รายงานวิจัยฉบับสมบูรณ์

โครงการการสกัดสมุนไพรไทยด้วยของไหลวิกฤตยวดยิ่งและของไหลกึ่งวิกฤตและการทำให้บริสุทธิ์ด้วยวิธีโครมาโตรกราฟีในระดับอุตสาหกรรม

สัญญาเลขที่ RSA5480012

โดย นางสาว อาทิวรรณ โชติพฤกษ์ และคณะ

มิถุนายน 2557

สัญญาเลขที่ RSA5480012

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คณะผู้วิจัย **สังกัด**

นางสาวอาทิวรรณ โชติพฤกษ์ **ภาควิชาวิศวกรรมเคมี จุฬาลงกรณ์มหาวิทยาลัย**

สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ.และสกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ RSA5480012

ชื่อโครงการ การการสกัดสมุนไพรไทยด้วยของไหลวิกฤตยวดยิ่งและของไหลกึ่งวิกฤตและการทำให้บริสุทธิ์ด้วยวิธีโครมาโตรกราฟีในระดับอุตสาหกรรม

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งานวิจัยมีวัตถุประสงค์ที่จะใช้เทคโนโลยีของไหลกึ่งวิกฤตในการพัฒนาผลิตภัณฑ์จากทรัพยากรทางการเกษตรของประเทศ และทำให้สารสกัดมีความบริสุทธิ์สูงขึ้นด้วยวิธีโครมาโตรกราฟี ในส่วนแรกคำถึงการพัฒนาสารสกัดจำพวกแคโรทินอยด์ และในส่วนที่สองเป็นการสกัดสารในกลุ่มฟินอลิกด้วยน้ำที่สภาวะกึ่งวิกฤต (อุณหภูมิระหว่าง 100 ถึง 375 องศาเซลเซียส โดยความดันสูงพอที่รักษาสภาวะของเหลว) เนื่องจากน้ำมีความปลอดภัย ราคาถูก และมีประสิทธิภาพในการสกัดและการทำปฏิกิริยาสูง

Abstract

This research aims to investigate suitable conditions for sub- and supercritical fluids extraction of natural materials in Thailand to produce value added products. The first part involves extraction of carotenoids and the purification of the compound by chromatography. The second part of the project involves subcritical water extraction of herbs and natural materials.

Executive Summary

ของไหลวิกฤตยวดยิ่งหมายถึงของของไหลใดๆ ที่มีอุณหภูมิและความดันสูงกว่าอุณหภูมิวิกฤตของของไหลนั้น โดยที่สภาวะวิกฤตหมายถึงสภาวะอุณหภูมิและความดันที่สสารจะไม่ปรากฏอยู่ในทั้งสภาวะไอหรือของเหลว ของไหลกึ่งวิกฤต (ของไหลที่สภาวะใกล้สภาวะวิกฤต) และของไหลวิกฤตยวดยิ่งนี้เอง สามารถนำมาประยุกต์ใช้กับกระบวนการที่สำคัญต่างๆ หลายชนิด เช่น กระบวนการสกัดสารสำคัญจากพืชสมุนไพร การผลิตเคมีภัณฑ์ ยาและเภสัชภัณฑ์ และอนุภาคพอลิเมอร์ขนาดเล็กเพื่อการประยุกต์ใช้ในอุตสาหกรรมต่างๆ นอกจากนี้เทคโนโลยีของไหลวิกฤตยิ่งยวดและของไหลกึ่งวิกฤตนี้ ยังใช้กับกระบวนการย่อยสลายของเสียจากโรงงานอุตสาหกรรมอีกด้วย กระบวนการต่างๆ ข้างต้นในของไหลกึ่งวิกฤตและของไหลวิกฤตยวดยิ่ง เป็นกระบวนการที่รวดเร็ว และปลอดภัย และไม่ต้องใช้ตัวเร่งปฏิกิริยา งานวิจัยมีวัตถุประสงค์ที่จะใช้เทคโนโลยีของไหลกึ่งวิกฤตในการพัฒนาผลิตภัณฑ์จากทรัพยากรทางการเกษตรของประเทศ และทำให้สารสกัดมีความบริสุทธิ์สูงขึ้นด้วยวิธีโครมาโตรกราฟี ในส่วนแรกคำถึงการพัฒนาสารสกัดจำพวกแคโรทินอยด์ และในส่วนที่สองเป็นการสกัดสารในกลุ่มฟินอลิกด้วยน้ำที่สภาวะกึ่งวิกฤต (อุณหภูมิระหว่าง 100 ถึง 375 องศาเซลเซียส โดยความดันสูงพอที่รักษาสภาวะของเหลว)

เนื้อหางานวิจัย

บทนำ

เป็นที่ทราบดีว่าเทคโนโลยีของไหลวิกฤตยิ่งยวดและของไหลกึ่งวิกฤตได้รับความสนใจและมีการประยุกต์ใช้เทคโนโลยีนี้ในกระบวนการทางอุตสาหกรรมต่างๆ อย่างกว้างขวาง เช่น กระบวนการสกัดสารสำคัญจากพืชสมุนไพร การผลิตเคมีภัณฑ์ ยาและเภสัชภัณฑ์ และอนุภาคพอลิเมอร์ขนาดเล็กเพื่อการประยุกต์ใช้ในอุตสาหกรรมต่างๆ นอกจากนี้เทคโนโลยีของไหลวิกฤตยิ่งยวดและของไหลกึ่งวิกฤตนี้ ยังใช้กับกระบวนการย่อยสลายของเสียจากโรงงานอุตสาหกรรมอีกด้วย กระบวนการต่างๆ ข้างต้นในของไหลกึ่งวิกฤตและของไหลวิกฤตยวดยิ่ง เป็นกระบวนการที่รวดเร็ว และปลอดภัย และไม่ต้องใช้ตัวเร่งปฏิกิริยา

ในโครงการที่เสนอขอรับทุนนี้ ผู้วิจัยจะศึกษาการใช้เทคโนโลยีของไหลวิกฤตยวดยิ่งและของไหลกึ่งวิกฤตในการพัฒนากระบวนการผลิตผลิตภัณฑ์ต่างๆ จากทรัพยากรทางการเกษตรของประเทศ โดยแบ่งงานวิจัยออกเป็นสองส่วน ในส่วนแรก จะศึกษาการใช้คาร์บอนไดออกไซด์วิกฤตยวดยิ่งในการสกัดสารที่มีขั้วต่ำ ได้แก่สารจำพวกแคโรทินอยด์ จากพืชสมุนไพรไทย เช่นสกัดสารลูทีน จากดอกดาวเรืองแห้ง

นอกจากนี้ เนื่องจากมูลค่าของสารสกัดยังขึ้นกับความบริสุทธิ์ของสาร ซึ่งเมื่อพิจารณาจากราคาของสารในกลุ่มแคโรทินอยด์แล้ว นับว่าเป็นการคุ้มค่าที่จะพัฒนากระบวนการทำให้บริสุทธิ์ของสารแคโรทินอยด์ ผู้วิจัยจึงเสนอที่จะศึกษาการทำให้บริสุทธิ์ของสารแคโรทินอยด์ที่สกัดได้โดยเฉพาะสารลูทีนบริสุทธิ์จากดอกดาวเรือง (เนื่องจากมีความพร้อมด้านวัตถุดิบของดอกดาวเรืองที่เพาะปลูกมากภายในประเทศ รวมถึงราคาของสารบริสุทธิ์ที่สูงถึงเกือบ 20,000 บาทต่อมิลิกรัม) เนื่องจากกระบวนการทำให้บริสุทธิ์ด้วยการตกผลึกที่ใช้ทั่วไปนั้น มีขั้นตอนหลายขั้นตอน ทำให้การพัฒนาขยายขนาดในระดับอุตสาหกรรมเป็นไปได้ค่อนข้างยาก ในส่วนนี้ผู้วิจัยเสนอที่จะพัฒนากระบวนการโครมาโครกราฟี โดยศึกษาสภาวะที่เหมาะสมต่อการแยก (เช่น ชนิดของ Stationary phase และ Mobile phase ขนาดอนุภาคของ Stationary phase และอัตราการไหลของ Mobile phase)

สำหรับการสกัดสารประเภทมีขั้ว การใช้คาร์บอนไดออกไซด์วิกฤตยวดยิ่งอาจไม่เหมาะสมเนื่องจากคาร์บอนไดออกไซด์เป็นสารที่มีขั้วต่ำ ดังนั้นในส่วนที่สองของข้อเสนอโครงการ ผู้วิจัยจึงเสนอที่จะใช้น้ำที่สภาวะกึ่งวิกฤต (อุณหภูมิระหว่าง 100 ถึง 375 องศาเซลเซียส โดยความดันสูงพอที่รักษาสภาวะของเหลว) เป็นตัวกลางในการสกัดสารสำคัญจากพืชไพรในกลุ่มนี้ จากงานวิจัยที่ผ่านมาพบว่า น้ำกึ่งวิกฤตสามารถสกัดสารกลุ่มแอนทราควิโนนส์จากรากของต้นยอ หรือสารกลุ่มฟินอลิคจากพืชสมุนไพรหลายชนิด เช่น มะระขี้นก หรือสมอไทยได้อย่างมีประสิทธิภาพ และนอกจากสมบัติด้านความมีขั้วที่ลดลงของน้ำกึ่งวิกฤตแล้ว น้ำกึ่งวิกฤตยังมีสมบัติการแตกตัวเป็นไอออนได้ดี จึงสามารถเป็นตัวกลางสำหรับปฏิกิริยาไฮโดรไลซิสของชีวมวลหรือกากของพืชผลทางการเกษตร เพื่อการผลิตผลิตภัณฑ์มูลค่าเพิ่ม งานวิจัยในอดีตพบว่าน้ำกึ่งวิกฤตสามารถนำมาใช้ในการสกัดโปรตีนและกรดอะมิโนจากรำข้าวได้อย่างมีประสิทธิภาพ ดังนั้นในส่วนที่สองของข้อเสนอโครงการนี้ จึงมีวัตถุประสงค์เพื่อศึกษาการสกัดสมุนไพรชนิดอื่นด้วยวิธีดังกล่าว รวมถึงศึกษาความเป็นไปได้ในการเพิ่มประสิทธิภาพของการสกัดด้วยการเตรียมวัตถุดิบในการสกัดโดยใช้เทคโนโลยีไมโครเวฟ

ผลของงานวิจัยนี้นำไปสู่การพัฒนากระบวนการที่ปลอดภัยต่อผู้บริโภคและสิ่งแวดล้อมในการเพิ่มมูลค่าให้กับพืชสมุนไพรไทยซึ่งเป็นทรัพยากรสำคัญของประเทศ รวมถึงวัตถุดิบที่เป็นผลพลอยได้ทางการเกษตร อีกทั้งยังเป็นการสร้างองค์ความรู้ใหม่ ที่จำเป็นต่อการประยุกต์เทคโนโลยีของไหลกึ่งวิกฤตและของไหลวิกฤตยวดยิ่งในกระบวนการดังกล่าว

วัตถุประสงค์

1 พัฒนาวิธีการสกัด และการทำให้สารบริสุทธิ์ของสารกลุ่มแคโรทินอยด์ด้วยวิธีโครมาโตรกราฟี

2 ศึกษาสภาวะที่เหมาะสม ในการสกัดสารกลุ่มฟินอลิกด้วยน้ำกึ่งวิกฤต

ระเบียบวิธีวิจัย

1 รวบรวมเอกสารวิจัย

2 ทดสอบวิธีการวิเคราะห์สาร

3 ทดลองการสกัดสารกลุ่มแคโรทินอยด์และทำให้สารบริสุทธิ์ด้วยวิธีโครมาโตรกราฟี

4 ทดลองสกัดสารกลุ่มฟินอลิกในรำข้าวและเปลือกพะยอมด้วยน้ำกึ่งวิกฤต

4 สรุปผลและทำรายงานและเตรียมผลงานตีพิมพ์ในวารสารนานาชาติ

ผลที่ได้จากงานวิจัย

ผลที่ได้จากงานวิจัยในโครงการนี้ ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติจำนวน 6 เรื่อง ดังต่อไปนี้

1. Boonnoun P., Opaskornkun T, Prasitchoke P., **Shotpruk A.** Purification of Free Lutein from Marigold Flowers by Liquid Chromatography, Engineering Journal 16(5), 145-156.
2. Boonnoun P., Nerome H., Machmudah S., Goto M., **Shotipruk A.** Supercritical Anti-solvent Micronization of Marigold-derived Lutein Dissolved in Dichloromethane and Ethanol, Journal of Supercritical Fluids 77 (2013), 103-109 (IF2012=2.732).
3. Boonnoun P., Nerome H., Machmudah S., Goto M., **Shotipruk A.** Supercritical Anti-solvent Micronization of Chromatography Purified Lutein Using Hexane and Ethyl Acetate Solvent Journal of Supercritical Fluids 80 (2013), 15-22 (IF2012=2.732).
4. Prommuak C. Pavasant P., Quitain A., Goto M., **Shotipruk A.** Simultaneous Production of Biodiesel and Free Lutein from *Chlorella vulgaris*. Chemical Engineering&Technology 36 (2013), 733-739 (IF 2012=1.366).
5. Wataniyakul P., Pavasant P., Goto M., **Shotipruk A.** Microwave Pretreatment of Defatted Rice Bran for Enhanced Recovery of Total Phenolics Compounds Extracted by Subcritical Water, Bioresource Technology124, 18-22 (IF 2012-4.750)**.**
6. Chainukool S., Goto M., Hannongbua S., Shotipruk A. Subcritical Water Extraction of Resveratrol from Barks of Shorea Roxburghii G. Don. Separation Science and Technology, Accepted (IF 2012=1.164).

เรื่องที่ 1-3 เป็นการพัฒนาสารลูทีนอิสระจากดอกดาวเรือง สารสกัดถูกนำมาทำให้มีความบริสุทธิ์สูงขึ้นด้วยวิธีโครมาโตกราฟี และทำให้เป็นอนุภาคขนาดเล็กในคาร์บอนไดออกไซด์วิกฤตยวดยิ่ง เรื่องที่ 4 เป็นการประยุกต์ขั้นตอนที่ได้ศึกษามาสำหรับการสกัดและแยกสารลูทีนอิสระจากดอกดาวเรืองกับการแยกสารลูทีนอิสระที่ได้จากสาหร่ายคลอเรลลา แวลการิส เรื่องที่ 5-6 เป็นการศึกษาการสกัดสารสำคัญจากพืชสมุนไพรและจากรำข้าวด้วยน้ำกึ่งวิกฤต

เรื่องที่ 1

ชื่อเรื่อง (อังกฤษ) Purification of Free Lutein from Marigold Flowers by Liquid Chromatography

ชื่อเรื่อง (ไทย) การทำให้บริสุทธิ์ของลูทีนอิสระจากดอกดาวเรืองด้วยโครมาโตรกราฟีของเหลว

ชื่อผู้เขียน (ไทย) ปณัฐพงศ์ บุญนวล[1], ธนวิช โอภาสกรกุล[1], พัฒานนท์ ประสิทธิ์โชค[2], โกโตะ โมโตโนบุ[3] และอาทิวรรณ โชติพฤกษ์[1]

ชื่อหน่วยงาน/สังกัดของผู้เขียน (ไทย)

1. ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพ 10330
2. บริษัท ปตท. โกลบอล เคมิคอล ระยอง
3. ภาควิชาชีวเคมีและเคมีประยุกต์ มหาวิทยาลัยกูมาโมโตะ ญี่ปุ่น

ชื่อวารสาร (อังกฤษ) Engineering Journal

ดาวเรืองเป็นแหล่งของแซนโทฟิลที่เรียกว่าลูทีน ซึ่งเป็นสารที่มีฤทธิ์ต้านอนุมูลอิสระและต้านมะเร็ง รวมถึงมีประโยชน์ต่อสายตา ทำให้มีการศึกษาการสกัดและการทำให้บริสุทธิ์ของสารลูทีนที่มีความเหมาะสมต่อการใช้งานในมนุษย์ โครมาโตรกราฟีของเหลวเป็นวิธีที่ใช้มากในการทำให้สารประกอบธรรมชาติมีความบริสุทธิ์ วัฏภาคเคลื่อนที่และวัฏภาคนิ่งเป็นปัจจัยสำคัญต่อการแยกด้วยวิธีนี้ ในงานวิจัยนี้จึงศึกษาการแยกสารลูทีนอิสระด้วยวิธีโครมาโตรกรฟี โดยใช้ซิลิกาเจล และตัวทำละลายผสมระหว่าเฮกเซนและเอทธิลอะซิเตตเป็นวัฏภาคนิ่งและวัฏภาคเคลื่อนที่ ตามลำดับ เริ่มจากการหาอัตราส่วนที่เหมาะสมของตัวทำละลายผสมโดยทดสอบกับโครมาโตรกราฟีแผ่นบาง พบว่าตัวทำละลายผสมที่อัตราส่วน 70:30 โดยปริมาตรของเฮกเซน:เอทธิลอะซิเตต เป็นตัวทำละลายที่เหมาะสมสำหรับการแยกลูทีนด้วยวิธีโครมาโตรกราฟีแบบนอร์มอบเฟส การทดลองการแยกด้วยวิธีโครมาโตรกราฟีในคอลัมน์ขนาดใหญ่ขึ้น พบว่าสามารถแยกสารลูทีนที่มีความบริสุทธิ์ได้ถึง 97.1 % โดยมีปริมาณผลได้เท่ากับ 60 % นอกจากนี้ เนื่องจากการแยกด้วยวิธีนี้อาศัยหลักการของการดูดซับของสารลูทีนบนซิลิกาเจล งานวิจัยนี้จึงศึกษาการดูดซับของระบบดังกล่าว ซึ่งพบว่ามีพฤติกรรมการดูดซับที่สมดุลเป็นไปตามแบบจำลองของแลงเมียร์

