส้นตรงในช่วง 0.01-60 ppm และขีดจำกัดต่ำสุดของการตรวจวัดที่ 3 ppb สำหรับกรดลิโปอิก และ 3.62 ppb สำหรับลิโปเอไมด์
8. ได้วิธีการและภาวะที่เหมาะสมสำหรับการตรวจวิเคราะห์กรดลิโปอิกในอาหารเสริมที่มีความถูกต้องแม่นยำ และจำเพาะ เปอร์เซ็นต์การกลับคืนของสารมาตรฐานที่เติมลงในสารตัวอย่างพบว่าได้ผลการทดลองในช่วง 94.4 ถึง 103.6 เปอร์เซ็นต์ และผลการทดลองที่ได้เป็นที่น่าพอใจมีความสอดคล้องกับค่าที่ได้จากวิธีมาตรฐาน
9. ตรวจสอบสมบัติทางเคมีไฟฟ้าของแอซิทิลซีสเทอีนโดยใช้ขั้วไฟฟ้าโบรอนโดปไดมอนด์ และเทคนิคไซคลิกโวลแทมเมทรี เปรียบเทียบกับขั้วไฟฟ้ากลาสสิคาร์บอน พบว่า แอซิทิลซีสเทอีนสามารถเกิดปฏิกิริยาแบบไม่ผันกลับที่ขั้วไฟฟ้าโบรอนโดปไดมอนด์ และขั้วไฟฟ้าโบรอนโดปไดมอนด์ให้ค่าสัญญาณของกระแสต่อพื้นหลังที่สูงกว่ากลาสิคาร์บอนอิเล็กโทรด pH ที่เหมาะสมที่ให้สัญญาณในการตรวจวัดสูงสุดสำหรับ แอซิทิลซีสเทอีน สารละลาย pH 9
10. หาภาวะที่เหมาะสมโดยใช้เทคนิคแอมเพอโรเมทรี ร่วมกับการตรวจวัดด้วยระบบโฟลว์อินเจคชันโดยใช้ขั้วไฟฟ้าโบรอนโดปไดมอนด์เป็นขั้วไฟฟ้าทำงาน จากผลการทดลอง พบว่าศักย์ไฟฟ้าที่เหมาะสม

ในการตรวจวัดแอซิทิลซีส ได้แก่ 0.85 โวลต์ และผลที่ได้จากเทคนิคโฟลว์อินเจคชันให้ค่าขีดจำกัดต่ำสุดสำหรับสารทั้งสี่ชนิด ในการตรวจวัดที่ 10 นาโนโมลาร์ (S/N ≈ 3) จากผลการทดลอง แอซิทิลซีสให้ช่วงความเข้มข้นที่เป็นเส้นตรงจาก 0.5 ถึง 50 ไมโครโมลาร์

11. ได้วิธีที่เหมาะสมมาใช้หาปริมาณแอซิทิลซีสในสารตัวอย่างยา ด้วยเทคนิคการเติมสารมาตรฐาน พบว่า ได้ร้อยละของการคืนกลับอยู่ในช่วง 97.4 – 103.6 ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ต่ำกว่า 3.5 เปอร์เซ็นต์ ซึ่งเป็นวิธีที่ง่าย ความถูกต้อง และแม่นยำ
12. ผลงานเรื่อง Reverse-Phase Liquid Chromatographic Determination of α-Lipoic Acid in Dietary Supplements Using a Boron-Doped Diamond Electrode กำลังอยู่ระหว่างการตอบรับให้ตีพิมพ์ในวารสาร Journal of Chromatography A (impact factor= 4.101) ดังเอกสารแนบ
13. ผลงานเรื่อง Highly Sensitive and Simple Method for the Determination of N-Acetyl-L-Cysteine in Drug Formulations using Boron-Doped Diamond Sensor อยู่ในระหว่างการส่งตีพิมพ์ในวารสารเรื่อง Bioelectrochemistry

CHAPTER I

INTRODUCTION

1.1. Introduction

Thiocompounds play an important role in biological and pharmacological process, and especially thiol-containing drugs hold a particular fascination. They form an important class of compounds with an extensive and interesting chemistry [1]. Thiol-containing drugs are well known to possess a multitude of roles within the clinical applications. In addition to the helpful effects of these medicines, they may have side effects that can be very serious. If any of the following side effects occur, the patient will be checked with doctor immediately. Therefore, these medicines are available only with doctor's prescription, in the following dosage form. Because of their side effects, the quality control of thiol-containing drugs is required to prevent the unwanted effects from using these medicines. Moreover, in pharmacokinetic study, their amounts in biological fluids can provide information to biomedical scientist with a versatile diagnostic handle through which the treatment. Successful exploitation of such an approach, however, is dependent on the availability of analytical techniques that can provide fast quantitative measurements of the various therapeutic concentrations.

From the literature survey, several methods have been used to determine thiol-containing drugs. Early, spectrophotometric methods [1] listed nearly 200 "specific" reagents and reactions for the thiol group. Many assays depend on the ability of the –SH group to form colored complexes with heavy metals. Therefore, many thiols spectrophotometric methods are available, e.g., cysteine gives a specific reaction with

ninhydrin in concentrated acid [2] and glutathione reacts with *o*-phthaladehyde giving a fluorescent product [3].

For electrochemical method, in theory, direct oxidation of the thiols at bare electrodes is the simplest approach to electrochemical measurement. However, large over potential are usually required before sufficient sensitivity can be attained. Moreover, in complex media, selectivity can often suffer as a consequence. Nevertheless, numerous substrates have been investigated for the following electrodes glassy carbon [4], carbon [5], graphite, platinum [6], gold [7], and silver [8]. Poor voltammetric responses are common among these electrodes and a comprehensive review on the electrochemical response to cysteine and cystine has been previously conducted [9]. Improvements in response have been sought and found with bismuth-doped lead dioxide [10] and conducting salts [11] providing enhanced responses to cysteine and glutathione respectively. Mercury has historically been among the more successful substrates for the study of thiols from both fundamental [12] and analytical perspective [13]. However, the method based on mercury has a limitation due to its toxicity and rapid deterioration of the electrode response.

Boron-doped diamond (BDD) thin film electrode has been realized as the outstanding electrode material for several electrochemical applications, including electrochemical waste treatment [14] and electroanalysis [15]. Diamond possesses several technologically important properties including extreme hardness, high electrical resistance, chemical inertness, high thermal conductivity, high electron and hole mobility, and optical transparency**.**

Several interesting and important electrochemical properties distinguish boron-doped diamond (BDD) thin films from conventional carbon electrodes. The films

exhibit voltammetric background currents and double-layer capacitances up to an order of magnitude lower than for glassy carbon [16].

α-Lipoic acid (1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid), or thioctic acid, is a natural sulfur-containing cofactor, which for example, occurs in four essential mitochondrial enzyme complexes. It is widely found in both prokaryotic and eukaryotic microorganisms as well as in animals and plants. It plays an essential role in various biological systems, such as improving mitochondrial function, increasing metabolic rate and decreasing oxidative damage [17-19]. Recently, α-lipoic acid has gained considerable attention as an 'ideal' or 'universal antioxidant' [20] and has been shown to provide protection against free radical attack both *in vitro* and *in vivo*. Furthermore, α-lipoic acid has been employed as therapy for many diseases associated with impaired energy utilization, such as type II diabetes, diabetic polyneuropathies [18], Azheimer-type dementia [21], neurodegenerative disorders, acquired immunodeficiency syndrome [22] and heavy metal poisoning [23] and was recently shown to display positive effects in the treatment of age-associated neurodegenerative disease and HIV infections [24].

In addition to its use as a drug, α-lipoic acid has become one of the most recognized and in-demand health supplements. With the medical importance of α-lipoic acid in both toxicological and acceptance criteria, and its potential to treat multiple diseases in the future, a fast, accurate, selective and sensitive method for quantifying the concentration of α-lipoic acid in dietary supplements, among other types of samples, is needed.

Various analytical methodologies have been developed for the determination of α-lipoic acid in a diverse variety of biological, drug and food samples. Chromatographic methods are widely used, and the substantiated approaches for the

determination of α-lipoic acid have been reviewed by Kataoda [25]. Gas chromatography coupled with detection by flame photometry [26-27] and mass spectrometries [28-30] are among the most regularly utilized techniques. However, these techniques are time-consuming because of the use of the derivatization approach.

Use of thin-layer chromatography has also been reported for the separation and determination of α-lipoic acid [31]. For densitometric analysis, derivatization with a palladium (II) chloride immersion reagent is necessary to overcome the poor absorbtivity of α-lipoic acid. A reversed phase HPLC [32-33] or capillarity electrophoresis (CE) coupled with ultraviolet detection [34-35] is another technique that is used in the determination of α-lipoic acid, but it typically provides a high minimum detection limit due to lack of a strong chromophore. In addition, the main hindrance of using CE is the adsorption of α-lipoic acid to the capillary wall, such that a washing step is required for further use after only a few injections. HPLC combined with other detection modes, such as fluorescence [36-39], mass spectrometry [40-42] or electrochemical detection [43-48], have also been developed to determine α-lipoic acid levels. Among these detectors, fluorescence-based methods have demonstrated low detection limits, though this depends on the fluorogenic labeling reagent. The detection limits obtained by fluorescent labels are typically low enough to detect α-lipoic acid in biological samples; however, these assays require laborious sample preparation steps and also have a high reagent cost. HPLC with mass spectrometry usually offers very low sensitivity and high selectivity, but there is high equipment cost and a significant amount of labor and analytical resources required, which can potentially cause substantial delays in obtaining results.

The use of an electrochemical detector is an alternative method for α-lipoic acid determination and has the benefits of simplicity, speed, sensitivity and low cost. A dual Hg-Au electrode in pulsed mode was used for the determination of nanomolar levels of α-lipoic acid, but loss of sensitivity over repeated or long analysis times and the necessity of renewal of the electrode are considerable drawbacks that offset the advantage of its sensitivity [49]. Alternative working electrodes that can be employed for the detection of α-lipoic acid are glassy carbon (GC) [46-47, 49], gold [45] and modified electrodes, including carbon paste electrodes modified with nickel(II)-cyclohexylbutyrate [50], lipoate-selective membranes [51] and GC electrodes modified with carbon nanotubes [52]. From the work with sensors in the literature, it can be deduced that a GC electrode suffers from a lack of sensitivity due to poisoning of the electrode surface at high oxidation potentials, whereas the gold electrode requires a pulsed amperometric mode. Lastly, the modified electrodes have disadvantages due to the different tedious preparation steps and lack of reproducibility of signal.

Therefore, efforts to extend electrochemical detection to α-lipoic acid have been challenging. Recently, boron-doped diamond (BDD) electrodes have been examined extensively in the field of electrochemical analysis. Compared to conventional electrodes, the BDD electrode offers many unique benefits, such as a low background, a wide working potential window, long-term response, high mechanical strength and a lack of adsorption [53-54]. With this excellent electrode material and ongoing research interest, the work reported here is an extension of our efforts in the determination of α-lipoic acid in dietary supplements with a BDD electrode following chromatographic resolution from matrixes.

Surprisingly, there is currently very little information about the determination of α-lipoic acid in health or dietary supplements and, to the best of our knowledge; there is no information about the determination of α-lipoic acid levels using a BDD electrode as the detector in chromatography. Therefore, this report is the first to use a BDD electrode for the quantification of α-lipoic acid following its chromatographic separation. The ultimate goal of this study is to investigate the feasibility of using the BDD electrode to detect α-lipoic acid and to compare the BDD sensor with the conventional HPLC-UV based method.

N-acetyl L-cysteine (NAC) is a small molecular weight thiol compound (M.W 163). It is an acetylated form of the amino acid L-cysteine. As early as 1970, NAC was shown as a source of sulfhydryl groups [55], in experiments, where NAC was given intravenously in Swiss mice and the protein and nonprotein sulfhydryl content of various tissues fractions was analyzed. The tissue protein sulfhydryl content was significantly higher with NAC. In addition to being a sulfhydryl source, NAC is also known as a thiol antioxidant due to its reactivity with oxidant species like $O_2^{\bullet}$, H_2O_2, and $OH^{\bullet}$ [56].

Initially used as a mucolytic agent [56], NAC now has proven to be a very important therapeutic agent in treatment of paracetomol poisoning [57], and as a cardioprotector against doxorubicin. It is also very beneficial in treatment of human immunodeficiency virus infection [58] and heart diseases. Presently with it was used in cancer treatment, NAC is one of the most promising chemopreventive agents. The LD_{50} of NAC is 7888 mg/kg in mice and greater than 6000 mg/kg in rats following oral administration [59]. Even though it is rapidly absorbed, the bioavailability of intact NAC is only about four to ten percent. Most of it gets incorporated into proteins and in the formation of other metabolites.

In spite of the wide spectrum of applications, very little is known about the exact mechanism of NAC action. NAC has long been suggested to exert its effect by increasing cysteine levels, and thereby act as a precursor of glutathione synthesis [60]. As a source of –SH groups, NAC can stimulate GSH synthesis and also enhance glutathione-S-transferease activity. As mentioned, NAC plays an important role in clinical research. Therefore, there is a pressing need to develop fast and sensitive method for the determination of NAC.

In the past, the determination of lipoic acid, dihydrolipoic acid, lipoamide and NAC has been performed by chromatography such as gas chromatography [61-65], high performance liquid chromatography [66-69] and capillary electrophoresis [70]. In a number of all mentioned application, the time delay in the derivatization steps are unacceptable. Moreover, the analyzing cost is high and analyzing instrument generally requires an experience operators. In order to improve on these weak points, electrochemical detection was taken to be a candidate for a novel analysis system.

In the next section, boron-doped diamond thin film electrode was utilized to investigate the electrochemical property of lipoic acid, lipoamide and NAC using cyclic voltammetry in comparing results with the glassy carbon electrode. For the following part, the BDD electrode will be applied as a detector in flow injection analysis system. The promising properties of BDD electrode coupled to the automatic FIA showed potential to detect very low concentration of lipoic acid, lipoamide and NAC compared to those of previous detection methods.

1.2 The Objective of research

The main objective of this research is to develop a new sensor for the determination of α-lipoic acid at low levels in food supplements and N-acetyl- L-cysteine in drugs samples by electrochemical detection. The use of flow injection and high performance liquid chromatography (HPLC) with amperometric detection is proposed for improvement the performance of methodology.

In the amperometric detection, a boron-doped diamond (BDD) electrode is used as sensor. In the process of developing the above mentioned method the following specific objectives of the study should therefore be described.

(1) To investigate the oxidation of α-lipoic acid as well as NAC using cyclic voltammetry at a BDD electrode and glassy carbon (GC) electrodes.
(2) To obtain the optimal parameters for ampermetric detection using BDD electrodes under HPLC system and/or flow injection system.
(3) To establish the optimal conditions for HPLC system and/or flow injection system.
(4) To study the validation of the method.
(5) To apply the proposed for real sample analysis.
(6) To compare the developed method to the standard method.

CHAPTER II

EXPERIMENTAL

2.1. Chemicals and Materials

All chemicals were analytical grade or better and used without further purification. All solutions and subsequent dilutions were prepared in deionized water. Phosphate buffers (pH 2.5-9.0), 0.1 M, were prepared from 0.1 M sodium dihydrogen phosphate (Merck) and 0.1 M disodium hydrogen phosphate (Fluka). Phosphate buffer (pH 2.5) was prepared from 0.1 M sodium dihydrogen phosphate and the pH was adjusted with orthophosphoric acid (85%, Carlo Erba). Phosphate buffer (pH 9), 0.1 M, was prepared from 0.1 M potassium dihydrogen phosphate and the pH was adjusted with 0.1 M sodium hydroxide.

2.2. Instruments and Equipments

The following were the list of instruments utilized in this work.

2.2.1 A Metler AT 200 or a Precisa of 40SM-200A (Switzerland) analytical balance was used for weighing chemicals in the preparation of standard and reagent solutions.

2.2.2 pH meter 744 (Metrohm) was used to measure the pH of prepared buffer solutions.

2.2.3 Milli-Q water system, model Millipore ZMQS 5 VOOY, Millipore, USA., was used to produce deionized-distilled water for preparing chemical and reagent solutions.

2.2.4 Autolab Potentiostat (PG-30, Methrom) was used to record the electrochemical response of analytes.

2.2.5 Diamond electrodes (compliment of Prof. Akira Fujisjima, Japan), the films were prepared by deposition of the BDD thin films on highly conductive n-Si (1 1 1) substrates by microwave plasma-assisted chemical vapour deposition. Deposition was usually carried out for 10 h to achieve a film thickness of approximately 30 μm. The nominal B/C atomic ratio in the gas phase was 1:100, and the typical boron-doping level in the film was approximately 10^{21} cm^{-3}.

2.2.6 Glassy carbon electrode (0.07 cm^2, Bioanalytical System Inc) was pretreated by polishing with alumina powder (1 and 0.05 micron, respectively) slurries in ultrapure water on felt pads and rinsed thoroughly with ultrapure water prior to use.

2.2.7 Ag/AgCl electrode (TCI) with a salt bridge was used as a reference electrode in batch analysis.

2.2.8 Ag/AgCl electrode (RE-3V aqueous reference electrode, Screw type) was used as a reference electrode in the flowing system.

2.2.9 Home-made platinum wire was used as a counter electrode.

2.2.10 Home made glass cell was used in batch analysis

2.2.11 Home made brass holder was used to hold diamond electrode and glass cell and for making an ohmic contact

2.2.12 O-ring viton (0.07 cm^2) was placed at the bottom home made glass cell to isolate and control the electrode surface.

2.2.13 Polishing set of 0.05 and 1 micron alumina powder slurry (Bioanalytical System Inc.) was used to polish the glassy carbon electrode.

2.2.14 Thin layer flow cell (Bioanalytical System Inc.) was used as flow through cell for flow injection system.

2.2.15 Teflon cell gasket (Bioanalytical System Inc.) was used to place against diamond electrode and flow cell.

2.2.16 Rheodyne injection valve, Model 7725 (Altech), with a 20 μL stainless steel injection loop (0.5 mm. i.d.) was used in the flow injection to deliver the sample solution into the system.

2.2.17 Peristaltic pump (Ismatic) was used to continuously deliver a carrier solution into the flow system.

2.2.18 A home made pulse dampener was used in series to reduce the pulsation introduced by the alternation of the roller of the peristaltic pump.

2.2.19 The HPLC system consisted of an HPLC compact pump model 2250 (Bischoff, Germany)

2.2.20 The chromatographic column (Luna 5u C18 column (150 mm x 4.6 mm i.d.) was obtained from Phenomenex and used as analytical comlumn

2.2.21 Auto pipette and tips were obtained from Eppendrof, Germany

2.2.22 0.2 μm Nylon membrane filter (Altech)

2.2.23 0.45 μm Nylon membrane syringe filter with polypropylene (PP) housing (Orange Scientific filter)

2.3. Preparation of Solutions

These successive parts included the preparation procedures of supporting electrolyte and standard solutions employed in this work.

2.3.1. 0.1 M Phosphate Buffer

The buffer solutions were prepared with boiled distilled, CO_2-free water. The weight of sodium dihyrogen phosphate and disodium hydrogen phosphate of various pH values were given in Table 2.1. Phosphate buffer (pH 2.5) was

prepared from 0.1 M potassium dihydrogen phosphate and the pH was adjusted with orthophosphoric acid (85%, Carlo Erba). Phosphate buffer (pH 9 and pH 10), 0.1 M, was prepared from 0.1 M potassium dihydrogen phosphate and the pH was adjusted with 0.1 M sodium hydroxide.

Table 2.1 Compositions of 0.1 M Phosphate Buffer Solutions

pH Values	$NaH_2PO_4.H_2O$(g)	$Na_2HPO_4.2H_2O$(g)	Volume of Stock Solution (mL)
2.5	6.8995	0	500
5.0	6.8443	0.0712	500
6.0	6.1350	0.9878	500
7.0	2.8495	5.2064	500
8.0	0.2553	8.5702	500
9.0*	0	8.8995	500
10.0*	0	8.8995	500

* The solutions were prepared from 0.1 M di-sodium hydrogen phosphate and pH was adjusted with 0.1 M sodium hydroxide.

2.3.2. Stock solution of α-lipoic acid

A stock standard solution of α-lipoic acid (500 μg/mL) was prepared by accurately weighing 5 mg of analyte and dissolving it in a 1:1 (v/v) ratio of acetonitrile: Milli Q water to 10 mL in a volumetric flask and storing it at 4 °C. The working solutions were prepared by suitable dilution of the stock standard solutions with the mobile phase.

2.3.3. Stock solution of NAC

The standard N-acetyl-L-cysteine (Sigma) solutions were freshly prepared with the phosphate buffer pH 9 prior to use.

2.4. Real Sample Analysis

2.4.1 α-lipoic acid samples

The average weight per capsule or tablet was calculated from the weight of 10 capsules or tablets. Five capsules or tablets were finely ground and homogenized in an agate mortar. The amount of the powdered mass analyte corresponding to one capsule or tablet was dissolved in the mobile phase, and the solution was diluted appropriately so that the concentration of α-lipoic acid in the final test solution was within the linear dynamic range (0.01 – 60 μg/mL). The solution was filtered through a 0.45-μm nylon membrane filter before injection into the HPLC-electrochemical system.

2.4.2 NAC samples

Tablets, powder containing NAC () were analyzed. The drug tablet was homogenized in an agar mortar. The amount of the powdered mass analyte was dissolved in 100 ml of 0.1 M phosphate buffer (pH 9). This solution was diluted in such a way that the concentration of NAC in the final test solution was within the linear dynamic range (0.5–50 μ M).