เรื่องที่ 2

ชื่อเรื่อง (อังกฤษ) Supercritical Anti-solvent Micronizaton of Marigold-derived Lutein Dissolved in Dichloromethane and Ethanol

ชื่อเรื่อง (ไทย) การทำให้เป็นอนุภาคขนาดเล็กของลูทีนที่ได้จากดาวเรืองที่ละลายในไดคลอโรมีเทนและเอทธานอล

ชื่อผู้เขียน (ไทย) ปณัฐพงศ์ บุญนวล ฮาซูกิ เนโรเม ซิต มาชมูดาห์ โมโตโนบุ โกโตะ และอาทิวรรณ โชติพฤกษ์

ชื่อหน่วยงาน/สังกัดของผู้เขียน (ไทย)

1. หน่วยปฏิบัติการวิศวกรรมเคมีเพื่อเพิ่มมูลค่าของทรัพยากรทางชีวภาพ ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพ 10330
2. บัณฑิตวิทยาลัยด้านวิศวกรรม มหาวิทยาลัยนาโกยา ญี่ปุ่น

ชื่อวารสาร (อังกฤษ) Journal of Supercritical Fluids

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการทำให้เป็นอนุภาคขนาดเล็กของลูทีนได้ได้จากดอกดาวเรืองด้วยการตกตะกอนออกจากตัวทำละลาย สารละลายลูทีนในไดคลอโรมีเทนหรือเอทธานอลถูกพ่นเข้าไปในคาร์บอนไดออกไซด์วิกฤตยวดยิ่งผ่านหัวฉีดที่มีแกนร่วมกันซึ่งอยู่ภายในห้องความดัน โดยศึกษาอิทธิพลของความดันและอัตราการไหลของคาร์บอนไดออกไซด์วิกฤตยวดยิ่งต่อขนาดเฉลี่ยและการกระจายตัวของขนาดของอนุภาค พบว่าขนาดเฉลี่ยของอนุภาคเล็กลงจาก 202.3 ไมโครเมตรสำหรับลูทนีที่ไม่ผ่านกระบวนการนี้จนมีขนาด1.58 ไมโครเมตรและ 902 นาโนเมตร สำหรับการทำให้เกิดอนุภาคขนาดเล็กโดยใช้ไดคลอโรมีเทนและเอทธานอล ตามำดับ ในทั้งสองระบบ ไม่พบว่าความดันและอัตราการไหลของคาร์บอนไดออกไซด์วิกฤตยวดยิ่งมีผลต่อรูปร่างลักษณะของอนุภาคอย่างมีนัยสำคัญ อย่างไรก็ตาม พบว่าความดันมีผลต่อขนาดและการกระจายตัวของอนุภาคของลูทีน

เรื่องที่ 3

ชื่อเรื่อง (อังกฤษ) Supercritical Anti-solvent Micronization of Chromatography Purified Marigold Lutein Using Hexane and Ethyl Acetate Solvent Mixture

ชื่อเรื่อง (ไทย) การทำให้เป็นอนุภาคขนาดเล็กของลูทีนที่ได้จากดาวเรืองโดยใช้ตัวทำละลายผสมเฮกเซนและเอธิลอะซิเตต

ชื่อผู้เขียน (ไทย) ปณัฐพงศ์ บุญนวล ฮาซูกิ เนโรเม ซิต มาชมูดาห์ โมโตโนบุ โกโตะ และอาทิวรรณ โชติพฤกษ์

ชื่อหน่วยงาน/สังกัดของผู้เขียน (ไทย)

1. หน่วยปฏิบัติการวิศวกรรมเคมีเพื่อเพิ่มมูลค่าของทรัพยากรทางชีวภาพ
   ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพ 10330
2. บัณฑิตวิทยาลัยด้านวิศวกรรม มหาวิทยาลัยนาโกยา ญี่ปุ่น

ชื่อวารสาร (อังกฤษ)Journal of Supercritical Fluids

งานวิจัยนี้มุ่งเน้นการทำให้เป็นอนุภาคขนาดเล็กของสารลูทีนบริสุทธิ์ที่ได้จากดอกดาวเรืองที่ละลายในตัวทำละลายผสมระหว่างเฮกเซนและเอทธิลอะซิเตต (70:30 โดยปริมาตร) ซึ่งเป็นตัวทำละลายที่ใช้เป็นเฟสเคลื่อนที่ในกระบวนการทำให้สารบริสุทธิ์ด้วยวิธีโครมาโตรกราฟี ผลการทดลองแสดงให้เห็นว่าความดันมีผลมากต่อลักษณะของอนุภาค การเพิ่มความเข้มข้นเริ่มต้นของลูทีนจาก 1.5 เป็น 3.2 มิลิกรัมต่อมิลิลิตร และการเพิ่มอัตราการไหลของคาร์บอนไดออกไซด์วิกฤตยวดยิ่งจาก 15 เป็น 25 มิลิลิตรต่อนาที ไม่มีผลต่อลักษณะของอนุภาค อย่างไรก็ตาม พบว่าขนาดอนุภาคเฉลี่ยลดลงจาก 2 เป็น 0.8 ไมโครมิเตอร์ เมื่อเพิ่มอัตราการไหลของคาร์บอนไดวิกฤตยวดยิ่ง รูปแบบของการหักเหของรังสีเอกซ์ของอนุภาคลูทีนแสดงให้เห็นว่าลูทีนมีลักษณะไม่เป็นโครงร่างผลึก ขณะที่ฟูเรียร์ ทรานส์ฟอร์ม สเปคโตรสโคปี ระบุว่าไม่มีการเปลี่ยนแปลงของโครงสร้างทางเคมีของสารลูทีนภายหลังจากกระบวนการทำให้เป็นอนุภาคขนาดเล็ก นอกจากนี้ความสามารถในการละลายของลูทีนในสารละลายน้ำมีค่าสูงขึ้นจากที่มีค่าการละลายน้อยมากก่อนผ่านกระบวนการทำให้เป็นอนุภาคขนาดเล็ก จนมีค่าการละลายประมาณ 20%

เรื่องที่ 4

ชื่อเรื่อง (อังกฤษ) Simultaneous Production of Biodiesel and Free Lutein from Chlorella vulgaris

ชื่อเรื่อง (ไทย) การผลิตไบโอดีเซลและลูทีนอิสระไปพร้อมกันจากคลอเรลลา วัลการิส

ชื่อผู้เขียน (ไทย) ฉัตรทิพย์ พรหมหมวก ประเสริฐ ภวสันต์ อาร์มานโด ที คิตาอิน โมโตโนบุ โกโตะ และ อาทิวรรณ โชติพฤกษ์

ชื่อหน่วยงาน/สังกัดของผู้เขียน (ไทย)

1. หน่วยปฏิบัติการวิศวกรรมเคมีเพื่อเพิ่มมูลค่าของทรัพยากรทางชีวภาพ ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพ 10330
2. ภาควิชาชีวเคมีและเคมีประยุกต์ มหาวิทยาลัยกูมาโมโตะ ญี่ปุ่น
3. บัณฑิตวิทยาลัยด้านวิศวกรรม มหาวิทยาลัยนาโกยา ญี่ปุ่น

ชื่อวารสาร (อังกฤษ) Chemical Engineering&Technology

ไบโอดีเซลและสารมูลค่าสูงลูทีนสามารถผลิตได้พร้อมกันจากสารสกัดไขมันของคลอเรลลา วัลการิส ตัวเร่งปฏิกิริยาด่างใช้ในทรานส์เอสเทอริฟิเคชันของไตรกลีเซอไรด์ ประพฤตตนเป็นสารตั้งต้นในการเปลี่ยนลูทีนเอสเทอร์ของกรดไขมันให้เป็นลูทีนอิสระ ผลได้ของไบโอดีเซลสูงสุดมีค่าเท่ากับ 33.6% โดยน้ำหนักของไขมันจากสาหร่าย เมื่อปฏิกิริยาเกิดขึ้น 4 ชั่วโมง และอัตราส่วนระหว่างเมทธานอลและชีวมวลเท่ากับ 16:1 โดยใช้ตัวเร่งปฏิริยาด่าง 6% ด่างและเมทธานอลในปริมาณที่เกินพอสำหรับไบโอดีเซลจะทำให้เกิดการสปอนนิฟิเคชันอย่างสมบูรณ์ของลูทีนเอสเทอร์ของกรดไขมันไปเป็นลูทีนอิสระ โดยให้ผลได้เท่ากับ 2.3% โดยน้ำหนักของไขมันจากสาหร่าย นอกจากนี้ กระบวนการสำหรับการแยกไบโอดีเซลและลูทีนอิสระออกจากผลิตภัณฑ์ของปฏิกิริยา ยังถูกเสนอขึ้น และท้ายที่สุด การประเมินทางเศรษฐศาสตร์เบื้องต้นแสดงให้เห็นว่ากระบวนการผลิตไบโอดีเซลและลูทีนอิสระจากสาหร่ายชนิดนี้มีความเป็นไปได้

เรื่องที่ 5

ชื่อเรื่อง (อังกฤษ) Microwave pretreatment of defatted rice bran for enhanced recovery of total phenolic compounds extracted by subcritical water

ชื่อเรื่อง (ไทย) การปรับสภาพของรำข้าวที่สกัดน้ำมันออกแล้วเพื่อการเพิ่มปริมาณสารประกอบฟินอลิครวมด้วยการสกัดด้วยน้ำกึ่งวิกฤต

ชื่อผู้เขียน (ไทย) ปิยาภรณ์ วทานิยะกุล, ประเสริฐ ภวสันต์ โกโตะ โมโตโนบุ และอาทิวรรณ โชติพฤกษ์

ชื่อหน่วยงาน/สังกัดของผู้เขียน (ไทย)

1. ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพ 10330
2. คณะวิศวกรรมศาสตร์ มหาวิทยาลัยนาโกยา ญี่ปุ่น

ชื่อวารสาร (อังกฤษ) Bioresource Technology

บทคัดย่อ (ไทย)

การปรับสภาพรำข้าวที่สกัดน้ำมันออกแล้วด้วยไมโครเวฟสามารถการเพิ่มปริมาณสารฟินอลิครวมที่สกัดได้ด้วยวิธีสกัดด้วยน้ำกึ่งวิกฤต โดยศึกษาผลของอุณหภูมิในการปรับสภาพด้วยไมโครเวฟ (60-100 °C) และระยะเวลาที่ใช้ (0-30 นาที) ต่อการสกัดรำข้าวด้วยน้ำกึ่งวิกฤตน้ำกึ่งวิกฤตในอัตราส่วนของรำข้าวต่อน้ำเท่ากับ 1:2 และ 1:5 สภาวะการปรับสภาพที่ดีที่สุดคือ 80 °C เป็นเวลา 10 นาที และอัตราส่วนระหว่างรำข้าวต่อน้ำเท่ากับ 1:2 ซึ่งเมื่อรำข้าวผ่านการปรับสภาพที่สภาวะนี้ จะส่งผลให้เวลาที่ใช้ในช่วงของการสกัดด้วยน้ำกึ่งวิกฤตลดลงเหลือเพียง 10 นาที (เมื่อสกัดด้วยน้ำกึ่งวิกฤตที่ 200 °C) เมื่อใช้สภาวะที่เหมาะสมที่สุดทั้งต่อการปรับปสภาพแต่การสกัด จะได้ผลได้ของฟินอลิครวมเท่ากับ 190.4±3.3 มิลิกรัมต่อกรัมรำข้าว ซึ่งเพิ่มขึ้นจากการสกัดรำข้าวโดยไม่ผ่านการปรับสภาพถึง 55% นอกจากนี้ความสามารถในการต้านอนุมูลอิสระของสารสกัดยังสูงขึ้นอีกด้วย ซึ่งเห็นได้ชัดจากค่า IC_{50} ที่มีค่าลดลง จาก 38.8±0.4 เป็น 27.7±0.5

เรื่องที่ 6

ชื่อเรื่อง (อังกฤษ) Subcritical Water Extraction of Resveratrol from Barks of Shorea Roxburghii G. Don

ชื่อเรื่อง (ไทย) การสกัดเรสเวอราทรอลจากลำต้นของ Shorea Roxbughii G. Don

ชื่อผู้เขียน (ไทย) สุมาลี ไชยนุกูล, โกโตะ โมโตโนบุ สุภา หารหนองบัว และอาทิวรรณ โชติพฤกษ์

ชื่อหน่วยงาน/สังกัดของผู้เขียน (ไทย)

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ชื่อวารสาร (อังกฤษ) Separation Science and Technology

งานวิจัยนี้ศึกษาการสกัดด้วยน้ำกึ่งวิกฤตของสารประกอบฟินอลิค เช่น ทรานส์เรสเวอราทรอลและสารประกอบของมันที่มีโมเลกุลน้ำตาลเกาะอยู่ หรือที่เรียกว่าทรานส์พิซีด จากลำต้นของพะยอม (*Shorea roxburghii G. Don.*) ผลของอุณหภูมิ (100-190 °C) และอัตราการไหลของน้ำ (2-4 มิลิลิตรต่อนาที) สำหรับการสกัด 6 ชั่วโมงที่ความดันคงที่ MPa ผลการทดลองถูกนำมาเปรียบเทียบกับผลที่ได้จากวิธีแบบดั้งเดิม โดยที่พบปริมาณของสารที่มีฤทธิ์สูงกว่าคือทรานส์เรสู่น้อย (0.68 – 13.01 μg/g DW) เมื่อเทียบกับปริมาณสารเรสเวอราทรอลที่มีโมเลกุลของน้ำตาลเกาะอยู่ ปริมาณสูงสุดที่สกัดได้เท่ากับ 301.70 ไมโครกรัมฟิซีดต่อกรัมน้ำหนักแห้ง โดยสกัดได้ที่อุณหภูมิ 190 องศาเซลเซียส และอัตราการไหล 3 มิลิลิตรต่อนาที ปริมาณของพิซีดที่สกัดได้ที่อุณหภูมิ 190 °C และเวลา 30 นาที คือ 130.88 ไมโครกรัมต่อกรัม ซึ่งถือว่ามีปริมาณมากกว่าที่ได้จากการสกัดด้วยตัวทำละลายอินทรีย์ และการสกัดโดยใช้ซอกเลตซึ่งได้เท่ากับ 68.37 และ 74.87, ตามลำดับ ถึงแม้ว่าปริมาณทรานส์พิซีดมีค่าสูงกว่า แต่สารทรานส์พิซีดที่ได้สามารถถูกเปลี่ยนให้เป็นทรานส์เรสเวอราทริล

สรุปงานวิจัย

จากผลการวิจัยพบว่าเทคโนโลยีการสกัดและการทำปฏิกิริยาในของไหลกึ่งวิกฤตและของไหลวิกฤตยวดยิ่งมีความเหมาะสมต่อการพัฒนาผลิตภัณฑ์มูลค่าเพิ่มจากพืชสมุนไพร ทั้งในส่วนของการสกัด และการทำให้สารสกัดมีอนุภาคขอนาดเล็ก โดยเฉพาะการคาร์บอนไดออกไซด์หรือน้ำที่สภาวะนี้ จะทำให้ได้ผลิตภัณฑ์ที่ปลอดภัยต่อผู้บริโภค อย่างไรก็ตาม ในการผลิตผลิตภัณฑ์มูลค่าเพิ่มจากพืชสมุนไพรหรือวัสดุธรรมลชาติ อาจยังจำเป็นต้องใช้กระบวนการนี้ร่วมกับกระบวนการแยกที่ใช้ตัวทำละลายอินทรีย์ เช่น กระบวนการโครมาโตกราฟีที่ใช้ตัวทำละลายอินทรีย์เป็นเฟสเคลื่อนที่

Output ที่ได้จากโครงการ ผลงานที่ได้ตีพิมพ์ลงวารสารวิชาการระดับนานาชาติ

1. Boonnoun P., Opaskornkun T, Prasitchoke P., **Shotpruk A.** Purification of Free Lutein from Marigold Flowers by Liquid Chromatography, Engineering Journal 16(5), 145-156.
2. Boonnoun P., Nerome H., Machmudah S., Goto M., **Shotipruk A.** Supercritical Anti-solvent Micronization of Marigold-derived Lutein Dissolved in Dichloromethane and Ethanol, Journal of Supercritical Fluids 77 (2013), 103-109 (IF2012=2.732).
3. Boonnoun P., Nerome H., Machmudah S., Goto M., **Shotipruk A.** Supercritical Anti-solvent Micronization of Chromatography Purified Lutein Using Hexane and Ethyl Acetate Solvent Journal of Supercritical Fluids 80 (2013), 15-22 (IF2012=2.732).
4. Prommuak C. Pavasant P., Quitain A., Goto M., **Shotipruk A.** Simultaneous Production of Biodiesel and Free Lutein from *Chlorella vulgaris*. Chemical Engineering&Technology 36 (2013), 733-739 (IF 2012=1.366).
5. Wataniyakul P., Pavasant P., Goto M., **Shotipruk A.** Microwave Pretreatment of Defatted Rice Bran for Enhanced Recovery of Total Phenolics Compounds Extracted by Subcritical Water, Bioresource Technology124, 18-22 (IF 2012-4.750)**.**
6. Chainukool S., Goto M., Hannongbua S., Shotipruk A. Subcritical Water Extraction of Resveratrol from Barks of Shorea Roxburghii G. Don. Separation Science and Technology, Accepted (IF 2012=1.164).