2.5. Procedures of Electrochemical Detection

2.5.1 Batch Analysis

2.5.1.1 Cyclic Voltammetry

The electrochemical measurements were performed in a single compartment glass cell using a potentiostat. Figure 3.1 showed an electrochemical cell for cyclic voltammetric experiment. An Ag/AgCl (A) was used as the reference electrode and platinum wire (B) was employed as the counter electrode. The boron-doped diamond electrodes (0.07 cm^2) were used as working electrodes (C). The working electrodes were pressed against a smooth ground joint at the bottom of the cell isolated by O-ring (area 0.07 cm^2). The exposed geometric area was 0.07 cm^2 Ohmic contact was made by placing the backside of the Si substrate on a brass plate.

Figure 2.1 The electrochemical cell for cyclic voltammetric study.

2.5.2. HPLC separation and apparatus

The HPLC-electrochemical measurement using a BDD electrode as an amperometric detector was carried out in the mobile phase (1:1 (v/v) ratio of 0.05 M potassium phosphate buffer (pH 2.5): acetonitrile) with an applied potential of 1.05 V versus Ag/AgCl and a flow rate of 1.0 mL/min. The HPLC system consisted of an HPLC compact pump model 2250 (Bischoff, Germany), a 20 μL sample loop injection (Rheodyne No. 7125, USA), a thin-layer flow cell (GL Sciences, Inc.), an amperometric detector and a data acquisition system (Eco-chemice Netherland). The chromatographic column was a C18 column (150 mm x 4.6 mm i.d.) from

Phenomenex and all chromatographic separations were performed at room temperature (~25 °C). A hydrodynamic voltammogram was obtained before the amperometric determination was performed. The peak current after each injection was recorded along with the corresponding background current. These data were plotted as a function of applied potential to obtain the hydrodynamic voltammograms. The amperometric measurements were carried out at the potential that provided the maximum signal-to-background (S/B) ratio in the hydrodynamic voltammograms.

2.5.3 Flow injection analysis with amperometric detection

The FIA system consisted of a thin layer flow cell (Bioanalytical System, Inc.), an injection port (Rheodyne7725) with a 20-μL injection loop, a peristaltic pump (Ismatec), and an electrochemical detector (PG100). The carrier stream, 0.1 M phosphate buffers (pH 9), was regulated by a reagent delivery module at a flow rate of 1 mL / min. A pulse dampener was used in series to reduce the pulsation introducing by the alternation of the roller of the peristaltic pump. The thin layer flow cell consisted of a silicone gasket as a spacer, Ag/AgCl as the reference electrode, stainless steel tube as an auxiliary electrode and outlet. The experiments were performed in a copper Faradaic cage to reduce electrical noise. A hydrodynamic voltammogram was obtained before the amperometric determination was carried out.

The peak current after each injection was recorded, together with the corresponding background current. These data were plotted as a function of applied potential to obtain hydrodynamic voltammograms. The amperometric measurements were carried out at the potential giving a maximum signal-to-background (S/B) ratio in the hydrodynamic voltammograms.

CHAPTER III
RESULTS AND DISCUSSION

In this chapter, details will be separated into two parts. The first part was the results and discussion from using boron-doped diamond thin film electrode for the determination of α-lipoic acid in food suppliments. The results obtained from batch system and HPLC analysis will be presented respectively. For the second part, the target analyte was changed from α-lipoic acid to N-acetyl-L-cysteine that to be analyzed by FIA amperometric system. The results obtained were categorized in the same steps as shown in the first part.

3.1. The development and validation of electrochemical techniques for the evaluation of lipoic acid and related compounds: Application in clinical and food samples

3.1.1. Electrooxidation of α-lipoic acid at the BDD electrode

The cyclic voltammograms of 1 mM α-lipoic acid and lipoamide at the BDD electrode in the mobile phase revealed a well-resolved oxidation wave for both compounds during the scan of potential towards the positive direction at the BDD electrode (**Figure 3.1**). However, compared to those obtained with the GC electrode , the charging currents at the BDD electrode were much lower, leading to a much larger signal-to-background ratio **(Figure 3.2)**. Interestingly, it was found that a slight fouling of the product occurred during consecutive scans with the BDD electrode in α-lipoic acid solutions. However, in contrast to the GC electrode, the background current was almost the same after rinsing with distilled water and immerging in the blank solution as the background current before exposure to the α-lipoic acid solution.

These results indicate that the BDD electrode offers greatly improved performance and substantially higher sensitivity than the GC electrode. Therefore, the electrochemical behavior of α-lipoic acid can be improved by this electrode.

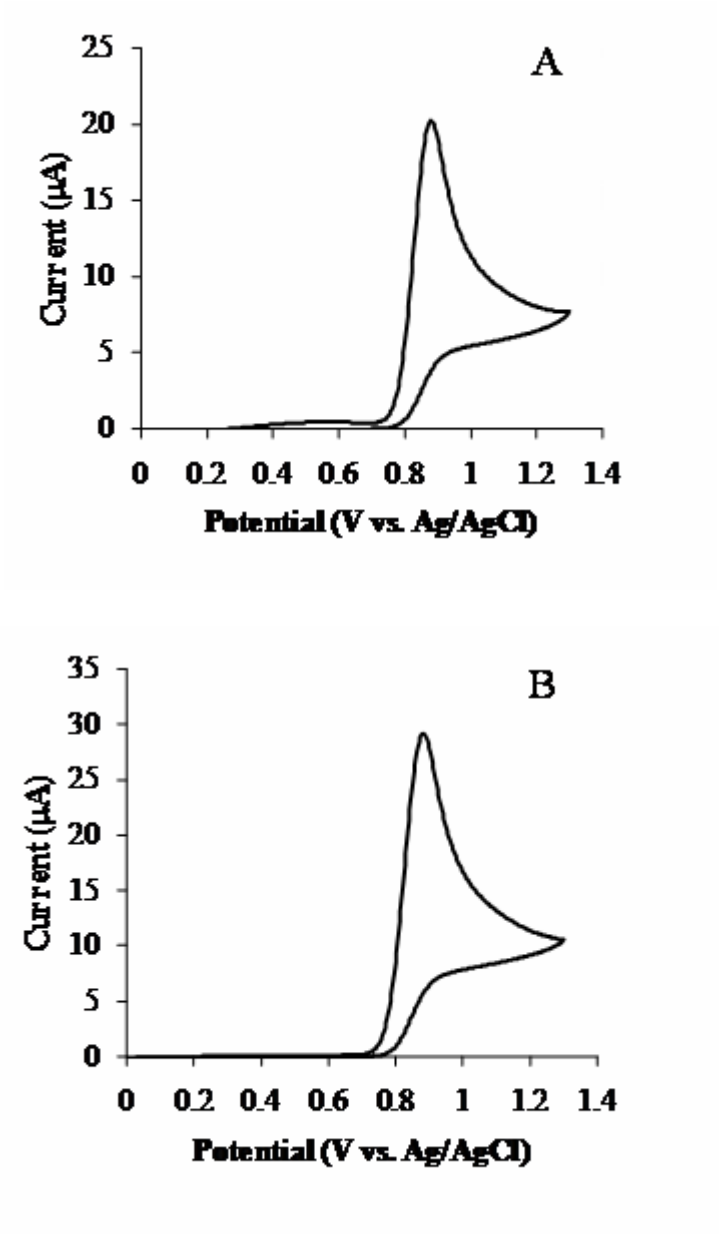

Figure 3.1. Cyclic voltammograms for the BDD electrode vs. Ag/AgCl of (A) 1.0 mM α-lipoic acid and (B) 1.0 mM lipoamide in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile. Sweep rate 100 mV/s, electrode area 0.07 cm^2. Voltammograms shown are representative of at least 5 independent repetitions.

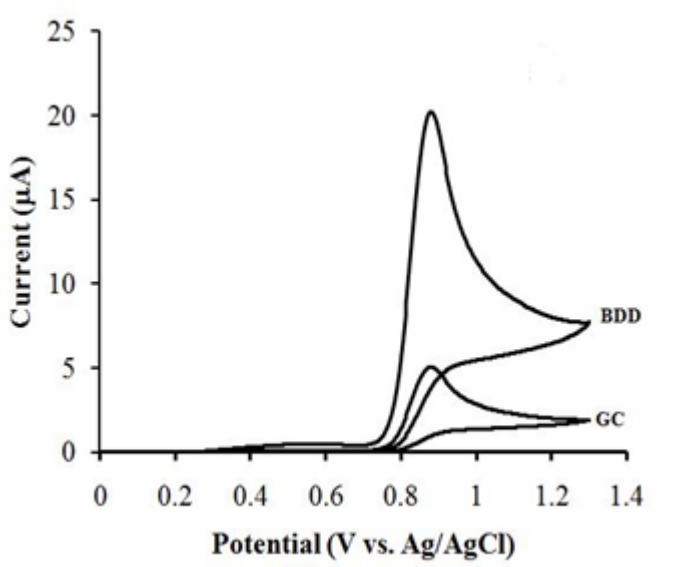

Figure 3.2 Cyclic voltammograms for a) GC electrode and b) BDD electrode versus Ag/AgCl in 1.0 mM lipoic acid in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile. Sweep rate, 50 mVs^{-1}; area of electrode, 0.07cm^2

To authenticate the adsorption of α-lipoic acid and lipoamide on the BDD electrode surface, the scan rate dependence was carried out within the range of 0.01 – 0.3 V/s (**Figure 3.3-3.4**). The anodic peak currents increased linearly with the square root of the scan rate over the examined range (linear regression $r^2 > 0.999$), supporting the theories that α–lipoic acid and lipoamide is slightly adsorbed on the electrode surface and that the reaction is controlled by the diffusion process.

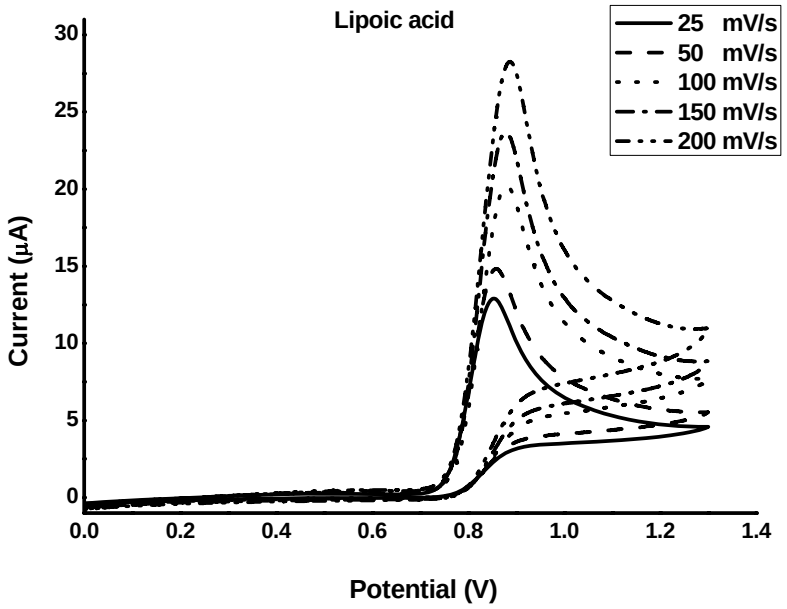

Figure 3.3 Cyclic voltammogram for 1 mM lipoic acid in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile at boron-doped diamond electrode for a series of potential sweep rates; area of electrode, 0.07cm^2.

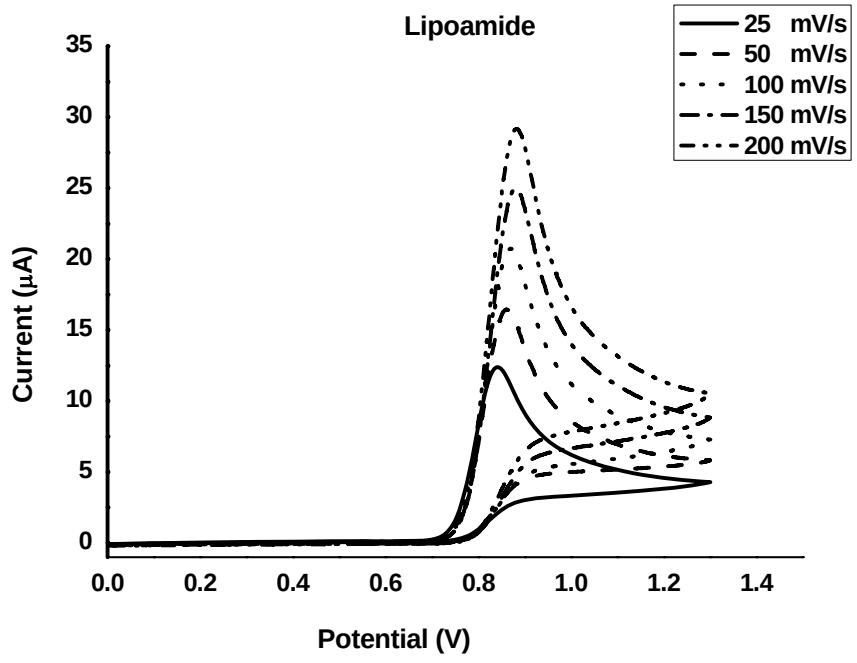

Figure 3.4 Cyclic voltammogram for 1 mM lipoamide in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile at boron-doped diamond electrode for a series of potential sweep rates; area of electrode, 0.07cm^2.

To study the influence of Concentration, the oxidation peak current was measured at the BDD electrode for the α-lipoic acid and lipoamide concentration range from 5 μM to 5 mM in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile at sweep rate 100 mV s^{-1}. Based on a series of cyclic voltammograms in which the concentration of α-lipoic acid and lipoamide were varied from 5 μM to 5 mM **(Figure 3.5-3.6),** a linear regression statically analysis of peak current (μA) versus concentration (mM) was obtained, linearly proportional in the range 0.2 mM to 1 mM ($r > 0.99$) for both α-lipoic acid and lipoamide, respectively. The response of the BDD electrode is appropriate for quantitative determination of lipoic acid and related compounds even in the micro-molar concentration range.

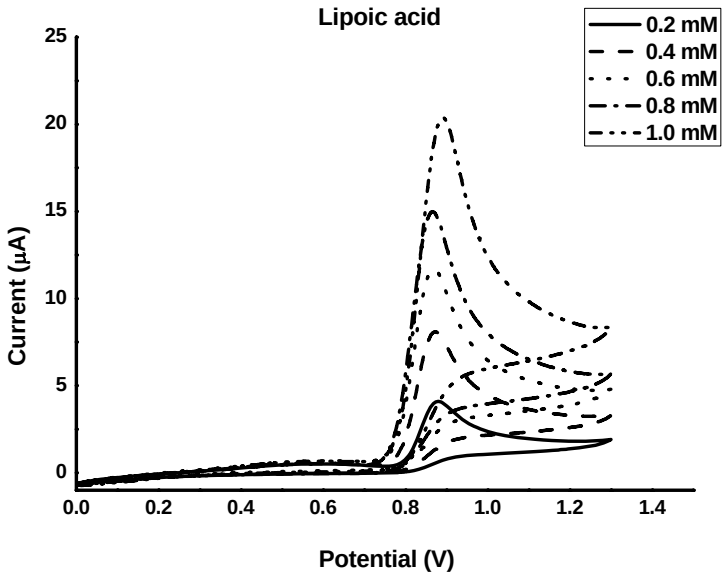

Figure 3.5 Cyclic voltammogram for α-lipoic acid in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile at boron-doped diamond electrode for a series of lipoamide concentrations.

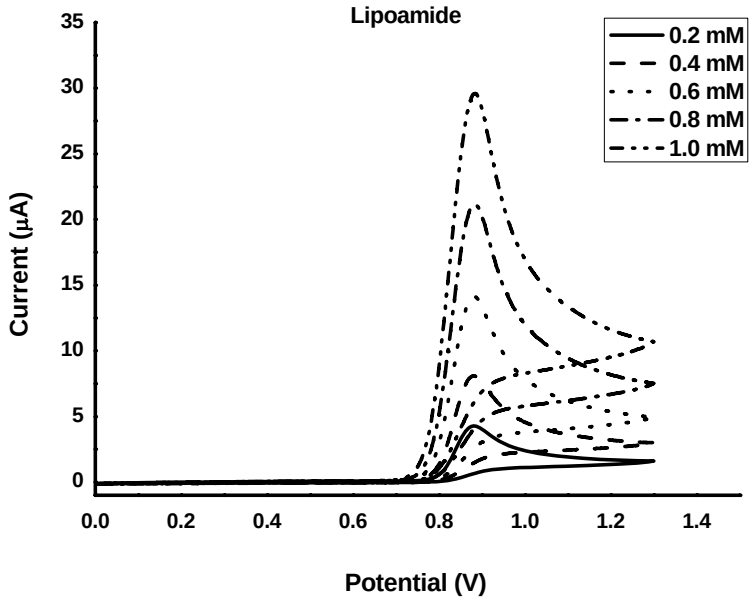

Figure 3.6 Cyclic voltammogram for lipoamide in a 1:1 (v/v) ratio of 0.05 M phosphate buffer (pH 2.5): acetonitrile at boron-doped diamond electrode for a series of lipoamide concentrations.

3.1.2. Liquid chromatographic separation

α-Lipoic acid and related compounds, such as lipoamide, were separated using a C18 packed column and an isocratic system. Initially, the mobile phase was investigated using different (v/v) ratios of 0.05 M phosphate buffer (pH 2.5): acetonitrile at a flow rate of 1.0 mL/ min, with optimization based upon obtaining a shorter run time without loss of purity (**Figure 3.7A**). The 0.05 M phosphate buffer (pH 2.5) was chosen as the most suitable solution for α-lipoic acid detection because it provided the lowest stable background current. As the percentage of acetonitrile in the mobile phase increased, a shorter elutation time was obtained; however, peak broadening increased, and lower resolution and sensitivity were observed, especially at and above 60% (v/v) acetonitrile. Thus, a 1:1 (v/v) ratio of 0.05 M phosphate

buffer (pH 2.5): acetonitrile was selected as the mobile phase, resulting in α-lipoic acid and lipoamide being well resolved as separate sharp peaks with the high signal. The chromatogram for the separation of a standard solution of lipoamide and α-lipoic acid under these conditions is presented in **Figure 3.7B**, where the retention times were 2.3 min and 4.1 min for lipoamide and α-lipoic acid, respectively. To our knowledge, this is the fastest reported separation time of α-lipoic acid and lipoamide with clear separation and indicates that α-lipoic acid and lipoamide can be determined very quickly by validated HPLC with BDD amperometric detection.

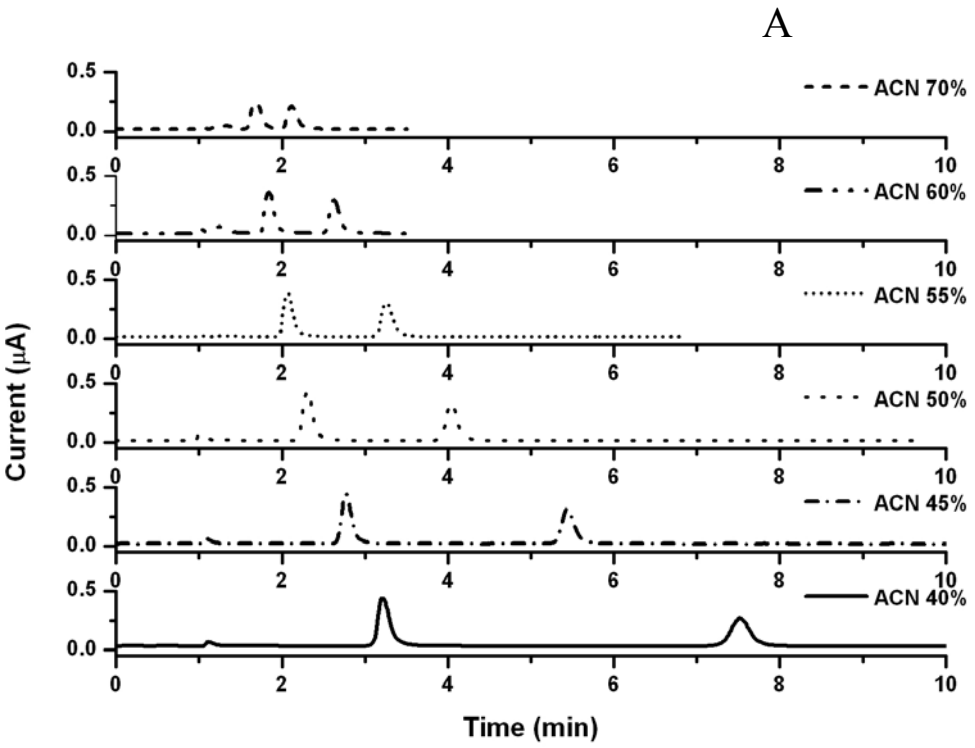

Figure 3.7A Representative HPLC–EC chromatograms of 10 ppm lipoamide and α-lipoic acid in a mobile phase (0.05 M phosphate buffer (pH 2.5): acteonitrile) comprised of (A) six different acetonitrile proportions

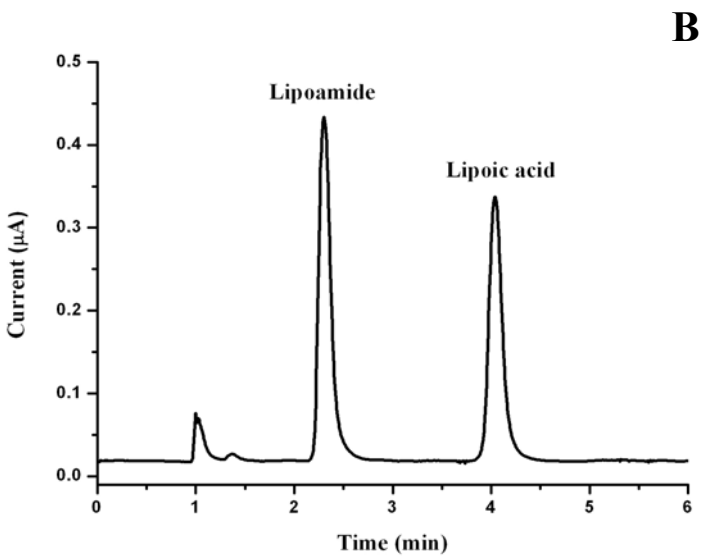

Figure 3.7B HPLC–EC chromatograms of 10 ppm lipoamide and α-lipoic acid in a mobile phase 1:1 (v/v) 0.05 M phosphate buffer (pH 2.5): acteonitrile. The detection potential was 1.05 V vs. Ag/AgCl using a BDD electrode. The injection volume was 20 μL, and the flow rate was 1.0 mL/min. Chromatograms shown are representative of at least 5 independent repetitions.