ภาคผนวก

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Article

Purification of Free Lutein from Marigold Flowers by Liquid Chromatography

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Abstract. Marigold is a rich source of a Xanthophyll called lutein, which shows anti-oxidant and anti-cancer activities and is beneficial to eye health. This has led to various extraction and purification studies to obtain the high purity free lutein suitable for human applications. Liquid chromatography is extensively used to purify high value natural compounds because the process results in high purity products. The suitable mobile phase and stationary phase are the key factors to achieve high purity. In this work, chromatographic separation of lutein extracted from marigold flowers was investigated, using silica gel and mixture of hexane:ethyl acetate as a stationary and a mobile phase, respectively. Initially, the suitable composition of hexane:ethyl acetate was determined using a thin layer chromatography. Hexane:ethyl acetate mixture at 70:30 volume ratio was found to be an appropriate mobile phase for a normal phase chromatographic separation of free lutein. Preliminary experiments on a semi-preparative and a preparative column carried out at the mobile phase flow rate of 10 ml/min suggested that as high as 97.1 % purity free lutien could be obtained with a 60 % approximate yield. Moreover, since the separation by chromatography arises from the adsorption of free lutein onto silica gel, thus to better understand the process, a batch adsorption study was carried out to obtain the equilibrium adsorption data. The isotherm plotted from these data was found to be reasonably described by Langmuir adsorption model.

Keywords: Preparative chromatographic purification, free lutein, marigold flower, adsorption isotherm.

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1. Introduction

Marigold flower is one of the richest sources of natural carotenoids. The major carotenoid in marigold is lutein, which has been reported to be beneficial in several aspects to human health such as supporting eyes and skin, and reducing the failure of the eyesight due to age-related macular degeneration (AMD), coronary heart disease and cancer [1]. Therefore, lutein has gained much interest due to its potential in nutraceutical and pharmaceutical applications.

In marigold flowers, lutein generally exists in the form of lutein fatty acid esters. Conventional method for marigold lutein fatty acid esters extraction is achieved by solvent extraction (generally using hexane). Alternatively, the environment friendly and non-toxic extraction solvent such as supercritical carbon dioxide (SC-CO_2) can also be used so as to provide milder extraction conditions [2]. Since only in its free form that lutein can be taken up by human body [3-4], marigold extract or marigold oleoresin must therefore be saponified with an alkali solution, i.e. KOH solution, to obtain free lutein [5]. Unfortunately, the saponified lutein mixture contains many impurities such as soap, oil, unreacted lutein fatty acid esters. Thus, a purification process is generally required to obtain purified lutein for human applications. Crystallization is a common process for purifying free lutein, however it results in rather low yield and purity. Although high purity could be achieved by re-crystallization, the process requires several steps, making it rather complicated, and thus lowering the overall yield [6].

Alternatively, liquid chromatography is extensively used to purify high value compounds from natural product. The development of an appropriate protocol (i.e., the optimization of mobile phase system) for chromatographic purification of a specific compound is generally performed via trial-and-error on an analytical High Performance Liquid Chromatography (HPLC). The isolation procedure on such analytical scales has been reported in existing literatures for free lutein derived from different raw materials, including fruits, vegetables and marigold flowers [7-8]. However, very few reports on chromatographic purification of lutein in larger semi-preparative and preparative scale were found.

In this work, normal phase chromatography with silica gel as a stationary phase was used for purifying free lutein from the saponified marigold oleoresin. Firstly, the suitable mobile phase system was investigated by using a thin layer chromatography (TLC), and with this suitable mobile phase, a preliminary study on an open column semi-preparative chromatography for lutein purification was conducted. Finally, a chromatographic separation was carried out on a preparative column to obtain the high purity free lutein. In addition, since the chromatographic separation arises from the partitioning of lutein between the mobile phase and the stationary phase by adsorption, the study on the adsorption equilibrium will provide better understanding of this process. Batch adsorption experiments were thus carried out to determine the adsorption isotherm of lutein on silica gel. The experimental results were fitted with the linearized forms of various adsorption models, and an appropriate model was proposed.

2. Materials and Methods

2.1. Materials and Chemicals

Dried marigold flowers were obtained from PTT Grobal Chemical Public Company limited (Rayong, Thailand). All the samples were finely powdered prior to use. Hexane (purity>99.5%) used for solvent extraction, recovery of total xanthophylls and mobile phase of semi-preparative, preparative liquid chromatography was supplied by Sigma-Aldrich. Chemicals used for saponification such as ethanol, potassium hydroxide and hydrochloric acid were purchased from Merck, USA. Diethyl ether was supplied by Merck, Thailand. Silica gel supplied by Merck, Thailand, was used as a chromatography column packing material. Ethyl acetate supplied by Merck, Thailand, was used as one of the components in the mobile phase of semi-preparative and preparative liquid chromatography. Lutein standards (analytical grade) were purchased from Sigma-Aldrich, Germany.

2.2. Solvent Extraction

The amount 100 grams of dried marigold powder was extracted with 500 ml of hexane in a 1 L beaker. The extraction was carried out for 4 h in a water bath whose temperature was controlled at 40°C. After extraction, the mixture was left to stand for 20 min at room temperature to allow the residue to settle. The supernatant was isolated and the caroteniods containing hexane solution was concentrated by a rotary

evaporator at 40 °C. The extract was then dried by a vacuum oven at 30 °C for 8 h. The remaining solid (marigold oleoresin) was collected and stored in a refrigerator at -20 °C for use in the next saponification step [6].

2.3. Saponification

The amount 0.6 of gram KOH was dissolved in 10 ml of ethanol in a 125 ml flask, into which one gram of marigold oleoresin was then added. The flask was shaken at 150 rpm and 50°C for 4 h. After the reaction was completed, 50 ml of ethanol was added into the saponified mixture, and this mixture was then transferred to a separation funnel, into which 100 ml of 5% Na_2SO_4 solution (in distilled water) and 80 ml of diethyl ether were added. All components were allowed to mix, and then separated into two phases. The upper phase (ether fraction) was collected as free lutein stock solution, while the lower phase was discarded water-soluble impurities still remained in the free lutein stock solution were extracted repeatedly with water until the water phase became colorless [9]. The resulting free lutein stock solution was then stored in a -20ºC refrigerator for use in a column chromatography.

2.4. Purification by Chromatography

2.4.1. Thin layer chromatography experiment

Silica gel coated thin layer chromatography plates (TLC silica gel 60, 25 Aluminium sheets 20 x 20 cm, Merck, USA) were used for screening for a proper mobile phase. Lutein stock solution was spotted onto TLC plates, each of which was then placed in a chamber containing the mobile phase of different composition. Mobile phases tested were mixtures at various compositions of hexane and ethyl acetate (at the ratios of 100:0, 90:10, 80:20, 70:30 and 60:40 hexane:ethyl acetate).

2.4.2. Column packing procedures

Silica gel was suspended in hexane to the slurry at a concentration of 5% (w/v). The suspension was then degassed overnight using a sonicator. The slurry was packed into a 8 mm × 240 mm semi-preparative open column or a 35 mm × 240 mm preparative chromatography column.

2.4.3. Semi-preparative open column chromatography

The amount 5 grams of silica gel slurry prepared as described above were packed into a glass column (8×240 mm) and 0.5 ml of lutein stock solution was then loaded into the semi-preparative glass column. Then mobile phase mixture of hexane: ethyl acetate (70:30 v/v) was allowed to flow by means of gravity. Fractions were collected on a one minute intervals for the HPLC analysis of free lutein content.

2.4.4. Preparative column chromatography

The amount 100 grams of silica gel slurry was packed into a glass column (35×240 mm). 10 ml of lutein stock solution was loaded to the column and eluted with the mixture of hexane: ethyl acetate (70:30 v/v). The sample was eluted from the bottom of the column by means of a peristaltic pump (Masterflex, model number 7523-60, Cole Parmer Thailand) at a flow rate of 10 ml/min. The fractions were collected at 10 minute intervals and were analyzed by HPLC.

2.5. Adsorption of Lutein on Silica Gel

A known concentration of free lutein stock solution was diluted to give a series of solutions of free lutein whose concentrations range from 2.0 to 12.0 $\mu g\ ml^{-1}$. Batch mode adsorption studies were carried out in a 125 ml covered conical flasks by first equilibrating an accurately weighed amount of silica gel (about 1 g) with 10 ml of sample solutions of known initial concentration of free lutein. The samples were agitated in an orbital shaker at 120 rpm and 30 °C for 30 min. After reach equilibrium, the contents of the flask were analyzed for the concentration of free lutein by a spectrophotometer. The concentration of the adsorbed free lutein was calculated by the difference of the known total amount of free lutein and the amount

measured in the solution after equilibrium. The amount of adsorbed solute (C_{ad}, μg free lutein/g dry silica) was calculated from Eq. (1).

$$C_{ad} = \frac{\left(V_0 C_0 - C_{qe}(V_0 + \Delta V)\right)}{W_d} \quad (1)$$

where the dilution volume, ΔV (ml) = $(W_w - W_d)/\rho$ in ml, W_w and W_d are the weights of wet and dry silica in g, respectively, V_0 is the volume of the solution (ml), C_0 and C_{eq} are the initial and equilibrium concentrations (μg/ml).

2.6. Analysis of Free Lutein by High Pressure Liquid Chromatography (HPLC)

The extracted, saponified and chromatography purified samples were analyzed by HPLC to identify luteins components in the samples. The reversed phase HPLC analysis was carried out using Agilant 1100, Lichrocart C-18 column (30 cm length), a Diode Array Detector Module 335 and an automatic injector. The mobile phase was a gradient solvent system composed of acetonitrile:methanol (9:1,v:v) (A) and ethyl acetate (B). The gradient system was run by linearly increasing solvent B from 0% to 100% over 30 min, at a flow rate of 1 ml/min. The sample injection volume was 20 μl and the detection wavelength was set at 450 nm [10].

2.7. Analysis of Saponified Sample by Liquid Chromatography Mass Spectrometery (LC-MS)

The saponified sample was analyzed by by HPLC to identify the luteins components. The reversed phase HPLC analysis was carried out using Agilant 1100, Lichrocart C-18 column (15 cm length), a Diode Array Detector Module 335 and an automatic injector. The mobile phase was a gradient solvent system of acetonitrile:methanol (9:1,v:v) (A) and ethyl acetate (B), from 0% to 100% of B using a linear gradient injected over 30 min, at a flow rate of 1 ml/min. The eluents were analyzed by Bruker Daltanic Model: Esquire 3000 to confirm mass of free lutein. MS was carried out in the positive ion measurement mode with a detection voltage of 1.6 kV, an APCI temperature of 400 °C, a curved desolvation line of 250 °C, and a block temperature of 200 °C. The flow rate of the nebulizer gas was 2.5 ml/min. Full scan spectra were obtained by scanning masses between m/z 200 and 800 [11].

2.8. Analysis of Purified Sample by Hydrogen Nuclear Magnetic Resonance Spectrometry (H-NMR)

Hydrogen Nuclear Magnetic Resonance Spectrometer (H-NMR) was acquired on a Varian INOVA model. All spectra were measured in $CDC1_3$ at 25°C with CP/MAS solid probe and Nano probe.

2.9. Analysis of Adsorption Isotherm

Adsorption data obtained from the experiment were fitted to the Langmuir and Freundlich models (shown in Table 1), to determine which of the models appropriately describes the results.

Table 1. Isotherm models.

Name	Equation	Linear form
Freundlich model	$C_{ad} = K_F C_{eq}^{1/n}$	$\log C_{ad} = \log K_F + \frac{1}{n}\log C_{eq}$
Langmuir model	$C_{ad} = \frac{K_L a_L C_{eq}}{1 + a_L C_{eq}}$	$\frac{1}{C_{ad}} = \frac{1}{K_L a_L C_{eq}} + \frac{1}{K_L}$

3. Results and Discussion

3.1. Liquid Chromatography Purification

HPLC and LC-MS analyses were used to determine the components of the saponified samples. The HPLC analysis results indicated that the solution consisted of two major compounds whose retention times were about 10 and 11 min, respectively (Fig. 1). The other impurities in saponified sample were detected at retention times of about 8 and 16 min. The identification of the compounds was then carried out by Liquid chromatography mass spectrometry (LC-MS), which indicated that the two compounds corresponding to the mass of 568 and 551 were, respectively, free lutein and the anhydrolutein, a lutein compound whose molecule was absent of an OH group (Fig. 2). This anhydrolutein might be a result of oxidization in the presence of oxygen by light or at moderate temperatures [12] during the extraction and saponification processes. LC-MS result of purified sample was compared to LC-MS result of lutein standard from Molnar's work [13]. From LC-MS results, purified sample contains free lutein, anhydrolutein and small amount of other impurities but lutein standard contains only free lutein and anhydrolutein.

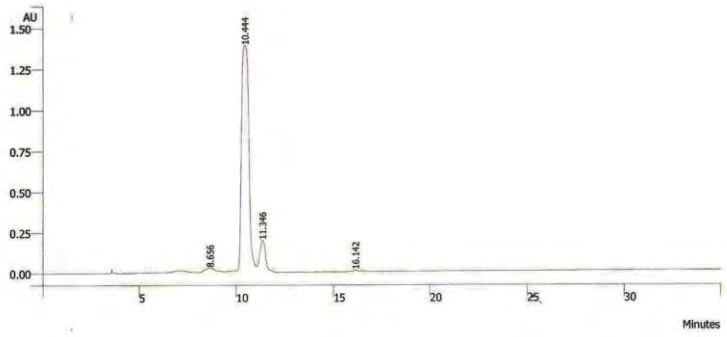

Fig. 1. HPLC chromatogram of saponified lutein sample.

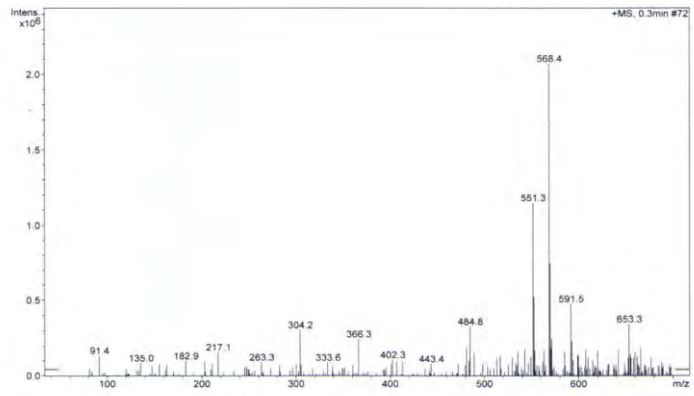

Fig. 2. LC-MS chromatogram of saponified lutein sample.

3.1.1. Screening of mobile phase by thin layer chromatography (TLC)

The hexane:ethyl acetate mixtures of various compositions were tested. The results shown in Fig. 3 indicated that the green spots of the samples were moved upwards from the base line when the mobile phase contains higher volume ratio of ethyl acetate. The most suitable ratio of the mobile phase was found to be 70:30 (hexane: ethyl acetate v/v), giving the clearest separation of the two major components.

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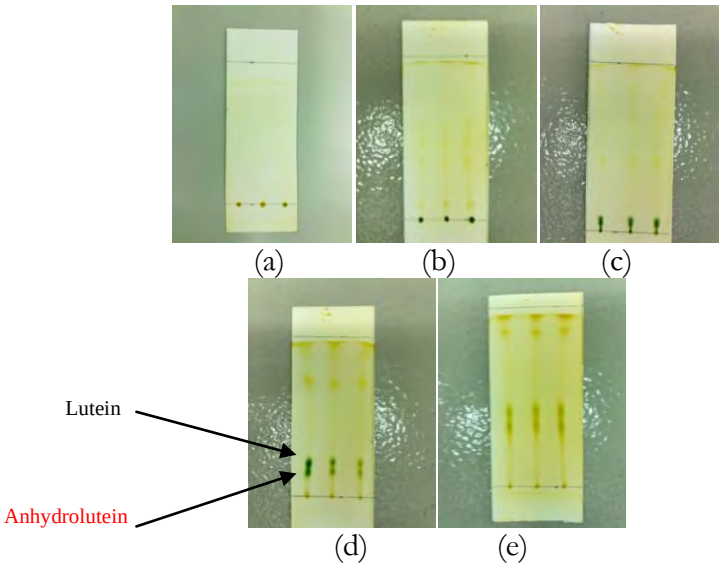


Fig. 3. Separations on TLC by hexane: ethyl acetate mobile phases at various compositions: (a) hexane: ethyl acetate = 100:0 v/v, (b) hexane: ethyl acetate = 90:10 v/v, (c) hexane: ethyl acetate = 80:20 v/v, (d) hexane: ethyl acetate = 70:30 v/v, (e) hexane: ethyl acetate = 60:40 v/v.

3.1.2. Chromatographic purification on semi-preparative and preparative columns

From the previous experiment, a mixture of hexane and ethyl acetate (70 : 30 v/v) shows the possibility to purify the free lutein from saponified solution. In this section, the 8 mm × 240 mm open column packed with silica and mixture of hexane and ethyl acetate (70 : 30 v/v) were used for preliminary study of free lutein purification. At this preliminary state, all the collected fractions were combined based on the color of the fractions that was observed visually on the semi-preparative columns as the samples were eluted, where two main color bands were observed: the yellow and the orange bands. The orange fractions eluted from the semi-preparative sample made up to relatively large amounts, which were expected to be mostly free lutein and its decomposed form mentioned above. Therefore the eluted fractions collected from this band were divided into 3 fractions and were analyzed by HPLC, the first and second combined fractions that were eluted from the semi-preparative column contain free lutein at rather high purity, whereas the last combined fractions of the same orange band contained the decomposed form of lutein, and thus has lower free lutein purity.