3.1.3. Hydrodynamic voltammetry

Hydrodynamic voltammetry was employed to optimize the detection potential of the α-lipoic acid from within a detection potential range of 0.8 V to 1.3 V versus Ag/AgCl. Analysis of the hydrodynamic voltammetric i-E curves of α-lipoic acid and lipoamide and the background current at each potential reveals that the oxidation current of α-lipoic acid and lipoamide, as well as the background current, were significantly affected by the detection potentials (**Figure 3.8A)**. Therefore, the net current after background subtraction (S/B) was considered and the S/B ratios were plotted against the potential curves **(Figure 3.8B)**, revealing that the signals increased when the potential increased up to 1.05 V vs. Ag/AgCl for both analytes. Thus, a detection potential at 1.05 V versus Ag/AgCl was selected as the optimal potential for

the amperometric detection of α-lipoic acid and lipoamide, following their HPLC separation.

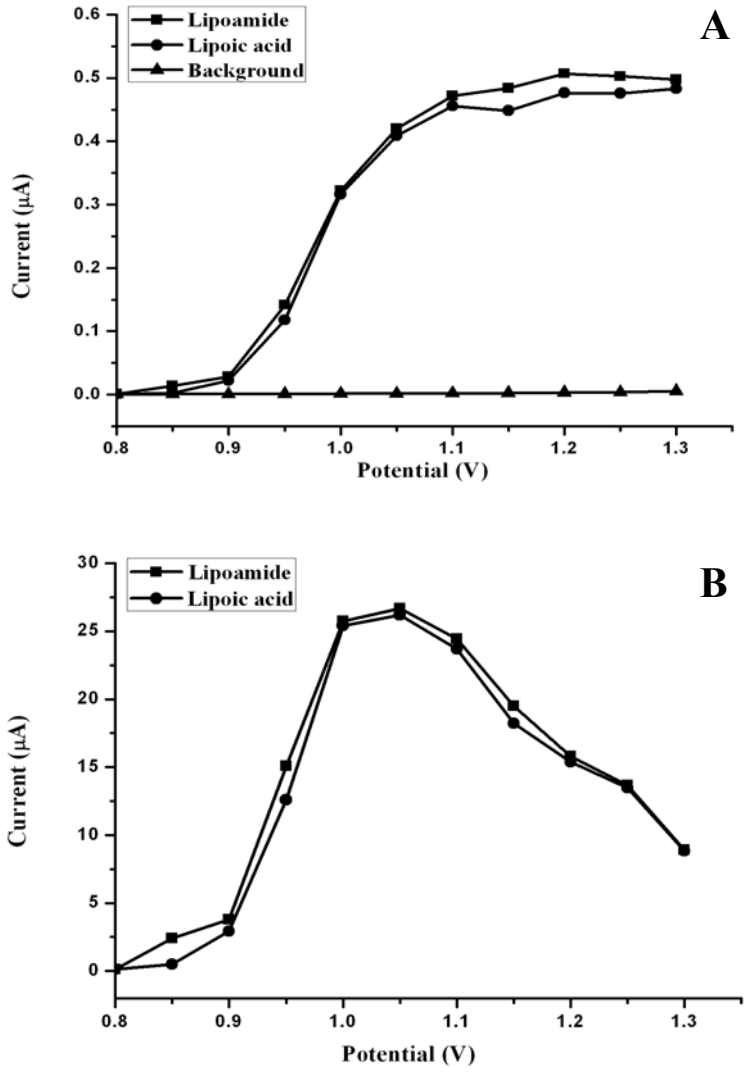

Figure 3.8 Hydrodynamic voltammetric results at the BDD electrode for a 10 ppm each mixture (20 μL total) of lipoic acid and lipoamide. (A) (■) lipoamide, (●) α-lipoic acid and (▲) background; (B) hydrodynamic voltammogram of signal-to-background ratios. The other conditions are the same as in Figure 3.7B. Data are shown as the mean ± 1 S.D. derived from 3 independent repetitions.

3.1.4. Method validation

3.1.4.1. Analytical performance

The calibration of the peak areas against concentrations generated linear functions for both of the analytes within the range of 0.01 and 120 ppm, and the coefficients of determination (r^2) were all higher than 0.99 (n = 5). The LOD and LOQ were calculated from $3S_{bl}/S$ and $10S_{bl}/S$, as described in the methods section, and are summarized in **Table 1.**

Table 1 Linearity, limit of detection (LOD) and limit of quantitation (LOQ) of the HPLC-EC method using a BDD electrode (n = 3)

Analyte	Linear range (ppm)	Slope (peak area) (units/ppm)	Intercept (μA)	R^2	LOD (ppb)	LOQ (ppb)
Lipoamide	0.01 - 60	0.3858	0.3452	0.9961	3.62	12.07
Lipoic acid	0.01 - 60	0.4029	0.2887	0.9971	3.00	10.00

When sensitivity was compared to that of previous studies, particularly in the electroanalysis field, it was found that the detection of α–lipoic acid with the BDD electrode was 1.6-, 6- and 380-fold more sensitive than the detection by HPLC-CEAD [44], lipoate-selective membrane sensors [51] and the GC electrodes [49], respectively. Though there are a few methods reported that provide a lower detection limit than the present method, those methods are tedious and complicated, requiring additional stages that cause a several-fold increase in the total analysis time, such as a derivatization step or modification of the electrode surface.

The precision of the analytical process was calculated by determining the RSD for the repeated injection of solutions containing the complete set of standard compounds. To evaluate the repeatability of the analytical process, three concentrations (0.5, 5.0 and 30.0 ppm) were studied. These spiked concentration levels were chosen in order to check the results obtained from low, medium and high concentrations with respect to the probable range of interest in food and dietary supplement samples.

The intra- and inter-day precision and recovery obtained from the proposed method are summarized in **Table 2**. The intra-day RSDs and recoveries of α–lipoic acid were found to vary over the ranges of 1.2 – 3.7% and 94.4 – 103.6%, respectively, while the inter-day RSDs and recovery of α–lipoic acid varied over the range of 1.4 – 4.1% and 93.0 – 102.7%, respectively.

Table 2 The intra- and inter-precisions and recoveries of the HPLC-BDD method (n = 3)

Samples	Spiked level (ppm)	Intra-day		Inter-day	
		Mean of % recovery (x ± SD)	RSD (%)	Mean of % recovery (x ± SD)	RSD (%)
Sample 1	0.5	97.5 ± 1.7	1.7	95.3 ± 3.6	3.5
	5	97.5 ± 3.6	3.7	98.3 ± 1.3	1.4
	30	100.4 ± 1.6	1.6	99.8 ± 1.8	1.8
Sample 2	0.5	100.4 ± 1.8	1.7	100.2 ± 0.7	1.7
	5	99.4 ± 1.6	1.6	101.5 ± 3.2	3.1
	30	100.8 ± 1.2	1.2	99.7 ± 1.8	1.8

Sample 3	0.5	94.4 ± 3.4	3.6	93.0 ± 2.4	2.6
	5	103.6 ± 2.7	2.7	100.0 ± 4.1	4.1
	30	99.8 ± 1.6	1.6	100.7 ± 1.4	1.4
Sample 4	0.5	97.4 ± 3.3	3.4	99.8 ± 2.7	2.7
	5	101.9 ± 2.1	2.1	100.8 ± 1.9	1.9
	30	100.3 ± 2.0	2.0	102.7 ± 3.4	3.2
Sample 5	0.5	99.8 ± 2.7	2.7	100.4 ± 1.8	3.6
	5	100.8 ± 1.9	1.9	99.4 ± 1.6	2.7
	30	102.7 ± 3.4	3.2	100.8 ± 1.2	2.3

3.1.4.2. Inference study

In order to check whether the matrix compounds reported on the label (carnitine and coenzyme Q10) influence the measured α–lipoic acid content, standard carnitine and coenzyme Q10 were analyzed by the proposed method. From **Figure 3.9(B–C)**, it can be seen that no signal of standard carnitine and coenzyme Q10 were observed. Therefore, no evidence of any interference effects on the determination of α-lipoic acid within the same samples was found under these optimized conditions **(Figure 3.9A)**.

3.1.5. Analysis of α-lipoic acid in dietary supplements

To verify the applicability of the BDD electrode and the methodology developed in the present study, target compounds, in terms of supplement samples from local Japanese supermarkets, were investigated by standard addition. Representative chromatograms obtained from the analysis of α–lipoic acid in two such

supplement samples, “1” and “2”, are illustrated in **Figure 3.10(A–B)**, in which the peaks were identified by comparison with the retention times of reference compounds following injection of standard solutions. In terms of consistency, note that the RSD (n = 3) for the determined content of α–lipoic acid in all supplements was lower than 4%.

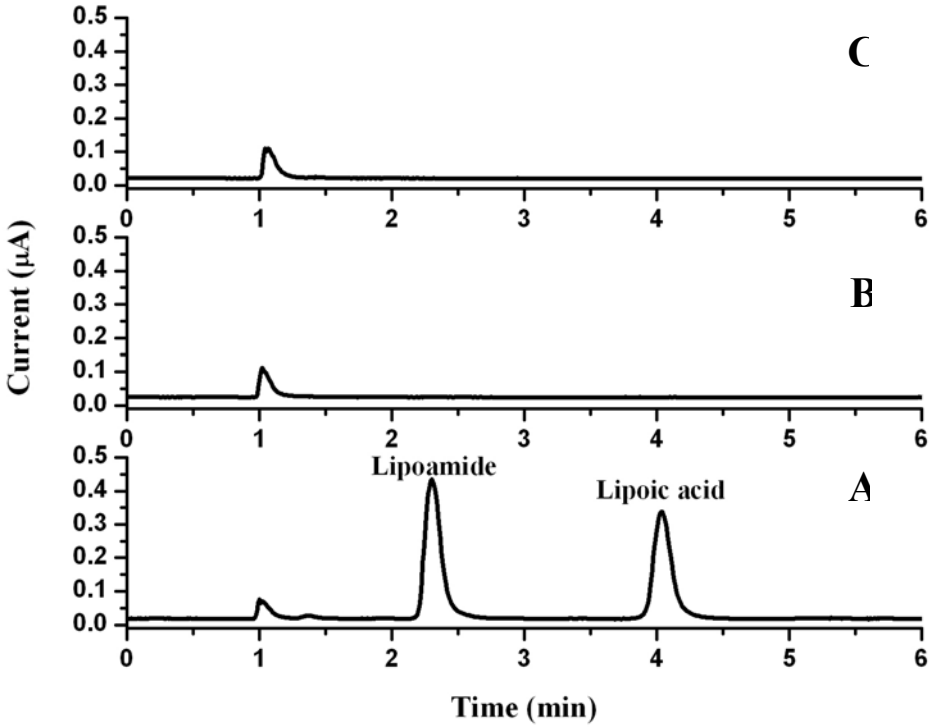

Figure 3.9. Representative HPLC–EC chromatograms of 10 ppm standard solutions (20 μL) of (A) lipoamide, α-lipoic acid, carnitine and coenzyme Q, (B) 10 ppm carnitine and (C) 10 ppm coenzyme Q. The mobile phase was 1:1 (v/v) 0.05 M potassium phosphate buffer (pH 2.5): acetonitrile. The detection potential was 1.05 V vs. Ag/AgCl using a BDD electrode and a flow rate of 1.0 mL/min. Chromatograms shown are representative of at least 5 independent repetitions.

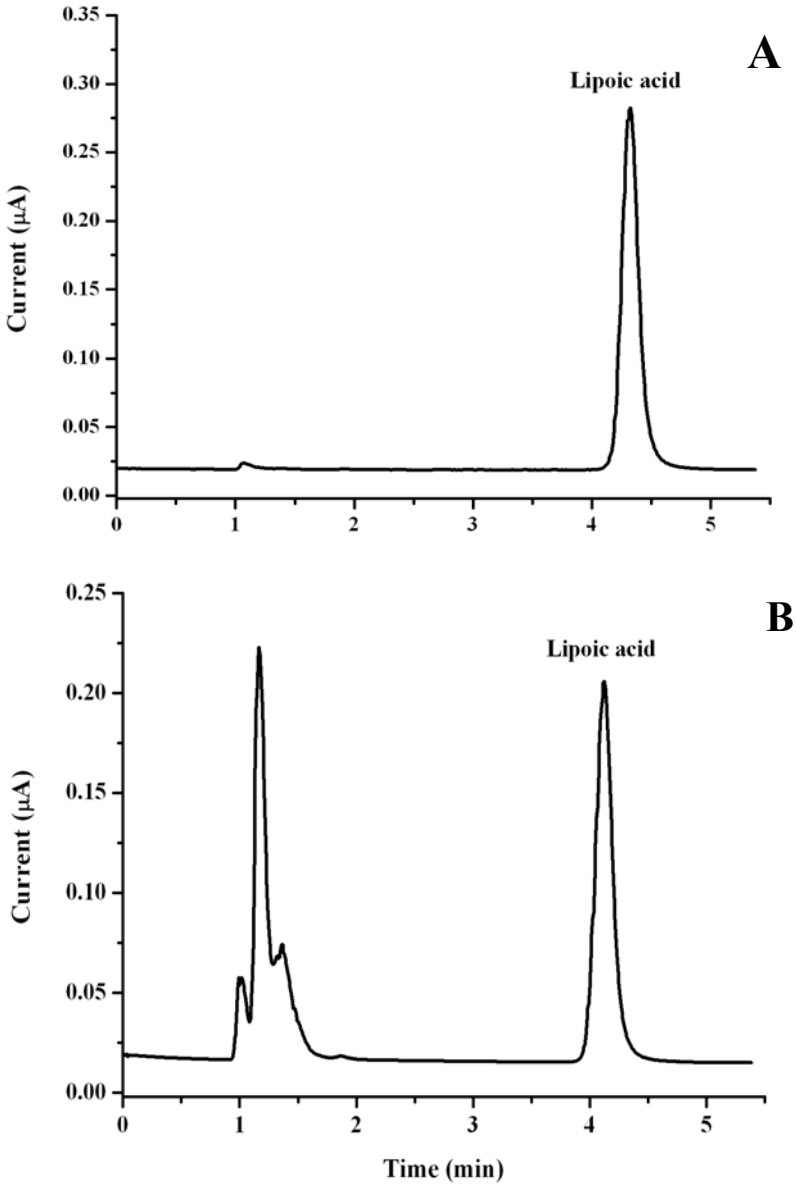

Figure 3.10 Representative HPLC-EC chromatograms of dietary supplement samples (A) '1' and (B) '2'. The mobile phase, detection potential, injection volume and flow rate were as detailed in Figure 3.9. Chromatograms shown are representative of at least 5 independent repetitions.

This quantitative evaluation revealed that the experimentally calculated values closely matched the manufacturer's claim in all five supplements examined (**Table 3**). These results were compared to those obtained by the HPLC-UV method [17] and statistically analyzed using the paired *t*-test at the 95% confidence interval. The paired two-tail test gave calculated *t* values (1.896) below the critical *t*-value (2.306), therefore accepting the null hypothesis. The data, summarized in **Table 3**, reveal that the results of HPLC coupled with BDD amperometry are of comparable accuracy to, and not significantly different from, those values obtained by the traditional HPLC-UV method.

Table 3 Determination of α-lipoic acid levels in different food supplement samples (n = 5) by the traditional HPLC-UV method and the developed HPLC-BDD electrode method reported here

Sample	Amount drug label (mg/capsule)	Amount found (mg/capsule) (x ± SD)		% Recovery	
		HPLC-BDD [a]	HPLC-UV [b]	HPLC-BDD [a]	HPLC-UV [b]
Sample 1	15	14.9 ± 0.2	14.6 ± 0.5	99.6 ± 1.3	97.3 ± 1.5
Sample 2	50	49.4 ± 0.8	49.1 ± 1.7	98.8 ± 1.7	98.2 ± 1.3
Sample 3	50	50.4 ± 1.7	51.7 ± 1.5	100.7 ± 3.5	103.4 ± 2.3
Sample 4	105	105.4 ± 2.5	104.4 ± 2.4	100.4 ± 2.4	99.4 ± 1.9
Sample 5	200	200.9 ± 1.4	201.9 ± 1.3	100.5 ± 2.7	101.0 ± 2.3

a = this developed method

b = Traditional method (Reference 33)

3.2 Highly sensitive method for the determination of N-Acetyl-L-Cysteine in drug formulations using boron-doped diamond sensor

3.2.1. Voltammetric Study

Cyclic voltammetry was initially used as means of examining and comparing the electrochemical signal of NAC at two different electrodes (BDD electrode and GC electrode). **Figure 3.11** showed details the voltammetric response obtained at BDD electrode when it was placed in a 0.1 M phosphate buffer (pH 9) solution, containing 1 mM NAC whilst **Figure 3.11B** details the response of the GC electrode under analogous conditions. Analysis of the cyclic voltammograms recorded prior to the addition of NAC reveals that the response at each electrode produces no appreciable oxidative signals over the potential range studied (0.0-1.2 V). Furthermore, a comparison of the background currents observed at each electrode reveals that the BDD electrode exhibits a lower capacitance consistent than GC electrodes. Upon the introduction of NAC to the solution the response at the BDD electrode shows a new well defined electrochemical signal emerging at +0.60 V which obtains a current plateau at +0.87 V. In contrast, the NAC response obtained at the GC electrode shows an increase in the oxidation current from +0.60 V. This current continues to rise with increasing potential but unlike the BDD electrode because there is no well-defined oxidation wave observed. Upon reversal of the scan direction, there are no new reductive processes observed in the potential range studied at each electrode. This is consistent with the electrochemically oxidized species of NAC undergoing a chemically irreversible reaction

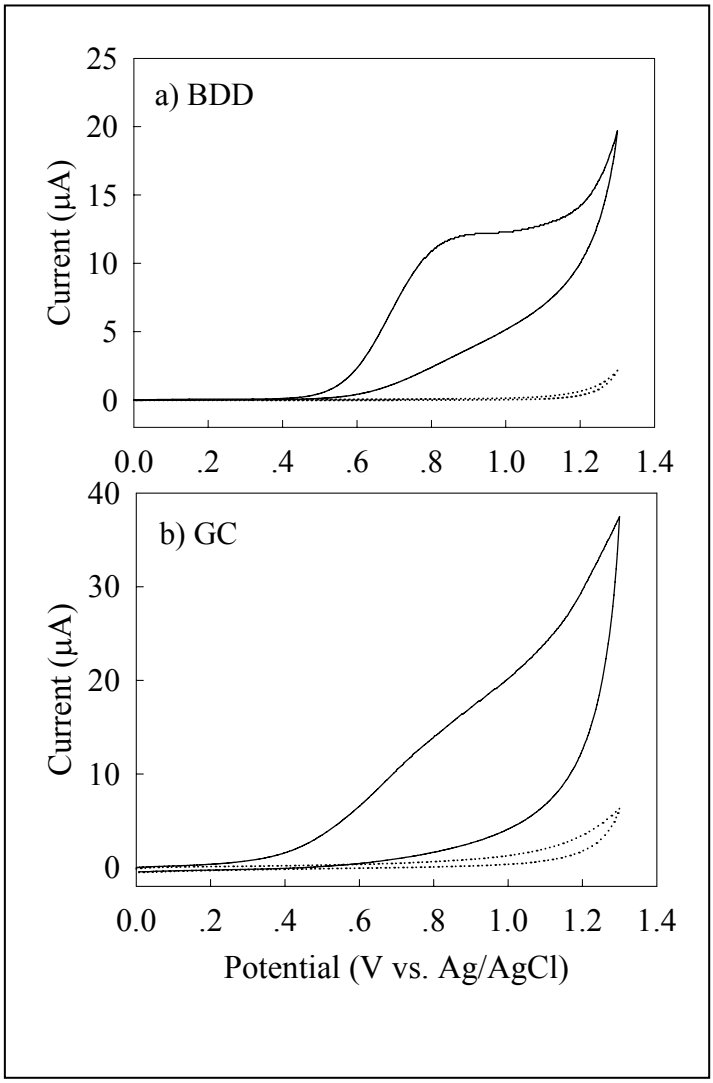

Figure 3.11 Cyclic voltammograms for a) BDD and b) GC versus Ag/AgCl in 1.0 mM NAC in 0.1 M phosphate buffer pH 9 (solid lines) and 0.1 M phosphate buffer pH 9 (dashed lines) Sweep rate, 50 mV/s; area of electrode, 0.07cm^2

3.2.2. Effect of pH value on the electrochemical behavior of NAC

In order to obtain the optimal electrochemical responses of NAC, it is important to examine the effect of buffer pH. Phosphate buffer, 0.1 M, was used as the supporting electrolyte for investigation the electrochemical property of NAC. The

experiments were performed at pH 2.5, 5.0, 6.0, 7.0, 8.0, and 9.0. The obtained results showed that NAC can be oxidized in neutral and alkali medium. The peak potential was shifted towards to more negative when the pH value increased as seen in **Table 4**. This remark can be explained using the electrooxidation of sulfur-containing compounds at BDD electrode. It involved the dissociation of the proton from the thiol group, followed by the electrochemical oxidation of NAC anion. So at higher pH value, NAC is easy to lose the proton and to produce more stable reduced from. The highest oxidation current peak was obtained from using phosphate buffer pH 9. Thus this pH was selected to use for all subsequent experiments.