From the feasibility of semi-preparative column to purify free lutein, the larger scale of preparative chromatography of free lutein was studied for purifying free lutein from the saponified product. Originally, the scale up factor was calculated from the diameter of semi-preparative and preparative chromatography columns as 19.14 (Eq. (2)) which was then used to estimate the operational conditions such as mass of packing material, mobile phase flow rate and sample loading volume. For example, the scale up factor was multiplied by the previous conditions on semi-preparative open column (10 gram of packing material, 2.5 ml/min of flow rate and 0.5 ml sample loading). Consequently, the conditions estimated from this factor for the preparative chromatography column were 100 gram of packing material (silica gel), 70:30 v/v of hexane: ethyl acetate mixture mobile phase, the flow rate was at at 50 ml/min and sample loading was 10 ml. However, due to the limit in the flow rate of the peristaltic pump currently employed in the study, the lower flow rate of 10 ml/min was used.

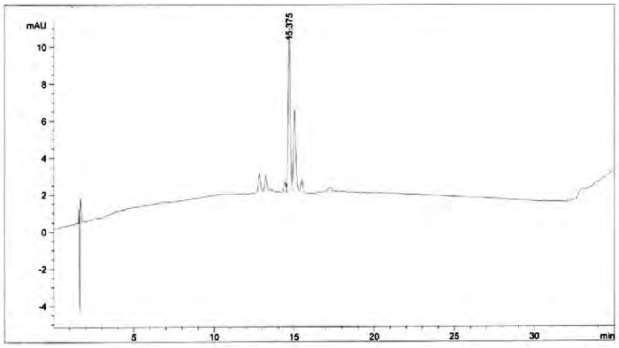
$$\text{Scale up factor} = \frac{\left(\frac{D_2}{2}\right)^2}{\left(\frac{D_1}{2}\right)^2} \tag{2}$$

Similar to semi-preparative purification, the two main color bands of yellow and the orange bands were observed. The purity, yield and amount of free lutein in each fractionated solutions is shown in Table 2. The first yellow bands were observed at fraction 2, 3 and 4 and the HPLC results show these fractions contain more impurities which were weakly adsorbed on silica gel, thus were eluted easily from the column (but eluted later from the HPLC reversed phase column) (Fig. 4(a)). The colorless fractions were observed at fraction 5, 6 and 7 and no compound could be detected by HPLC from these fractions. The orange

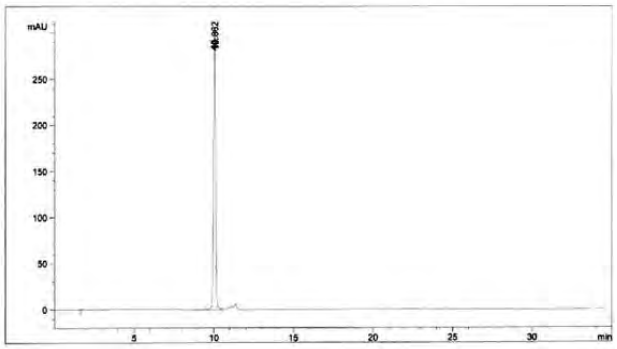
bands were observed in fraction 8 and 9 and the HPLC results show the higher purity of free lutein at 97.1% (based on the HPLC peak area ratios) (Fig. 4(b)). Moreover, yield of free lutein which calculated base on the amount of free lutein in stock solution before chromatographic purification was about 61%. The mixed solution of free lutein and anhydrolutein was observed at fraction 10 to 17 (Fig. 4(c)). The purity and yield of free lutein in these fractions were about 65% and 27% respectively. After the chromatographic separation, the column was then washed by 100% ethyl acetate. This washed solution was also analyzed and was found to contain the impurities which were most strongly adsorbed on silica and also a small amount of free lutein (Fig. 4(d)).

Table 2. Purity, yield and amount of free lutein in each fractionation solutions.

Fraction number	**Fraction color**	**Amount of free lutein detected by HPLC (mg)**	**% Yield of free lutein (%)**	**Purity of free lutein (%)**
1	Colorless	Not detected	0	0
2 - 4	Yellow	Not detected	0	0
5 - 7	Colorless	Not detected	0	0
8 - 9	Orange	6.43±0.38	61.78±3.63	97.1±1.85
10 - 17	Yellow	2.68±0.38	25.72±3.63	66.25±2.75
Washed solution	Light yellow	1.3±0.02	12.7±0.02	5±0.04

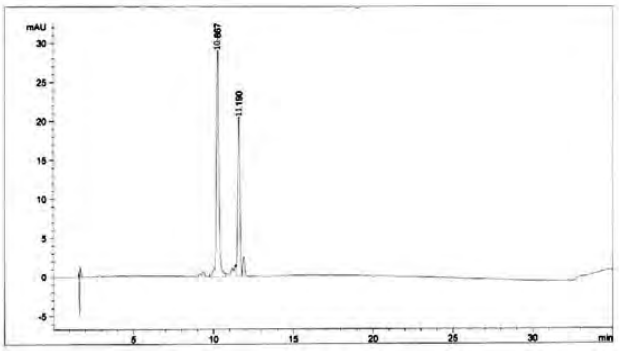


(a)



(b)

DOI:10.4186/ej.2012.16.5.145



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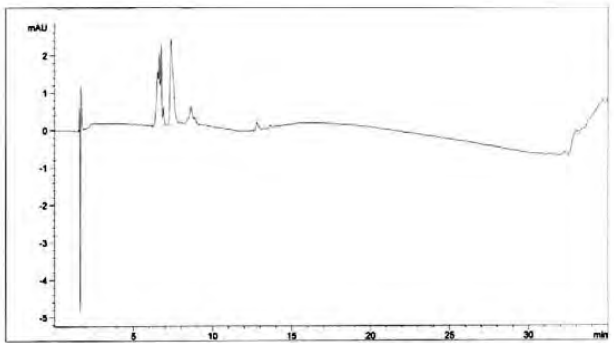

(d)

Fig. 4. HPLC chromatograms of lutein sample after liquid chromatography purification; (a) fraction 2-4, (b) fraction 8-9, (c) fraction 10-17, and (d) washed solution.

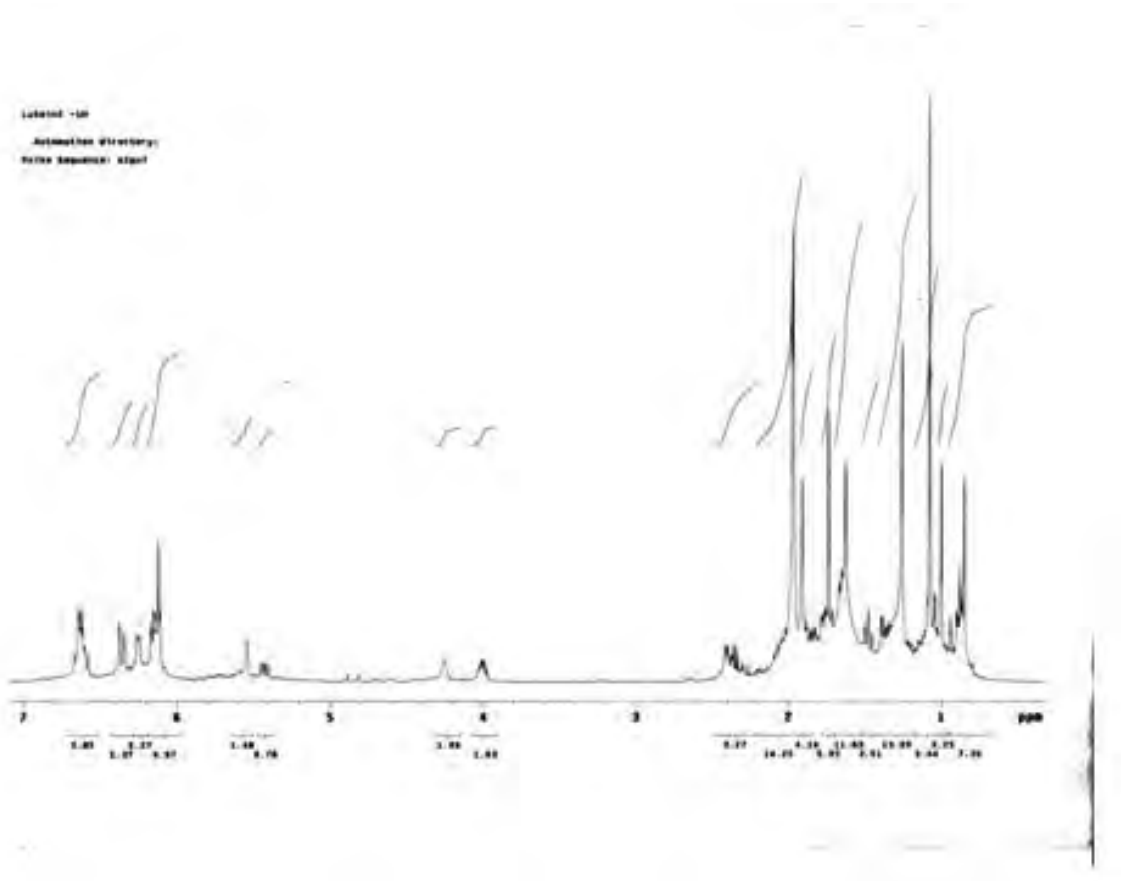

Fig. 5. H-NMR chromatograms of lutein sample after chromatographic purification.

To confirm structure of free lutein after chromatographic purification, the purified free lutein sample was analyzed by H-NMR. The H-NMR result in Fig. 5 showed the similar peak pattern to those of Aman's work [14] and Khachik's work [15]. From the result, it can be concluded that the purified free lutein sample contained only free lutein, free lutein's stereoisomers and anhydrolutein. The H-NMR result thus verifies the HPLC analysis and the calculation of free lutein purify based on the HPLC peak area reported in Table 2.

3.2. Adsorption of Lutein on Silica Gel

To determine the time required for lutein to reach equilibrium adsorption on silica gel, a preliminary batch adsorption study was conducted at initial free lutein concentration of 12.0 μg/ml and 1 g of silica at 30 °C. The result showed that adsorption has quickly reached equilibrium within about 5 min. However, to ample time for all samples to attain equilibrium, 30 min of agitation was allowed during the subsequent adsorption experiment.

3.2.1. Profile of equilibrium isotherm

Adsorption isotherm of free lutein on silica gel is shown in Fig. 5. Two isotherm equations: Freundlich and Langmuir equations have been tested in the present study to establish appropriate correlation for the equilibrium curves of the adsorption data. The data fitted to the linearized models are shown in Figs. 6-7.

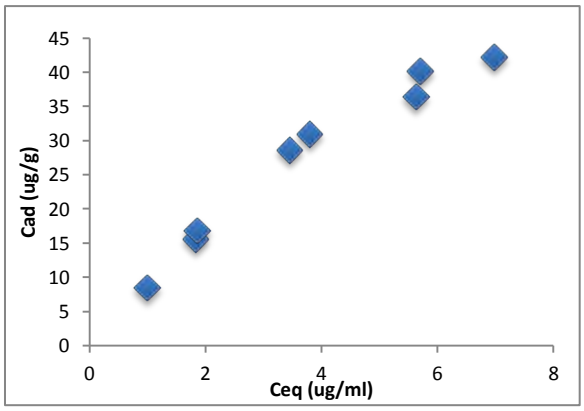

Fig. 6. Adsorption isotherm of free lutein on silica at 30°C.

Comparing the two models, it would appear that the Langmuir isotherm can better predict the equilibrium adsorption results for free lutein on silica gel as indicated by higher R^2 (R^2= 0.992).

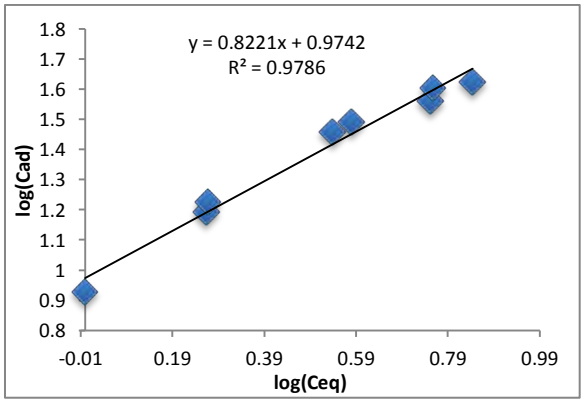

Fig. 7. Fit of Freundlich model to experimental data.

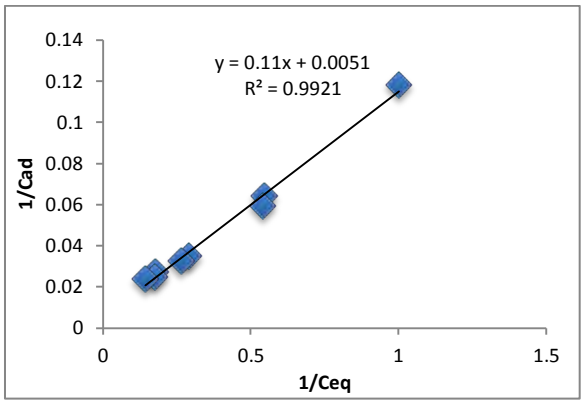

Fig. 8. Fit of Langmuir model to experimental data.

4. Conclusions

Screening for suitability of mobile phase composition of different ratios of hexane:ethyl acetate mixture using thin layer chromatography, Hexane: ethyl acetate mixture at 70:30 volume ratio was found to be an appropriate mobile phase on the normal phase chromatography system. Semi-preparative column chromatography by using 70:30 volume ratio of hexane:ethyl acetate as mobile phase showed the feasibility for purifying free lutein from saponified solution. The scale up factor was then used to estimate the operational conditions for preparative column chromatography such as mass of packing material, mobile phase flow rate and sample loading volume. At the most suitable condition, preparative chromatography could produce high purity free lutein (>95%). Langmuir adsorption model was found to reasonably describe the equilibrium adsorption data of free lutein on silica gel. Nevertheless, experiments at extended range of concentrations would be needed to more accurately determine the maximum adsorption capacity.

Acknowledgements

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References

[1] M. Wang, R. Tsao, S. Zhang, Z. Dong, R. Yang, J. Gong, and Y. Pei, "Antioxidant activity, mutagenicity/anti-mutagenicity, and clastogenicity/anti clastogenicity of lutein from marigold flowers," *Food and Chemical Toxicology* , vol. 44, pp. 1522–1529, 2006.

[2] W. Palumpitag, P. Prasitchoke, M. Goto, and A. Shotipruk, "Supercritical carbon dioxide extraction of marigold lutein fatty acid esters: Effects of cosolvents and saponification conditions," *Separation Science and Technology*, vol. 46, no. 4, , pp. 605-610, Jan. 2011.

[3] F. Khachik, G. Englert, R. Gary, J. Beecher, and C. Smith, "Isolation, structural elucidation, and partial synthesis of lutein dehydration products in extracts from human plasma," *Journal of Chromatography B*, vol. 670, pp. 219-233, 1995.

[4] F. Khachik and M. Beltsville, "Process for extraction, purification of lutein, zeaxanthin and rare carotenoids from marigold flowers and plants," United States Patent no. 6262284, 2001.

[5] B. Olmedilla, F. Granado, I. Blanco, and M. Vaquero, "Lutein, but notalpha-tocopherol, supplementation improves visual function in patients with age-related cataracts: A 2-year double blind, *placebo-controlled pilot study*," *Nutrition*, vol. 19, pp. 21–24, 2003.

[6] J. Vechpanich and A. Shotipruk, "Recovery of free lutein from tagetes erecta: Determination of suitable saponification and crystallization conditions," *Separation Science and Technology*, vol. 46, pp. 265-271, 2011.

[7] P. Alisa, R. Helen, and J. J. Elizabeth, "Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products," *Journal of Food Composition and Analysis*, vol. 22, pp. 9–15, 2009.

[8] G. Dias, G. F. C. Filomena, and O. Luisa, "Carotenoids in traditional Portuguese fruits and vegetables," *Food Chemistry*, vol. 113, pp. 808–815, 2009.
[9] S. Shibata, C. Ishihara, and C. Matsumoto, "Improved separation method for highly purified lutein from chlorella powder using jet mill and flash column chromatography on silica gel," *Journal of Agricultural and Food Chem*istry, vol. 52, pp. 6283-6286 , 2004.
[10] R. Piccaglia, M. Marotti, and S. Grandi, "Lutein and lutein ester content in different types of Tagetes patula and T. Erecta," *Industrial Crops and Products*, vol. 8, pp. 45–51, 1998.
[11] N. Kiyotaka, K. Takehiro, H. Keijiro, A. Akira, K. Fumiko, S. Phumon, T. Tsuyoshi, A. Hiroyuki, and M. Teruo, "Development of a high-performance liquid chromatography-based assay for carotenoids in human red blood cells: Application to clinical studies," *Analytical Biochemistry*, vol. 381, pp. 129–134, 2008.
[12] N. E. Craft and J. E. Soares, "Relative solubility, stability and absorptivity of lutein and β-carotene in organic solvents," *Journal of Agricultural and Food Chemistry*, vol. 40, pp. 431-434, 1992.
[13] P. Molnar, Z. Szabo, E. Osz, P. Olah, G. Toth, and J. Deli, "Separation and identification of lutein derivatives in processed foods," *Chromatographia*, vol. 60, pp. S101-S105, 2004.
[14] R. Aman, J. Biehl, R. Carle, J. Conrad, U. Beifuss, and A. Schieber, "Application of HPLC coupled with DAD, APcI-MS and NMR to the analysis of lutein and zeaxanthin stereoisomers in thermally processed vegetables," *Food Chemistry*, vol. 92, pp. 753–763, 2005.
[15] F. Khachik, G. Englert, G. R. Beecher, and J. C. Smith Jr., "Isolation, structural elucidation, and partial synthesis of lutein dehydration products in extracts from human plasma," *Journal of Chromatography B*, vol. 670, pp. 219-233, 1995.