Table 4 Oxidation potential and current obtained from various pH of solution

pH solution	**1 mM NAC**	
	Potential (V vs. Ag/AgCl)	**Current (μA)**
2.5	-	-
5.0	-	-
6.0	-	-
7.0	-	-
8.0	0.92	9.75
9.0	0.85	12.5

3.2.3. Scan rate and Concentration dependence

In order to verify that this oxidative reaction is diffusion controlled, a study into the effects of scan rate on the electrochemical signal was undertaken. The corresponding voltammetric responses varied with scan rate with the current plateau increasing linearly with the square root of the scan rate ($R^2 > 0.995$) as shown in

Figure 3.12. Such dependence indicated that the oxidation of NAC is indeed diffusion controlled.

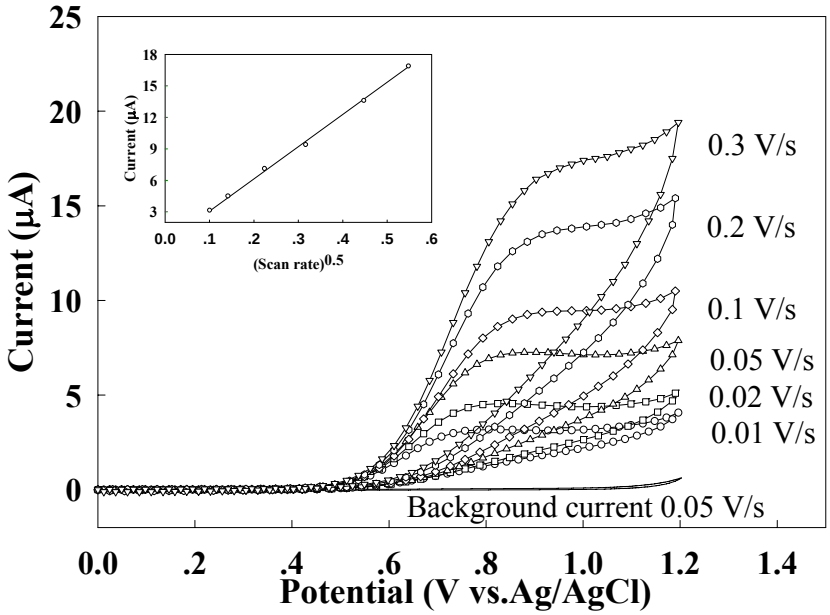

Figure 3.12 Cyclic voltammogram for 1 mM NAC in 0.1 M phosphate buffer (pH 9) at boron-doped diamond electrode for a series of potential sweep rates; area of electrode, 0.07 cm^2. The calibration curve of relationship between current (μA) and $(\text{sweep rate})^{0.5}$ was also in the inset of this figure.

Next, the analytical utility of using the BDD electrode was examined. The variation in the voltammetric currents was analyzed as a function of the NAC concentrations. The response of the BDD electrode was found to be linear over the concentration range 0.025−3.0 mM and produced a limit of detection at S/B ≥3 of 25 μM. The data was shown in **Figure 3.13**.

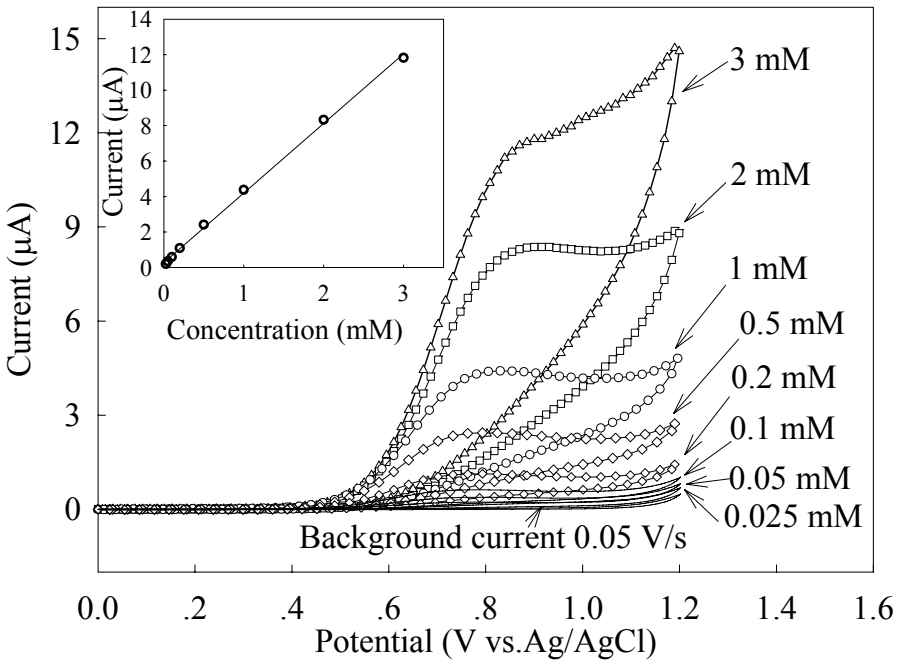

Figure 3.13 Cyclic voltammogram for NAC in 0.1 M phosphate buffer (pH 9) at boron-doped diamond electrode for a series of NAC concentrations. The potential sweep rate was 50mV/ s; area of electrode, 0.07cm^2. The calibration curve is shown in the inset.

3.2.4. Hydrodynamic voltammetry

Hydrodynamic voltammetry is a suitable method to obtain the appropriate potential applied to the FIA/amperometic detection. In this study, standard solution of NAC was repetitively injected while the FIA/amperometry operating potential was increased from 0.50 V to 1.00 V in 0.1 V increments. **Figure 3.14(a)** displayed the hydrodynamic voltammogram of the standard solution containing 100 μM NAC. When the applied potential increased the current response of NAC also increased, and no reached to maximum value. Hence, the S/B ratio was calculated from the **Figure 3.14(a)** at each point to create a graph as a function of S/B

ratios and applied potential (**Figure 3.14(b)**) to obtain the maxima potential point. To get the best sensitivity and signal to noise ratio, +0.85V was chosen as an optimal detection potential.

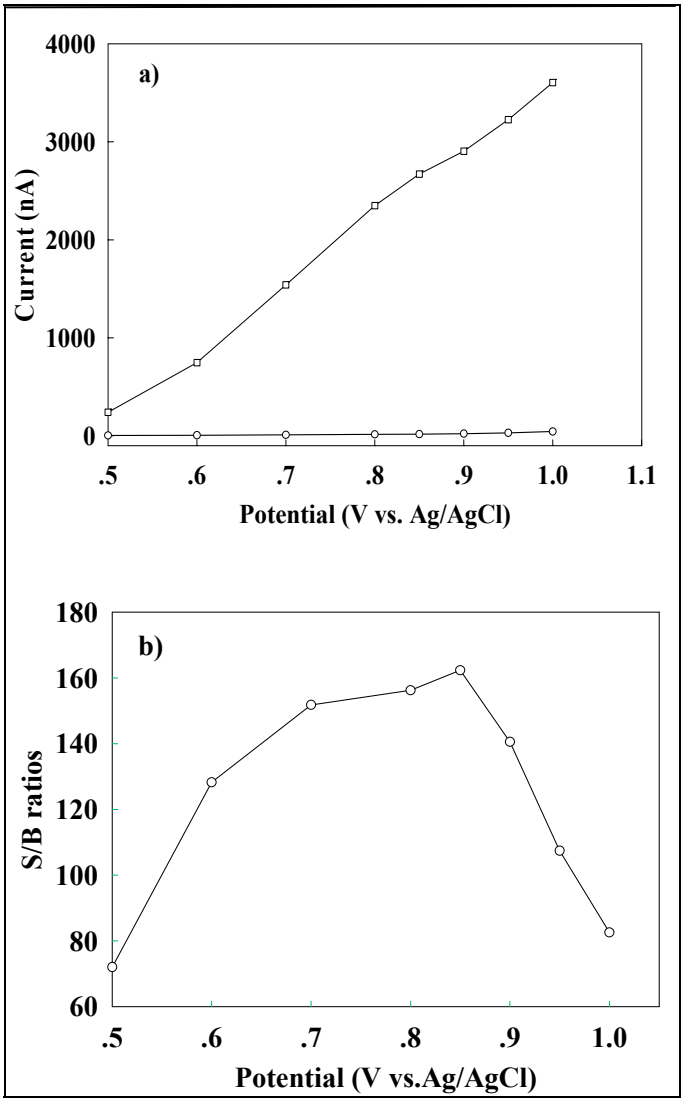

Figure 3.14 (a) Hydrodynamic voltammogram of (-○-) 0.1 M phosphate buffer (pH 9, background current) and (-□-) 100 μM of NAC in 0.1 M phosphate buffer (pH 9) with four injections of analysis, using 0.1 M phosphate buffer (pH 9) as a carrier solution. (b) Hydrodynamic of signal- to- background ratio. The flow rate was 1 mL/ min.

3.2.5. Flow injection analysis with amperometric detection

After optimizing the suitable detection potential for the FIA procedure, amperometric measurements were carried out in phosphate buffer (pH 9) containing different NAC concentration in order to obtain the analytical curve. **Figure 3.15** illustrated the FI current-time response for different NAC concentrations. A linear relationship between the current values (at +0.85 V) obtained and the NAC concentrations exhibited from 0.5 μM to 50 μM (see inside **Figure 3.15**); with a correlation coefficient of 0.9980. To higher concentrations than 50 μM occurs the deviation of linearity. The detection limit was 10 nM (S/N = 3).