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Supercritical anti-solvent micronization of marigold-derived lutein dissolved in dichloromethane and ethanol

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ABSTRACT

This work aims to study supercritical anti-solvent (SAS) micronization of lutein derived from marigold flowers. Lutein solution in dichloromethane (DCM) or ethanol was atomized into the stream of supercritical carbon dioxide (SC-CO_2) through a concentric nozzle in a pressurized vessel. The effects of pressure and SC-CO_2 flow rate on morphology, mean particle size (MPS) and particle size distribution (PSD) were investigated. The reduction in lutein MPS from 202.3 μm of unprocessed lutein to 1.58 μm and 902 nm could be achieved by SAS micronization using DCM and ethanol, respectively. In both solvent systems, no significant effects of pressure and SC-CO_2 flow rate on particle morphology were observed. However, pressure was found to have a significant effect on MPS and PSDs of lutein particles.

1. Introduction

Lutein ($C_{40}H_{56}O_2$, MW = 568.87) whose molecular structure is shown in Fig. 1, is an active pharmaceutical ingredient (API) that have received considerable interest as it possesses several beneficial properties such as preventing age-related macular degeneration and having high antioxidant activity [1,2]. The compound has been shown to have an amorphous structure, and have the melting point of 177 °C and the heat of fusion of 27,500 kJ/mol [3]. Natural sources of lutein include various fruits and vegetables, many of which are taken as human diet. However, marigold flower is well perceived as the most important natural source of lutein currently produced commercially. Lutein is highly soluble in organic solvents such as tetrahydrofuran (THF) and chloroform [4] but almost insoluble in water. However, same as other drugs and APIs, lutein must be dissolved in water in order to exert their effects [5].

One approach to improve water solubility of drugs and APIs is by micronization of these compounds which leads to increased particle surface area [6]. Lutein made into nano-sized particles has been shown to have 76% solubility in water [7]. However, some problems arise when employing conventional micronization processes including the degradation of thermal labile compounds by frictional heat, such as in milling and grinding, and contamination with toxic solvents used in chemical method such as re-crystallization [8].

Nowadays, techniques of particle micronization employing supercritical fluids are widely studied as they require mild operating temperatures, making the processes particularly suitable for heat-sensitive compounds. Of these techniques, supercritical anti-solvent (SAS) micronization is one of the simplest processes and is suitable for compounds that have rather low solubility in supercritical fluids. In a typical SAS process, a solution in an organic solvent is flown through a nozzle into a chamber simultaneously with a supercritical fluid, which acts as an anti-solvent. Mass transfer between the solution and the fluid thus occurs, leading to the supersaturation state, which then results in the formation of the solute particles. The particle morphology, size and size distribution are influenced by several factors such as experimental set up (nozzle type and nozzle inner diameter) and the nature of the organic solvent, as well as the operating conditions (pressure, temperature, and solution flow rate). Accurate prediction of the effects of the process variables to particle size and morphology is however difficult. This is due to the fact that SAS micronization involves several processes such as the solvent jet disintegration or solvent dispersion as it flows through the nozzle, mass transfer between the liquid jet and the supercritical anti-solvent, as well as particle nucleation and growth. Nevertheless, consideration of the mechanisms involved, together with the fluid phase equilibrium provides some basic guidelines of how particle size and morphology may be controlled [9–16].

In this study, the SAS technique using CO_2 as an anti-solvent was employed for micronization of marigold derived lutein particles, due to its near zero solubility in CO_2 at our operating conditions

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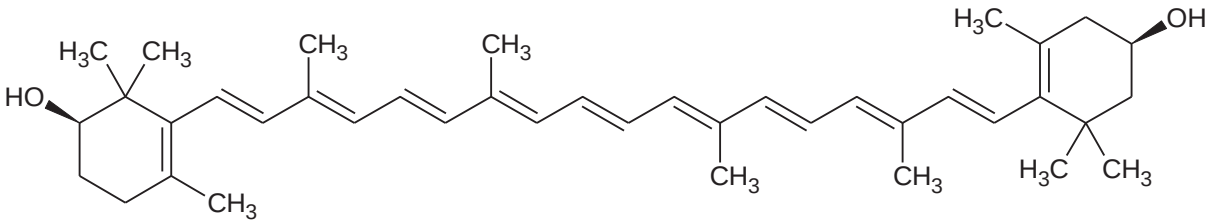

Fig. 1. Chemical structure of lutein.

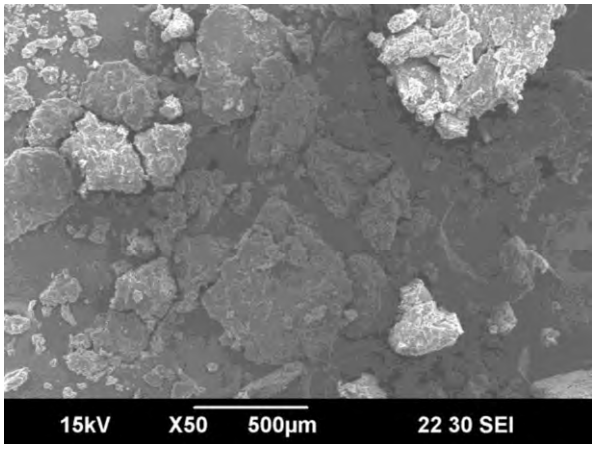

Fig. 2. SEM image of un-processed lutein sample.

[17]. Because their binary phase equilibrium data with CO_2 are available in literature [18], dichloromethane (DCM) and ethanol were chosen as solvents in which lutein was dissolved prior to the SAS experiments. The effects of micronization operating conditions including pressure and supercritical carbon dioxide (SC-CO_2) flow rate on the morphology, mean particle size (MPS) and particle size distribution (PSD) of the resulting particles were investigated.

2. Materials and methods

2.1. Materials and chemicals

Dried powdered marigold flower sample was provided by PTT Global Chemical Public Company Limited (Rayong, Thailand). Hexane (purity > 99.5%) was supplied by Sigma–Aldrich. Ethanol and potassium hydroxide (KOH) used for sample preparation step were purchased from Merck, USA. Diethyl ether, ethyl acetate and sodium sulfate (Na_2SO_4) were supplied by Merck Ltd., Thailand. Liquid CO_2 was supplied by Uchimura Sanso Co. Ltd. (Osaka, Japan) with a purity of 99.97%. DCM and ethanol used for SAS precipitation were supplied by Wako Pure Chemical Industries Inc. (Tokyo, Japan).

2.2. Preparation of lutein sample

Following the procedure described in Vechpanich and Shotipruk [19], 100 g of dried marigold powder was extracted with 500 ml of hexane for 4 h at 40 °C in a stirred vessel whose temperature was controlled by a water bath. The system was then left to stand for 20 min at room temperature to allow the residue to settle and separate from the extract. Hexane in the extract was evaporated under vacuum at 40 °C, and the concentrated extract was further dried in a vacuum oven at 30 °C for 8 h. The dried marigold extract which is hereby called marigold oleoresin was further subjected to saponification to convert lutein in the esterified forms to the free lutein. 0.6 g KOH was dissolved in 10 ml of ethanol in a 125 ml Erlenmeyer flask, into which one gram of marigold oleoresin was then added. The flask was shaken at 150 rpm at 50 °C for 4 h. After the reaction was completed, 50 ml of ethanol was added into the saponified solution, and this solution was then transferred to a separation funnel, into which 100 ml of 5% Na_2SO_4 solution (in distilled water) and 80 ml of diethyl ether were added. All components were mixed thoroughly and the mixture was then allowed to be separated into two phases. The upper phase (the ether fraction) was collected and was then extracted with water to remove water-soluble impurities. After repeated extractions with water until the water phase became colorless, the ether upper phases were collected and

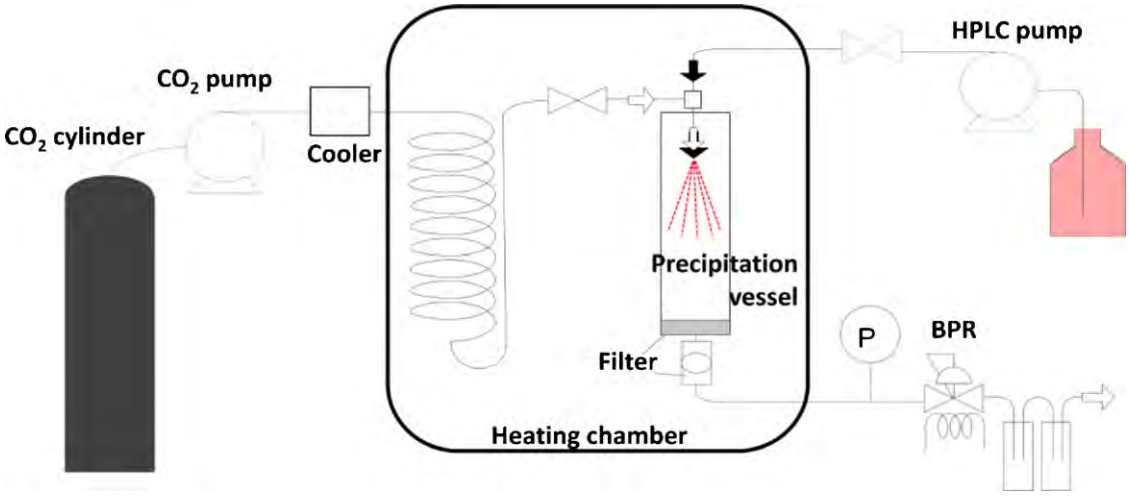

Fig. 3. Schematic of supercritical antisolvent apparatus.

Table 1
Operating conditions for SAS micronization of lutein using DCM as solvent.

Sample number	P (MPa)	T (°C)	Solution flow (ml/min)	CO_2 flow (ml/min)	Density of CO_2 (kg/m^3)	Re of CO_2	Re of DCM	CO_2 molar fraction[a]	Mean particle size
1	8	55	0.25	20	205	8261	489	0.935	–
2	10	55	0.25	20	303	9543	482	0.935	2.05 μm
3	12	55	0.25	20	504	12,149	475	0.935	2.49 μm
4	8	55	0.25	25	205	10,326	489	0.947	–
5	10	55	0.25	25	303	11,929	482	0.947	1.58 μm
6	12	55	0.25	25	504	15,186	475	0.947	1.94 μm

[a] CO_2 molar fraction is based on the binary DCM–CO_2 system.

Table 2
Operating conditions for SAS micronization of lutein using ethanol as solvent.

Sample number	P (MPa)	T (°C)	Solution flow (ml/min)	CO_2 flow (ml/min)	Density of CO_2 (kg/m^3)	Re of CO_2	Re of ethanol	CO_2 molar fraction[a]	Mean particle size
7	8	55	0.25	20	205	8261	40.4	0.93	–
8	10	55	0.25	20	303	9543	40.2	0.93	3.41 μm
9	12	55	0.25	20	504	12,149	40.0	0.93	1.58 μm
10	8	55	0.25	25	205	10,326	40.4	0.943	–
11	10	55	0.25	25	303	11,929	40.2	0.943	1.08 μm
12	12	55	0.25	25	504	15,186	40.0	0.943	902 nm

[a] CO_2 molar fraction is based on the binary ethanol–CO_2 system.

combined to obtain the lutein stock solution [20]. This solution was then dried overnight in a vacuum oven. The SEM image of the resulting dried sample is shown in Fig. 2 and the MPS was determined to be 202.3 μm. The dried sample was tightly wrapped and stored in a freezer for use in SAS micronization experiments.

2.3. SAS micronization of lutein

The apparatus for SAS micronization of lutein is shown in Fig. 3, which consists of an LC-8A preparative liquid chromatography pump (Shimadzu, Japan), a PU-980 intelligent HPLC pump (Jasco, Japan), a cooler (Eyela Cool Ace CA 1100, Japan), a precipitation vessel (SUS316 cell, inner diameter: 3 cm, length: 17.0 cm, volume: 120.1 cm^3, P-max: 30 MPa) and a double-tube nozzle (inner diameter: 1/16 and 1/8 in.) through which the CO_2 and lutein solution were delivered. The precipitation vessel was housed in a heating chamber whose temperature was controlled by air convection (AKICO, Japan; T-max: 100 °C). The experiments begin with pumping liquid CO_2 through a cooler at 0 °C, to ensure the liquefaction of the gas to prevent cavitation, before being delivered to a precipitation vessel at a specified flow rate through the annular cross-sectional surface of the concentric nozzle. The pressure

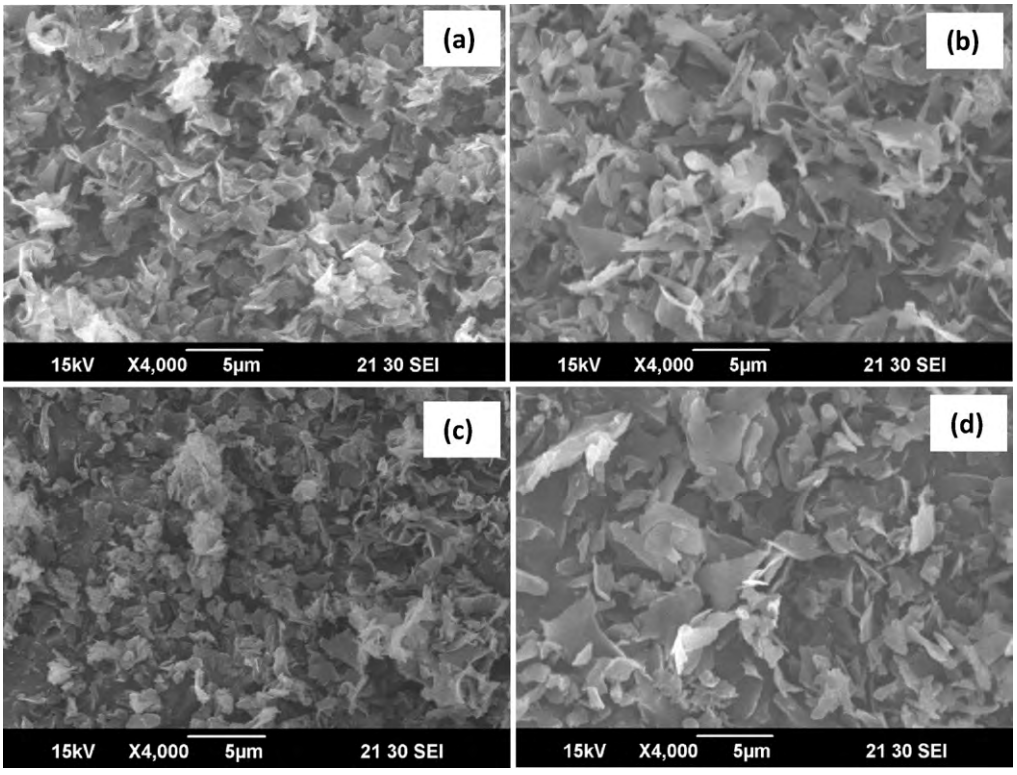

Fig. 4. SEM images of SAS micronized lutein samples using DCM as solvent at 55 °C, 0.25 ml/min solution flow rate, and at pressure (a) 10 MPa, (b) 12 MPa for 20 ml/min CO_2 flow rate, and (c) 10 MPa, (d) 12 MPa for 25 ml/min CO_2 flow rate.

of the precipitation vessel was set to a desired value by a back-pressure regulator (AKICO, Japan). The temperature of the heating chamber was then set to a fixed value of 55 °C. The initial lutein solution in an organic solvent was prepared by dissolving lutein in DCM or ethanol with continuous stirring for 30 min. Although lutein solubility in DCM and ethanol at ambient temperature has been reported to be 0.8 and 0.3 mg/ml, respectively [21], at the same temperature as the SAS operating temperature (55 °C), lutein solution of higher concentration of 1 mg/ml could be prepared. The lutein solution was then pumped to the precipitation vessel by the HPLC pump, through the inner tube of the concentric nozzle. Precipitated lutein particles were trapped by a filter (SUS316, Swagelok) fixed at the bottom of the vessel. After the micronization process was completed, the flow of the lutein solution was stopped but that of pure SC-CO_2 was continued for an additional hour to ensure that all the residual solvent was removed from the lutein particles. No residual solvent could be detected by the gas chromatography (GC) analysis of the solution of micronized sample in chloroform. This was also confirmed by constantly weighing the micronized lutein sample that was placed in a rotary vacuum evaporator at 40 °C at every 30 min interval for 3 h. The SAS micronization experiments were conducted 2–3 times at some selected conditions to test the reproducibility of the results. The conditions employed for the SAS experiments with DCM and ethanol as solvents, respectively, are shown in Tables 1 and 2, along with information on CO_2 density, Reynolds' numbers (*Re*) for solvent and CO_2 flows and the MPS of the resulted particles.

2.4. Evaluation of particle morphology, MPS and PSD

A JEOL model JSM-6390LV scanning electron microscope (SEM) was used to examine the morphology and size of the particles. Conductive double-sided tape was used to fix the particles to the specimen holder. A thin layer of gold was sputtered onto the sample using an ion sputtering device (JEOL model JFC-1100E). In determining the MPS and PSD, 500 particles were measured using Image J analytical software.

3. Results and discussion

3.1. SAS micronization of lutein dissolved in DCM

SAS micronization at various operating conditions of lutein dissolved in DCM resulted in particles of various MPS, PSDs, and morphologies. The effect of pressure (8, 10 and 12 MPa) on the morphology, MPS and PSD of lutein could be drawn from the sample numbers 1–3, whose operating conditions are listed in Table 1. At 8 MPa, no particles could be collected. This could be explained by considering the phase equilibrium data of all three components involved in the SAS process. Although the ternary data for lutein, DCM, and CO_2 are not available in literature, binary phase equilibrium data of CO_2 and DCM [18] could be used initially to address the feasibility of the process. The fact that the operating condition at 8 MPa and 55 °C is located below the critical point of the binary mixture caused the liquid and vapor phases to coexist. Lutein then remained dissolved in the liquid organic solvent, which was then carried over by the flowing stream of carbon dioxide, through the filter, out of the micronization chamber, without forming any particles. On the other hand, the pressures of 10 MPa and 12 MPa were above the mixture critical point in which the single supercritical region is reached. At these conditions, SC-CO_2 became dissolved into the organic phase, causing the state of supersaturation, thus resulting in the production of fine particles (Fig. 4(a) and (b)). In both cases, rather than being spherical, lutein particles have flake-like morphology, which can probably be explained by the competition of the diffusion rate of SC-CO_2 into organic solvent droplet as the solvent flowed out of the nozzle and the nucleation rate of the lutein particles. In this case, it was likely that the rate of SC-CO_2 diffusion was lower than that of particle nucleation, causing the nucleation to start as soon as the diffusion front forms, and not far from the droplet surface. Thus, the flake-like particles whose morphology is similar to the droplet surface were formed [22]. The results in Table 1 also indicated that the increase in pressure leads to the increase in lutein MPS. For the process operated at the SC-CO_2 flow rate of 20 ml/min, the increase in MPS from 2.05 to 2.49 μm was observed as the pressure increased from 10 MPa to 12 MPa, while for the SC-CO_2 flow rate of 25 ml/min, particle MPS increased from 1.58 to 1.94 μm with the same increased pressure. It should be noted that although the flake-like morphology might lead to overestimation of particle size when viewed under SEM, at all conditions, flake-like particles were obtained, thus general trend could still be drawn from the results of this present study. In addition to higher MPS, as shown in Fig. 5, wider PSDs were observed at higher pressures. These results could largely be due to the effect of pressure on the characteristics of jet disintegration [23–25]. Generally, depending on the flow rate and operating conditions such as temperature and pressure, three regimes of liquid phase dispersion are observed: (i) the dripping regime, in which the droplets are formed at the outlet of the nozzle; (ii) the laminar regime, in which the solvent flows smoothly and continuously before a break-up zone where uniform size droplets are formed; (iii) the turbulent regime, in which the jet

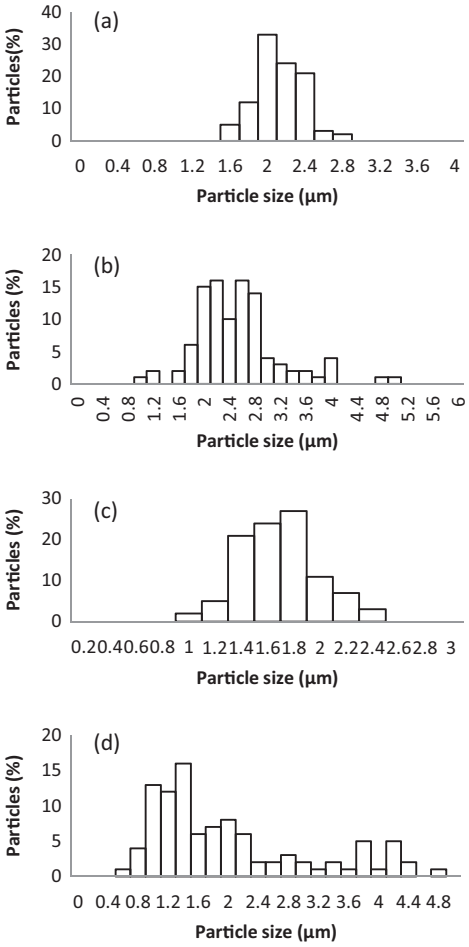

Fig. 5. PSD of SAS micronized lutein samples using DCM as solvent at 55 °C, 0.25 ml/min solution flow rate, and at pressure (a) 10 MPa, (b) 12 MPa for 20 ml/min CO_2 flow rate, and (c) 10 MPa, (d) 12 MPa for 25 ml/min CO_2 flow rate.

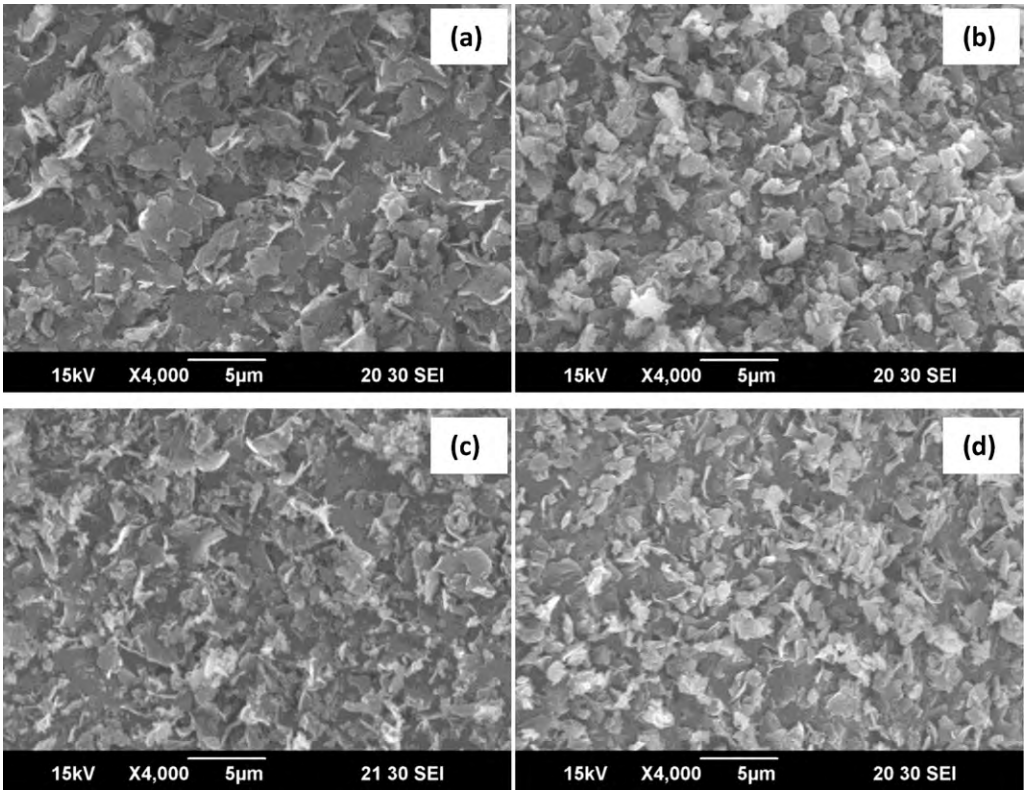

Fig. 6. SEM images of SAS micronized lutein samples using ethanol as solvent at 55 °C, 0.25 ml/min solution flow rate and pressure (a) 10 MPa, (b) 12 MPa for 20 ml/min CO_2 flow rate and (c) 10 MPa, (d) 12 MPa for 25 ml/min CO_2 flow rate.

surface becomes irregular and the resulting non-uniform droplets are formed as stretched or small broken droplets [16].

In general, the ratio of inertia force and viscous force of liquid flow, known as a Reynolds' number ($Re = \rho DU/\mu$; where ρ is density, D is nozzle diameter, U is liquid velocity and μ is liquid viscosity) can be use to estimate flow regime. The *Re* numbers of DCM at both pressures of 10–12 MPa were calculated to be about 400 (as shown in Table 1) which lie in the laminar flow regime. However *Re* alone could not describe such complicated system of SAS micronization. Indeed the jet hydrodynamics is determined by another important dimensionless number, namely a Ohnesorge number ($Oh = (\mu/(D\rho\sigma)^{1/2})$; where σ is interfacial tension), which relates the viscous force and the interfacial tension [26]. However, since the data for the transient interfacial tension of our system at supercritical conditions is not available, it was not possible to estimate the *Oh* numbers for the systems in this study. Despite the unavailibity of *Oh* numbers, previous literature has shown that the interfacial tension of DCM–CO_2 and ethanol–CO_2 systems decreased with increasing pressure, and this in turn caused the transition in the modes of jet dispersion [16]. At low pressure, and thus high system interfacial tension, the smooth and continuous solvent flow (laminar regime) is maintained, which results in the formation of symmetric jet and uniform drops of solvent. At higher pressure, solvent flow becomes irregular (turbulent) due to the low value of interfacial tension, thus non-uniform drops are formed. The increase in MPS and wide PSD as the pressure increased from 10 MPa to 12 MPa for this case suggested therefore that a transition from laminar to turbulent regime could have occurred.

The effects of SC-CO_2 flow rate on lutein particle morphology and MPS were observed for the SC-CO_2 flow rates of 20 and 25 ml/min at a fixed lutein solution flow rate of 0.25 ml/min. As shown in Fig. 4(a)–(d), increasing SC-CO_2 flow rate shows no significant effect on particle morphology, that is, similar flake-like particles were observed at both SC-CO_2 flow conditions. However, the MPS of lutein decreased as the SC-CO_2 flow rate increased as shown in Table 1 (sample numbers 2, 3, 5 and 6). As SC-CO_2 flow rate increased, supersaturation was reached more readily as CO_2 diffuse into the droplet, resulting in precipitation of fine particles.

3.2. SAS micronization of lutein dissolved in ethanol

Similar to SAS micronization with DCM as a solvent, no particles could be formed by SAS micronization of lutien dissolved in ethanol at 8 MPa and 55 °C. Based on the binary phase equilibrium data reported by Tsivintzelis et al. [18], this operating condition again was below the critical point of ethanol and CO_2 mixture and thus lie within the two phases region. Operated at higher pressures, SAS micronization at 10 MPa and 12 MPa resulted in flake-like lutein particles (Fig. 6(a) and (b)). On the contrary to the previous results with DCM as a solvent, when ethanol was used as a solvent, the increase in pressure leads to the decrease in lutein MPS. The decrease of MPS from 3.41 to 1.58 μm and from 1.08 μm to 902 nm was observed for the SC-CO_2 flow rates of 20 ml/min and 25 ml/min, respectively. Moreover, narrower PSDs were observed at higher pressures (as shown in Fig. 7). The *Re* numbers for ethanol flow at both 10 and 12 MPa were about 40 (as shown in Table 2), which was similar to the system with DCM, which lie in the laminar flow regime. The decrease in MPS at higher pressure of 12 MPa however suggested that the jet dispersion characteristics of this system might be different. As the interfacial tension of ethanol–CO_2 system was reported to be lower than that of DCM–CO_2 at ambient condition (about 22 mN/m versus 28 mN/m) [16], it might be drawn from this data that at higher pressure of 12 MPa, the ethanol–CO_2 interfacial tension would also be lower and would possibly approach zero. When this is the case, jet dispersion behavior did not follow any of the three flow regimes described earlier, instead no

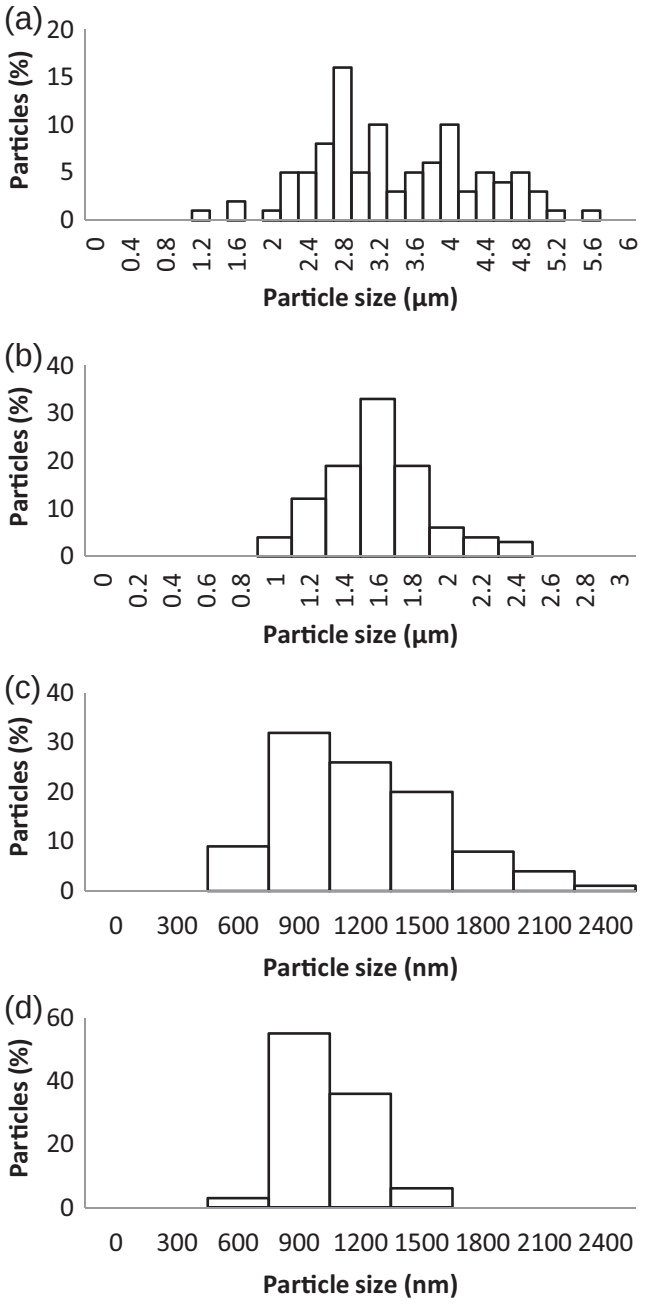

Fig. 7. PSD of SAS micronized lutein samples using ethanol as solvent at 55 °C, 0.25 ml/min solution flow rate, and (a) 10 MPa, (b) 12 MPa for 20 ml/min CO_2 flow rate, and (c) 10 MPa, (d) 12 MPa for 25 ml/min CO_2 flow rate.

distinct droplets are formed and the particle formation on the other hand occurred in favor of gas-phase nucleation and growth within the dispersed gas plume, resulting in very fine nano-sized particles [26]. The decrease in lutein MPS and narrower range of PSDs observed at higher pressures in these experiments was possibly the results of this shift from droplet formation to gas mixing.

The effect of SC-CO_2 flow rate on morphology and MPS of lutein was carried out at 20 and 25 ml/min. Similar to the previous results with DCM, an increase in SC-CO_2 flow rate showed no significant effect on particle morphology: flake-like lutein particles were observed as shown in Fig. 6(a)–(d). The decrease in MPS of lutein particles could be obtained when the SC-CO_2 flow rate is increased as shown in Table 2 (sample numbers 8, 9, 11 and 12).

4. Conclusions

Supercritical anti-solvent (SAS) micronization using DCM and ethanol was shown to effectively produce fine particles of lutein derived from marigold flowers. In both solvent systems, the increase in pressure and SC-CO_2 flow rate did not have significant effects on lutein particle morphology. However, significant effects of increasing pressure on MPS and PSDs of lutein particles were observed. The increase in SC-CO_2 flow rate from 20 to 25 ml/min shows the reduction of MPS in both DCM and ethanol system. It is noted some loss of product was observed in this study and this was attributed to the relatively large filter pore diameter (1 μm). Thus, filter with small pore size should be used in future study. In addition, in order to further gain better insights into the entire process, the investigations on interfacial tension and other hydrodynamic parameters such as jet breakup lengths and diffusion lengths should be carried out.