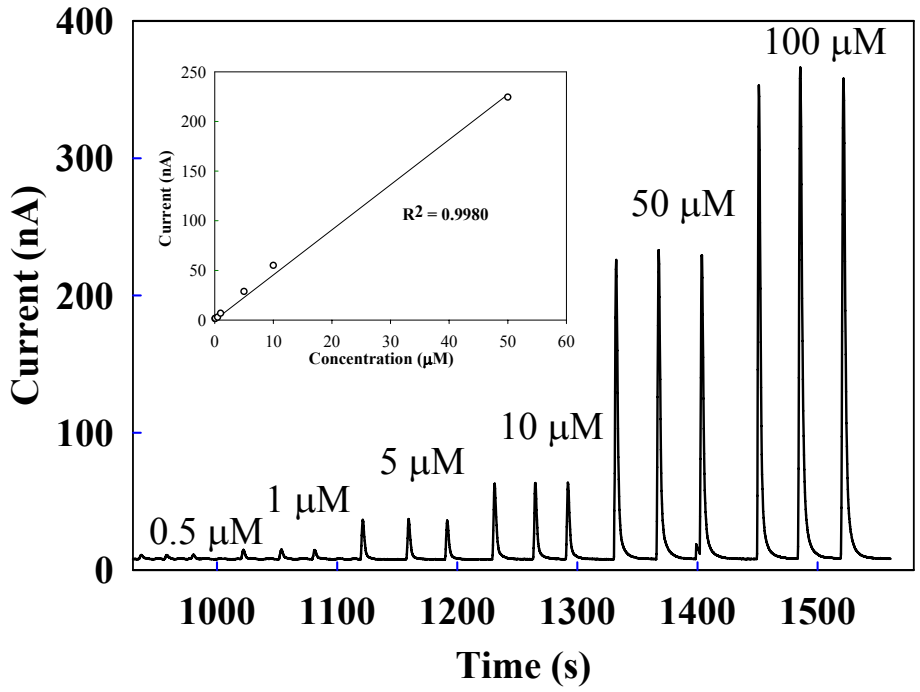

Figure 3.15 Flow injection analysis with amperometric detection results for a diamond electrode using 20-μL of NAC at various concentrations. The mobile phase was 0.1 M phosphate buffer (pH 9) at a flow rate of 1mL/ min. The calibration curve is shown in the inset.

3.2.6. Detection of NAC in commercially available drugs

In order to investigate the analytical of proposed method, FIA amperometric procedure was applied for the determination of NAC in commercially available drug as seen in **Table 5**. The NAC content was determined by standard addition method. Representative FIA results were shown in **Figure 3.17**. The recovery of this method was determined by measuring the percentage of recovery after sample solutions have been spiked with known amounts of standard compounds. **Table 6** showed the recovery for intra-day and inter-day from the determination of NAC using this proposed method.

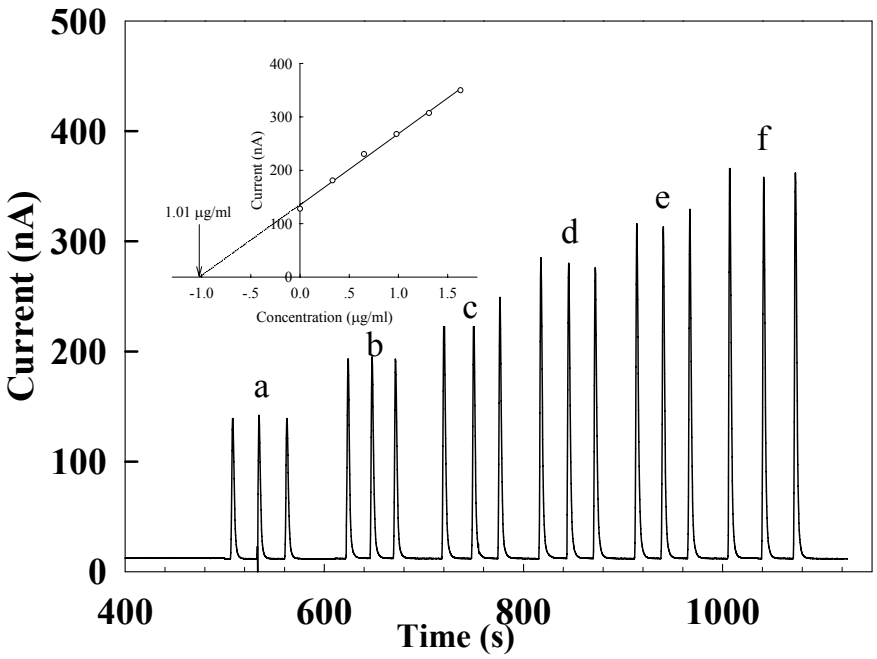

Figure 3.17 Flow injection analysis results for determination of NAC in commercially available tablets. Using the standard addition for the added NAC: (a) 0.00 µg/mL, (b) 0.33 µg/mL, (c) 0.65 µg/mL, (d) 0.98 µg/mL, (e) 1.31 µg/mL, (f) 1.63 µg/mL.

Table 5 The intra- and inter-precisions and recoveries of the FIA-BDD method (n = 5)

Samples	Spiked level (μg/mL)	Intra-day		Inter-day	
		Mean of % recovery (x ± SD)	RSD (%)	Mean of % recovery (x ± SD)	RSD (%)
Sample 1	0.33	97.8 ± 1.7	2.7	95.3 ± 3.6	3.5
	0.67	97.5 ± 3.6	3.7	98.3 ± 1.3	3.4
	1.31	100.4 ± 1.6	1.6	99.8 ± 1.8	1.8
Sample 2	0.33	100.4 ± 1.8	3.7	100.2 ± 0.7	3.8
	0.67	99.4 ± 1.6	2.6	101.5 ± 3.2	3.1
	1.31	100.8 ± 1.2	1.2	99.7 ± 1.8	1.8
Sample 3	0.33	94.4 ± 3.4	3.6	93.0 ± 2.4	2.6
	0.67	103.6 ± 2.7	2.7	100.0 ± 4.1	4.1
	1.31	99.8 ± 1.6	1.6	100.7 ± 1.4	1.9
Sample 4	0.33	97.4 ± 3.3	3.4	99.8 ± 2.7	2.7
	0.67	101.9 ± 2.1	2.1	100.8 ± 1.9	2.9
	1.31	100.3 ± 2.0	2.0	102.7 ± 3.4	3.2
Sample 5	0.33	99.8 ± 2.7	2.7	100.4 ± 1.8	3.6
	0.67	100.8 ± 1.9	1.9	99.4 ± 1.6	2.7
	1.31	102.7 ± 3.4	3.2	100.8 ± 1.2	3.3

The precision of the method was obtained on the basis of intra-assay. Three concentrations of added solution (0.33 μg/mL, 0.65 μg/mL, 1.31 μg/mL μg / mL) were chosen. Results obtained from ten injections were within 3.7 % of the relative standard deviation (RSD). The RSD values for day-to-day assays of NAC were also

investigated. It was found that in the same laboratory within one week the RSD values did not different more than 5 %.

Table 2 Determination of NAC levels in different drug samples (n = 5) by the traditional HPLC-UV method and the developed FIA-BDD electrode method reported here

Sample	Amount drug label (mg/tablet)	Amount found (mg/capsule) (x ± SD)		% Recovery	
		FIA-BDD [a]	HPLC-UV [b]	FIA-BDD [a]	HPLC-UV [b]
Sample 1	600	589.9 ± 0.2	587.4 ± 0.5	98.3 ± 1.3	97.9 ± 1.5
Sample 2	200	195.8 ± 0.8	197.2 ± 1.7	97.9 ± 1.7	98.6 ± 1.3
Sample 3	200	199.4 ± 1.7	200.9 ± 1.5	99.7 ± 3.5	104.5 ± 2.3
Sample 4	600	595.4 ± 2.5	593.6 ± 2.4	99.2 ± 2.4	98.9 ± 1.9
Sample 5	200	200.9 ± 1.4	201.9 ± 1.3	100.5 ± 2.7	101.0 ± 2.3

a = this developed method

b = Traditional method (Reference 71)

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

The major work in this project was focused on the use of electrochemical boron-doped diamond detectors for flowing liquid system consisting of flow injection analysis and high performance liquid chromatography. While understanding the behavior of electrochemical detection process, boron-doped diamond thin film electrode was tested for α-lipoic acid and related compounds as well as N-acetyl-L-cysteine. BDD have been widely used as electrode for many electrochemical applications. The superior electrochemical properties were low background current leads to the enhancement of the S/B ratios, wide working potential window in the aqueous solution and slightly adsorption of polar molecules.

4.1 The development and validation of electrochemical techniques for the evaluation of α-lipoic acid and related compounds: Application in clinical and food samples

The innovative concept for coupling of HPLC to a BDD electrode for the determination of α-lipoic acid in dietary supplements is reported. Particular effort was focused on the use of the BDD electrode as an alternative detector to overcome the drawback of using metal, GC or modified electrodes. With the superior electrochemical properties of the BDD electrode, it not only allows highly sensitive detection but also simplicity and cost-effectiveness due to the fact that α-lipoic acid can be detected amperometrically without derivatization or the use of a pulse waveform. Compared to other validated chromatographic methods, the total assay

could be completed in a several-fold shorter time period and yet could successfully determine α-lipoic acid contents in dietary supplements without extraction or concentration. Indeed, the good agreement in the determination of α-lipoic acid concentrations in supplement samples by the developed method and the standard HPLC-UV method further vouches for the simplicity and straightforwardness of this technique. In conclusion, HPLC coupled with BDD amperometry provides an attractive alternative method for the determination of α-lipoic acid in food supplement samples, among others, and may also be useful for biomedical and clinical investigation of α-lipoic acid levels.

4.2 Highly sensitive method for the determination of N-Acetyl-L-Cysteine in drug formulations using boron-doped diamond sensor

The results of this investigation show that BDD electrode is attractive material for further investigations concerning the determination of NAC. Cyclic voltammetric studies show the superiority of BDD electrode over GC electrode in terms of reproducibility, sensitivity and low background current (capacitive current). The anodic peak currents increase compared with that on a GC electrode. The effect of the scan rate on the peak current shows that the electrochemical process of NAC on BDD electrode was controlled by the diffusion of the species.

Experiments in FIA were performed to characterize the BDD electrode as an amperometric sensor for the detection of NAC. It can be successfully adopted to detect NAC. The method showed to be fast, simple and precise among other interesting features of the method proposed which make it applicable to NAC analysis and quality control of pharmaceutical samples. The results indicated that this method

has been satisfactorily applied to the determination of NAC in commercially available drug with corresponding to the label.

Suggestion for Future Work

Based on its range of superior characteristics for sensitive electroanalytical measurements, BDD as an electrode material and electrochemical sensor material may constitute one of the more important developments in this area, which further support the development of new applications from flowing liquid system.

Compared to already existing detection schemes, electrochemical detection was shown to be excellent in view of the integrated system requirements (such as low power, low-cost, sensitivity, and selectivity etc). The majority of the research described is still basic work and further studies are needed for application to practical chemical and/or biochemical analysis system.

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