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References

[1] G.W. Burton, Antioxidant action of carotenoid, J. Nutrition 119 (1989) 109.
[2] F. Granado, B. Olmedilla, I. Blanco, Review article: nutritional and clinical relevance of lutein in human health, J. Nutrition 90 (2003) 487.
[3] F. Miguel, A. Martın, F. Mattea, M.J. Cocero, Precipitation of lutein and coprecipitation of lutein and poly-lactic acid with the supercritical anti-solvent process, Chemical Engineering and Processing 47 (2008) 1594–1602.
[4] M. Reza, L. Schafer, C. Lambert, D.E. Breithaupt, H.K. Biesalski, J. Frank, Solubility, uptake and biocompatibility of lutein and zeaxanthin delivered to cultured human retinal pigment epithelial cells in tween40 micelles, European J. Nutrition 46 (2007) 79–86.
[5] C.I. Park, M.S. Shin, H. Kim, Micronization of arbutine using supercritical antisolvent, Korean J. Chemical Engineering 25 (3) (2008) 581–584.
[6] M. Perrut, J. Jung, F. Leboeuf, Enhancement of dissolution rate of poorly soluble active ingredients by supercritical fluid processes. Part II: preparation of composite particles, International J. Pharmaceutics 288 (2005) 11–16.
[7] K. Mitri, R. Shegokar, S. Gohla, C. Anselmi, R.H. Muller, Lutein nanocrystals as antioxidant formulation for oral and dermal delivery, International J. Pharmaceutics 420 (2011) 141–146.
[8] K.T. Kwon, M.S. Uddin, G.W. Jung, B.S. Chun, Preparation of micro particles of functional pigments by gas-saturated solution process using supercritical carbon dioxide and polyethylene glycol, Korean J. Chemical Engineering 28 (10) (2011) 2044–2049.
[9] S.D. Yeo, M.S. Kim, J.C. Lee, Recrystallization of sulfathiazole and chlorpropamide using the supercritical fluid antisolvent process, J. Supercritical Fluids 25 (2003) 143–154.
[10] Y. Wang, Y. Wang, J. Yang, R. Pfeffer, R. Dave, B. Michniak, The application of a supercritical antisolvent process for sustained drug delivery, Powder Technology 164 (2006) 94–102.
[11] M.S. Kim, S. Lee, J.S. Park, J.S. Woo, S.-J. Hwang, Micronization of cilostazol using supercritical antisolvent (SAS) process: effect of process parameters, Powder Technology 177 (2007) 64–70.
[12] M. Bahrami, S. Ranjbarian, Production of micro- and nano-composite particles by supercritical carbon dioxide, J. Supercritical Fluids 40 (2007) 263–283.
[13] G. Caputo, E. Reverchon, Use of urea as habit modifier in the supercritical antisolvent micronization of sulfathiazole, Industrial & Engineering Chemistry Research 46 (12) (2007) 4265–4272.
[14] E. Reverchon, R. Adami, G. Caputo, I. De Marco, Spherical microparticles production by supercritical antisolvent precipitation: Interpretation of results, J. Supercritical Fluids 47 (2008) 70–84.
[15] K. Byrappa, S. Ohara, T. Adschiri, Nanoparticles synthesis using supercritical fluid technology – towards biomedical applications, Advanced Drug Delivery Reviews 60 (2008) 299–327.
[16] E. Badens, O. Boutin, G. Charbit, Laminar jet dispersion and jet atomization in pressurized carbon dioxide, J. Supercritical Fluids 36 (2005) 81–90.
[17] M.S. Gomez-Prieto, M.L. Ruiz del Castillo, G. Flores, G. Santa-Marıa, G.P. Blanch, Application of Chrastil's model to the extraction in SC-CO_2 of β-carotene and lutein in *Mentha spicata* L., J. Supercritical Fluids 43 (2007) 32–36.

[18] I. Tsivintzelis, D. Missopolinou, K. Kalogiannis, C. Panayiotou, Phase compositions and saturated densities for the binary systems of carbon dioxide with ethanol and dichloromethane, Fluid Phase Equilibria 224 (2004) 89–96.
[19] J. Vechpanich, A. Shotipruk, Recovery of free lutein from *Tagetes erecta*: determination of suitable saponification and crystallization conditions, Separation Science and Technology 46 (2011) 265–271.
[20] S. Shibata, C. Ishihara, C. Matsumoto, Improved separation method for highly purified lutein from chlorella powder using jet mill and flash column chromatography on silica gel, J. Agricultural and Food Chemistry 52 (2004) 6283–6286.
[21] E. Craft, Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents, J. Agricultural and Food Chemistry 40 (1992) 431–434.
[22] S.S. Dukhin, Y. Shen, R. Dave, R. Pfeffer, Droplet mass transfer, intradroplet nucleation and submicron particle production in two-phase flow of solvent-supercritical antisolvent emulsion, Colloids and Surfaces 261 (2005) 163–176.
[23] A. Braeuer, R. Adami, S. Dowy, M. Rossmann, A. Leipertz, Observation of liquid solution volume expansion during particle precipitation in the supercritical CO_2 antisolvent process, J. Supercritical Fluids 56 (2011) 121–124.
[24] A. Gokhale, B. Khusid, R.N. Dave, R. Pfeffer, Effect of solvent strength and operating pressure on the formation of submicrometer polymer particles in supercritical microjets, J. Supercritical Fluids 43 (2007) 341–356.
[25] B.Y. Shekunov, J. Baldyga, Particle formation by mixing with supercritical antisolvent at high Reynolds numbers, P. York, Chemical Engineering Science 56 (2001) 2421–2433.
[26] E. Reverchon, I. De Marco, Mechanisms controlling supercritical antisolvent precipitate morphology, Chemical Engineering Journal 169 (2011) 358–370.

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Supercritical anti-solvent micronization of chromatography purified marigold lutein using hexane and ethyl acetate solvent mixture

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ABSTRACT

This work aims to study supercritical anti-solvent micronization of marigold derived purified lutein that was dissolved in the mixture of hexane and ethyl acetate (70:30 v/v), the solvent used as the mobile phase for chromatographic purification. The results show significant effect of pressure on the morphology of micronized lutein particles. The increase in lutein initial concentration from 1.5 mg/ml to 3.2 mg/ml and the increase in SC-CO_2 flow rate from 15 ml/min to 25 ml/min show no significant effects on the morphology of lutein particles. However, the reduction of mean particle size from about 2 μm to 0.8 μm was observed by increasing SC-CO_2 flow rate. The X-ray diffraction patterns of the micronized lutein particles show apparent amorphous nature, while the Fourier transform infrared spectroscopy results show that no chemical structural changes occurred. Moreover, the solubility of the micronized lutein particles in aqueous solution was found to increase significantly from being almost insoluble to having approximately 20% solubility

1. Introduction

Lutein ($C_{40}H_{56}O_2$, MW = 568.87) whose molecular structure is shown in Fig. 1 is one of the most important active pharmaceutical ingredients (APIs), which is now widely used as natural colorants. In addition, due to its high antioxidant activity, the compound exhibits other positive effects such as reducing the failure of the eyesight due to age-related macular degeneration, as well as fighting against coronary heart disease and cancers [1,2]. Although lutein can be found in various fruits and vegetables, one of the richest natural sources of lutein is known to be marigold flowers [3]. Therefore, a number of studies have been conducted on extraction and purification of lutein from this source [4–6]. Recently, we successfully employed preparative chromatography technique to purify marigold derived lutein to the purity up to 97%. In this process, silica gel was used as a stationary phase and a mixture of hexane:ethyl acetate (70:30 v/v) was used as a mobile phase [7].

Despite having high medicinal activities, many APIs and lutein alike, are of low bioavailability due largely to their low solubility in water [8]. An improvement in aqueous solubility of an API can generally be achieved by micronizing it to smaller particles. As a result, the surface area increases, and thus the dissolution rate increases [9,10]. This not only helps lower the dosage requirement, it can also reduce any possible side effects associated with up-taking the compound [11]. However, there are some concerns over the conventional particle micronization such as grinding, milling, chemical precipitation and spray drying. APIs undergoing mechanical processes could be degraded by friction heat; and those undergoing chemical processes may contain toxic organic solvent residues.

Alternatively, supercritical fluids, especially carbon dioxide (CO_2), have recently played an important role in the processing of natural compounds, polymers, drugs and APIs. Micronization of particles with supercritical carbon dioxide (SC-CO_2) requires mild operating temperature; and is therefore suitable for thermally labile compounds. In addition, SC-CO_2 can easily be separated from the particles as it returns to gas phase at ambient temperature and pressure, leaving a solvent-free product [12]. Among several particle micronization techniques using supercritical fluids, supercritical anti-solvent (SAS) micronization has been widely studied due to the effectiveness of the method in making fine particles from various sources of compounds [13–17]. In a typical SAS process with SC-CO_2 used as an anti-solvent, the interested solute is first dissolved in an organic solvent, which is then flown simultaneously with SC-CO_2 through a nozzle. The solution becomes supersaturated, and the solute is thus forced to precipitate into fine particles. Previous research has demonstrated that beside the process conditions such as temperature, pressure, as well as solution and SC-CO_2 flow rates, the type of organic solvents in which the

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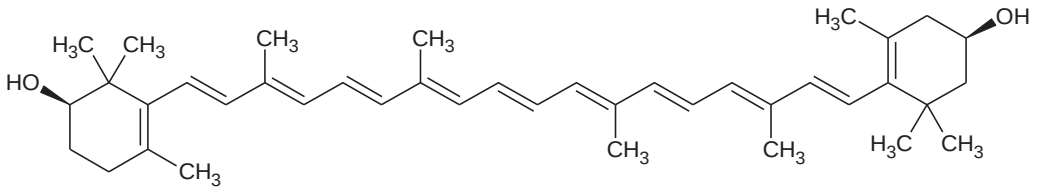

Fig. 1. Chemical structure of lutein ($C_{40}H_{56}O_2$, M.W. = 568.87).

solute is initially dissolved also plays a key role in the formation of the particles by the SAS process [18–20]. Common organic solvents used include ethanol and dichloromethane and their equilibrium data with CO_2 have been determined [21]. We have indeed demonstrated that SAS micronization of lutein was possible using ethanol and dichloromethane at some specified process conditions [22].

In this work, the feasibility of employing SAS technique was determined for micronization of marigold derived purified lutein that was dissolved in the mixture of hexane and ethyl acetate (70:30 v/v), the solvent used as the mobile phase for the prior chromatographic purification step [7]. This solvent system was hardly used in previous SAS micronization study, and to our knowledge, the phase equilibrium data with CO_2 are not available. Here, the effects of SAS micronization conditions including pressure, the initial concentration of lutein in the solvent and the supercritical carbon dioxide (SC-CO_2) flow rate were determined on particle morphology, mean particle size (MPS) and particle size distribution (PSD). In addition, the crystallinity of the micronized particles was examined from the X-ray diffraction (XRD) patterns and any chemical structural changes of the micronized lutein particles compared to un-processed lutein were examined by the analysis of Fourier transform infrared (FTIR) spectrum. Furthermore, the solubility in aqueous solution of micronized lutein and un-processed lutein were also investigated. The success of SAS micronization using the same solvent system as the mobile phase of the chromatography process will imply that a step of solvent evaporation from the eluted samples and re-dissolving the dried sample into another organic solvent can be omitted. By this, not only the process cost can be reduced, the degradation of lutein during complicated processing steps can also be minimized.

2. Materials and methods

2.1. Materials and chemicals

Dried powdered marigold flower sample was provided by PTT Global Chemical Public Company Limited (Rayong, Thailand). Hexane (purity > 99.5%) used for sample preparation step was supplied by Sigma–Aldrich. Ethanol (95% purity) and potassium hydroxide (KOH, purity > 99%) were purchased from Merck, USA. Diethyl ether (purity > 99%), ethyl acetate (purity > 99%) and sodium sulfate (Na_2SO_4, purity > 99%) were supplied by Merck Ltd., Thailand. Liquid CO_2 was supplied by Uchimura Sanso Co. Ltd. (Osaka, Japan) with a purity of 99.97%. Hexane (purity > 99.5%) and ethyl acetate (purity > 99%) used for SAS precipitation were supplied by Wako Pure Chemical Industries Inc. (Tokyo, Japan). Lutein standards (purity > 90%) were purchased from Sigma–Aldrich, Germany.

2.2. Sample preparation

Following the procedure described in Vechpanich and Shotipruk [6], 100 g of dried marigold powder was extracted with 500 ml of hexane for 4 h at 40 °C in a stirred vessel whose temperature was controlled by a water bath. The system was left to stand for 20 min at room temperature to allow the residue to settle and separate from the extract. Hexane in the extract was evaporated under vacuum at 40 °C, and the concentrated extract was further dried in a vacuum oven at 30 °C for 8 h. The dried marigold extract which is hereby called marigold oleoresin was further subjected to saponification to convert lutein in the esterified forms to the free lutein. 0.6 g KOH was dissolved in 10 ml of ethanol in a 125 ml Erlenmeyer flask, into which one gram of marigold oleoresin was then added. The flask was shaken at 150 rpm at 50 °C for 4 h. After the reaction was completed, 50 ml of ethanol was added into the saponified solution, and this solution was then transferred to a separation funnel, into which 100 ml of 5% Na_2SO_4 solution (in distilled water) and 80 ml of diethyl ether were added. All components were mixed thoroughly and the mixture was then allowed to be separated into two phases. The upper phase (the ether fraction) was collected and was then extracted with water to remove water-soluble impurities. After repeated extractions with water until the water phase became colorless, the ether upper phases were collected and combined to obtain the lutein stock solution [23]. This solution was then dried overnight in a vacuum oven. The dried sample was tightly wrapped and stored in a freezer for use in SAS micronization experiments. This sample if further purified by chromatography according to Boonnoun et al. [7], lutein of up to 97% purity would be obtained. In this micronization study however, we employed the extracted and saponified lutein prepared as described earlier. The analysis of the sample by HPLC following the method given in Boonnoun et al. [7] indicated that the purity of the sample used in this study was about 89%.

2.3. SAS micronization of lutein

The apparatus for SAS micronization of lutein is shown in Fig. 2, which consists of an LC-8A preparative liquid chromatography pump (Shimadzu, Japan), a PU-980 intelligent HPLC pump (Jasco, Japan), a cooler (Eyela Cool Ace CA 1100, Japan), a precipitation vessel (SUS316 cell, inner diameter: 3 cm, length: 17.0 cm, volume: 120.1 cm^3, *P*-max: 30 MPa) and a double-tube nozzle (inner diameter: 1/16 and 1/8 in.) through which the CO_2 and lutein solution were delivered. The precipitation vessel was housed in a heating chamber whose temperature was controlled by air convection (AKICO, Japan *T*-max: 100 °C). The experiments begin with pumping liquid CO_2 through a cooler at 0 °C, to ensure the liquefaction of the gas to prevent cavitation, before being delivered to a precipitation vessel at a specified flow rate through the annular cross-sectional surface of the concentric nozzle. The pressure of the precipitation vessel was set to a desired value by a back-pressure regulator (AKICO, Japan). The temperature of the heating chamber was then set to a fixed value of 50 °C, after which the lutein solution in mixture of hexane and ethyl acetate (70:30 v/v) was then pumped to the precipitation vessel by the HPLC pump, through the inner tube of the concentric nozzle. Precipitated lutein particles were trapped by a filter (SUS316, Swagelok) whose nominal pore diameter was 1 μm fixed at the bottom of the vessel. After the micronization process was completed, the flow of the lutein solution was stopped but that of SC-CO_2 was continued for an additional hour to ensure that all the residual solvent was removed from the lutein particles. At some selected conditions, SAS micronization of lutein was conducted 2–3 times to test the repeatability of the

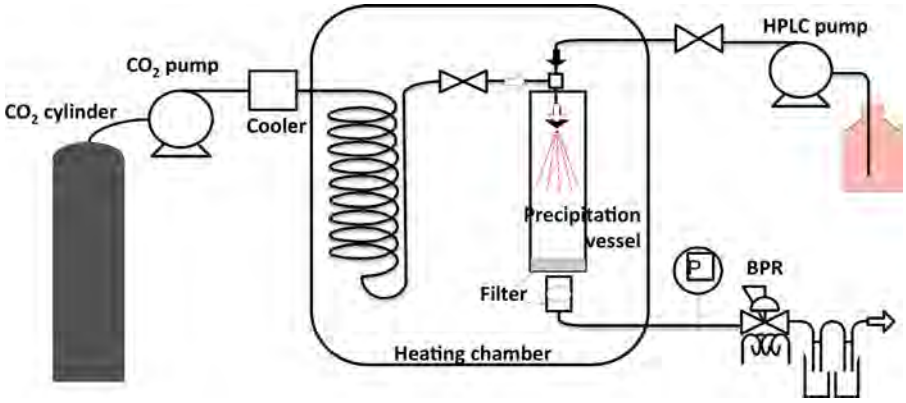

Fig. 2. Schematic diagram of SAS micronization apparatus.

Table 1
Operating conditions for SAS micronization of free lutein and resulting mean particle size (MPS) and yield.

Sample number	P (MPa)	T (°C)	C_0 (mg/ml)	Lutein solution[a] flow rate (ml/min)	CO_2 flow rate (ml/min)	CO_2 molar fraction[b]	Mean particle size (μm)	Yield (%)
1	6.5	50	2.5	0.5	20	0.931	–	0.0
2	8	50	2.5	0.5	20	0.931	2.1	58
3	12	50	2.5	0.5	20	0.931	Agg	30
4	10	50	1.5	0.5	20	0.931	Agg	21
5	10	50	2.5	0.5	20	0.931	Agg	23
6	10	50	3.2	0.5	20	0.931	Agg	27
7	8	50	2.5	0.25	15	0.953	2.2	67
8	8	50	2.5	0.25	20	0.964	2.0	31
9	8	50	2.5	0.25	25	0.971	0.8	27

Agg = agglomerated particles.
[a] Lutein dissolved in the mixture of hexane and ethyl acetate (70:30 v/v).
[b] CO_2 molar fraction is based on the tertiary hexane–ethyl acetate–CO_2 system.

results. The purity of micronized samples was determined to be the same as before SAS micronization (89% purity). The conditions employed for the SAS experiments are shown in Table 1, along with the MPS and yields of the resulted particles. The yields of lutein from SAS micronization was calculated based on Eq. (1).

$$\text{Yield of lutein} = \frac{\text{weight of free lutein final product (collected at filter)}}{\text{dry weight of free lutein before SAS precipitation}} \times 100\% \quad (1)$$

2.4. Evaluation of particle morphology, MPS, and PSD of micronized lutein samples

A JEOL model JSM-6390LV scanning electron microscope (SEM) was used to examine the morphology particles. Conductive double-sided tape was used to fix the particles to the specimen holder. A thin layer of gold was sputtered onto the sample using an ion sputtering device (JEOL model JFC-1100E). The sizes of lutein particles were analyzed. To determine the mean particle size (MPS) and particle size distribution (PSD), lutein samples were suspended into DI water and 1000 particles were measured three times by HORIBA Laser Scattering Particle Size Distribution Analyzer model LA-950.

2.5. Particle characterization

2.5.1. X-ray powder diffraction (XRD)

X-ray scattering measurements were performed on a Rigaku RAD-1B Discover diffractometer. The dried sample powders were prepared in a 0.5 mm thick specimen holder. Background (air scattering) was measured for the same sampling time, typically 30 min, and was subtracted from each measurement.

2.5.2. Fourier transform infrared spectrometer (FT-IR)

Micronized lutein FTIR spectra was recorded between 4000 and 400 cm^{-1} in transmission/absorbance mode on FTIR Spectrum One spectrometer (Perkin-Elmer, Norwalk, USA).

2.5.3. Dissolution test

The dissolution test was performed for processed and unprocessed lutein by SAS micronization using a USP II rotating paddle apparatus Pharmatest PTW SIII (Pharma Test, Germany) at 37 °C at rotating speed of 100 rpm in 500 ml of a buffer prepared by mixing 50 ml of 0.2 M KCl with 85 ml of 0.2 M HCl at pH 1.2. 15 mg of lutein samples were then placed in the basket which was connected

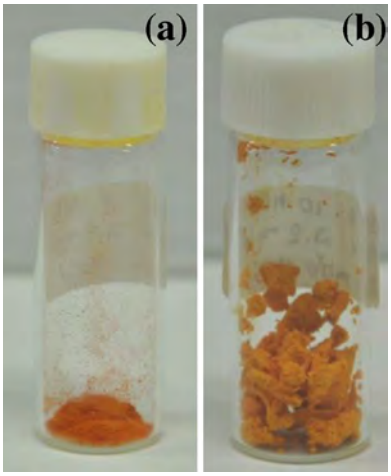

Fig. 3. SAS micronized lutein samples: (a) fine powder obtained at 8 MPa, (b) agglomerated particles obtained at 10 and 12 MPa.

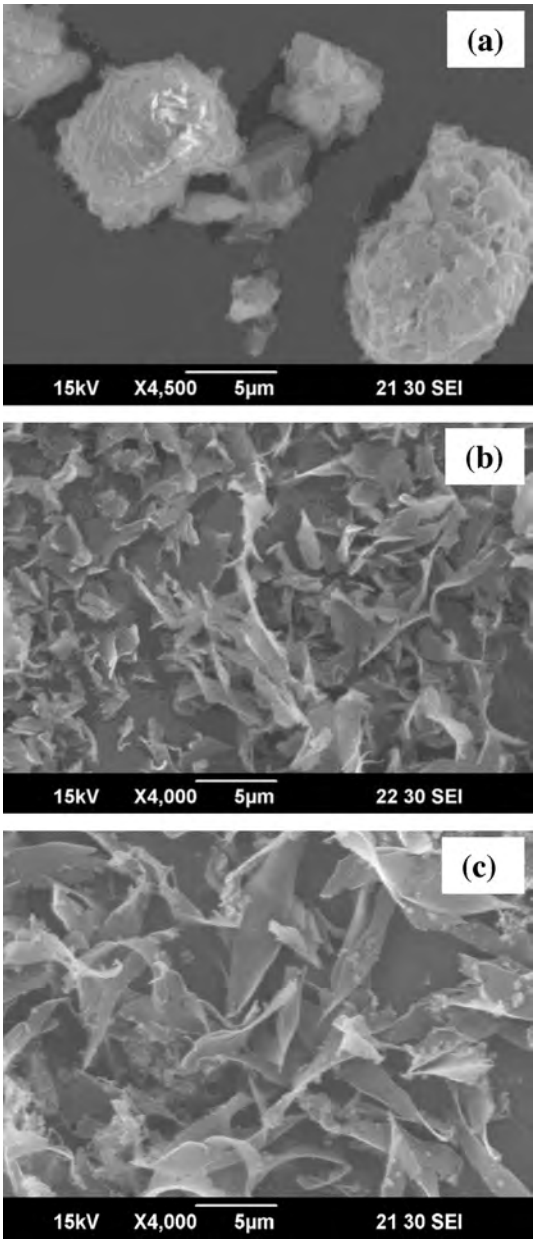

Fig. 4. SEM images of SAS micronized lutein samples obtained at 50 °C, 0.5 ml/min solution flow rate, 20 ml/min CO_2 flow rate, 2.5 mg/ml lutein concentration, and at various pressures (a) 8 MPa, (b) 10 MPa, (c) 12 MPa (sample numbers 2, 5, and 3).

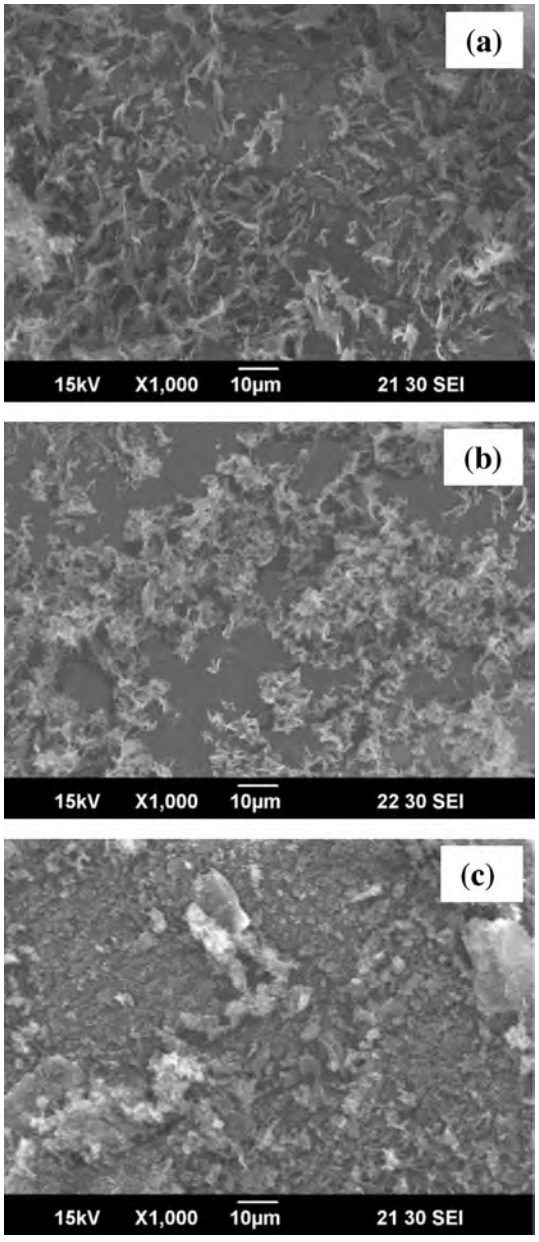

Fig. 5. SEM images of SAS micronized lutein samples obtained at 50 °C, 10 MPa, 0.5 ml/min solution flow rate, 20 ml/min CO_2 flow rate, and various lutein concentrations (a) 1.5 mg/ml, (b) 2.5 mg/ml, (c) 3.2 mg/ml (sample numbers 4, 5, and 6).

to rotating paddle apparatus. Then 5 ml of liquid samples were withdrawn at selected time intervals of 5, 15, 30, 45 and 60 min. Aliquots were filtered through 0.22 μm filters and assayed by a UV spectrophotometer at 450 nm [24].

3. Results and discussion

3.1. Micronization of lutein by supercritical anti solvent (SAS)

3.1.1. Effect of pressure

The effect of pressure on the morphology of micronized lutein could be drawn from the sample numbers 1, 2, 3 and 5, listed in Table 1, which were obtained at 6.5, 8, 12 and 10 MPa, respectively. At 6.5 MPa however, no particles could be collected. It was possible that the condition at 6.5 MPa and 50 °C was below the mixture critical condition, thus CO_2 did not act as an anti-solvent. Thus the two phases coexisted in the system at this condition, in which the lutein remained dissolved in the organic liquid phase, which was then carried over out of system by the flowing stream of CO_2. As the pressure of the system increases to 8 MPa, the single supercritical region may have been reached, the solubility of lutein in organic solvent decreased, due to an increase of SC-CO_2 concentration in the organic liquid phase [25]. As a result, the lutein solution immediately reached the supersaturation state, resulting in the production of fine particles at 8 MPa or higher pressures. However, the resulted lutein particles have different appearances. As shown in Fig. 3(a) and (b), fine powder with the MPS of 2.1 μm was obtained at 8 MPa, while particles obtained at 10 and 12 MPa tended to be agglomerated. Due to the agglomeration of the particles, it was difficult to measure the MPS at these conditions. Therefore, the effect of pressure on MPS and PSDs of micronized lutein could not be drawn. In addition, the agglomerated particles are likely to adhere to the wall of the precipitation vessel, resulting in much smaller yield (30%),

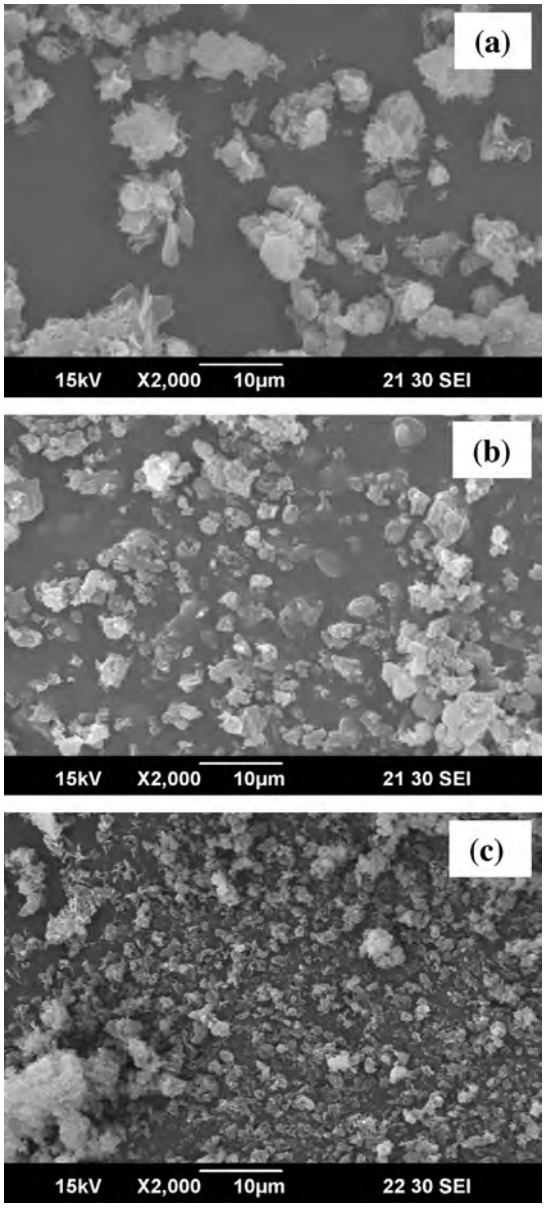

Fig. 6. SEM images of SAS micronized lutein samples obtained at 50 °C, 8 MPa, 0.25 ml/min solution flow rate, 2.5 mg/ml lutein concentration, and various CO_2 flow rates (a) 15 ml/min, (b) 20 ml/min, (c) 25 ml/min (sample numbers 7, 8, and 9).

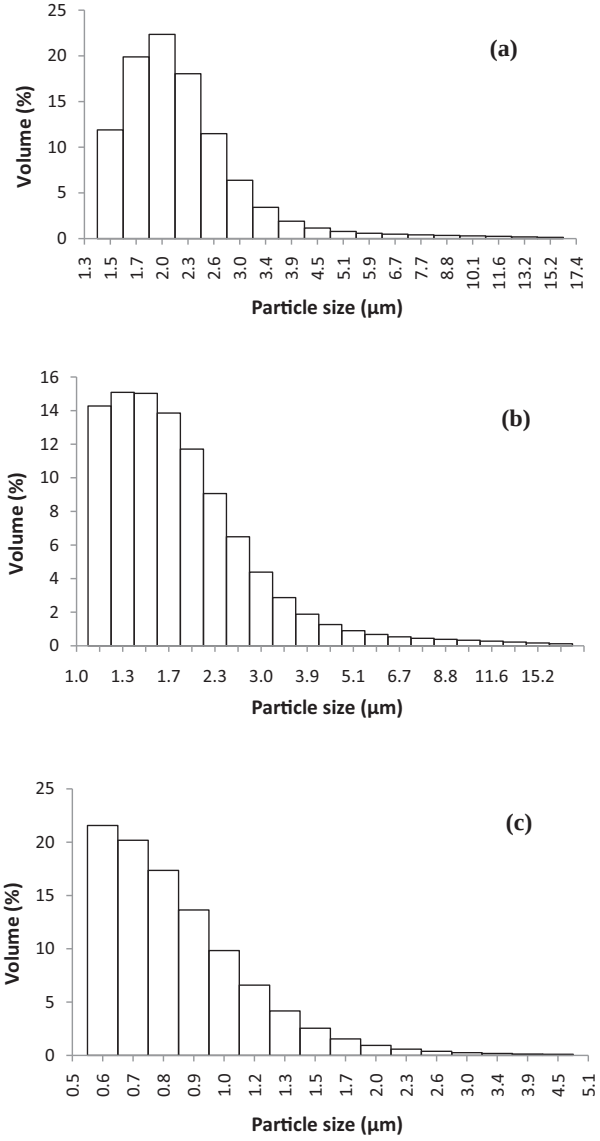

Fig. 7. PSD of SAS micronized lutein samples obtained at 50 °C, 8 MPa, 0.25 ml/min solution flow rate, 2.5 mg/ml lutein concentration, and various CO_2 flow rates (a) 15 ml/min, (b) 20 ml/min, (c) 25 ml/min (sample numbers 7, 8, and 9).

compared with the non-agglomerated particles obtained at 8 MPa (58%), whose loss was mainly attributed to the loss of particles of smaller size than the filter pores (1 μm). At 8 MPa, the resulted particles possess the ellipsoid to spheroid morphology as shown in Fig. 4(a). At 10 and 12 MPa on the other hand, the flake-like and the twisted leaf-like particles were obtained, respectively (Fig. 4(b) and (c)).

The different morphologies could largely be due to the effect of pressure on the characteristics of jet disintegration [19,26,27]. This jet disintegration occurs at the early stage in the process as organic solvent flows out of the nozzle. The jet flow behavior is connected to the presence of two distinct fluid phases because there is no mass transfer between the organic solvent and anti-solvent at this stage. Therefore, it could be classified by principal regime of liquid phase dispersion observed for liquid–liquid and liquid–gas systems [28]. Although phase equilibrium behavior, on the other hand, comes in to play at a later stage, it determines the success of the SAS process, and the micronization conditions must lie above the mixture critical point as previously described. Generally, depending on the flow rate and operating conditions such as temperature and pressure, three regimes of liquid phase dispersion are observed: (i) the dripping regime, in which the droplets are formed at the outlet of the nozzle; (ii) the laminar regime, in which the solvent was flowed smoothly and continuously before a break-up zone where uniform size droplets are formed; (iii) the turbulent regime, in which the jet surface becomes irregular [28] and the resulting non-uniform droplets are formed as stretched and small broken droplets. The ellipsoid–spheroid morphologies observed at 8 MPa suggested that the jet disintegration occurred at the laminar regime. As the pressure increased to 10 and 12 MPa, the organic solvent and CO_2 interfacial surface tension decreased, the jet disintegration entered the turbulent regime [28], resulting in irregular jet surface. As a result, non-uniform flake-like and twisted leaf-like particles were formed.

3.1.2. *Effect of lutein initial concentration*

The effect of lutein initial concentration (1.5, 2.5 and 3.2 mg/ml) on the particle morphology could be observed from sample

numbers 4, 5 and 6 for the fixed pressure of 10 MPa and the fixed SC-CO_2 flow rate of 20 ml/min. The SEM images of the resulted particles are shown in Fig. 5(a–c). There seemed to be no differences in the morphologies of the particles obtained with different lutein initial concentrations. In all cases, the flake-like particles of lutein were obtained. However, as mentioned above, the particles formed at 10 MPa tended to be agglomerated. As a result, large lumps of particles were seen for the samples formed from the lutein solution as the initial concentration increased.

3.1.3. Effect of SC-CO_2 flow rate

The effect of SC-CO_2 flow rate on morphology, MPS and PSD of micronized lutein could be observed from sample numbers 7, 8 and 9 for the fixed pressure of 8 MPa and the fixed lutein initial concentration of 2.5 mg/ml. As shown in Fig. 6, at 8 MPa, similar ellipsoid–spheroid shape morphology of micronized lutein particles were observed for all SC-CO_2 flow rates (15, 20 and 25 ml/min). However, as SC-CO_2 flow rate increased, MPS decreased from 2.2 μm to 0.8 μm. At higher SC-CO_2 flow rates, supersaturation was reached more readily as CO_2 quickly diffuse into the droplet, resulting in precipitation of very fine particles.

Moreover, the size distributions of the particles obtained at various SC-CO_2 flow rates are shown in Fig. 7. It can be inferred from Fig. 7 that some particles whose size was smaller than the filter pores were lost as it passed through the filter. Considerable loss was observed particularly at the SC-CO_2 flow rate of 25 ml/min, in which particles of the smallest MPS were formed giving the yield of only 27%. Despite this, it could still be drawn from Fig. 7 that slightly wider lutein PSD was observed when the SC-CO_2 flow rate increased from 15 ml/min to 20 ml/min, while increasing SC-CO_2 from 20 ml/min to 25 ml/min, on the other hand, resulted in narrower PSD. The larger PSD as the SC-CO_2 flow rate increased initially from 15 ml/min to 20 ml/min could be due to the interference by the increased aerodynamic force resulted from the increasing of SC-CO_2 flow rate, thus the liquid phase dispersion became irregular and the uniform size of droplet cannot be formed. When the SC-CO_2 flow rate increased to 25 ml/min however, much smaller particles were formed, thus the PSD became narrower.

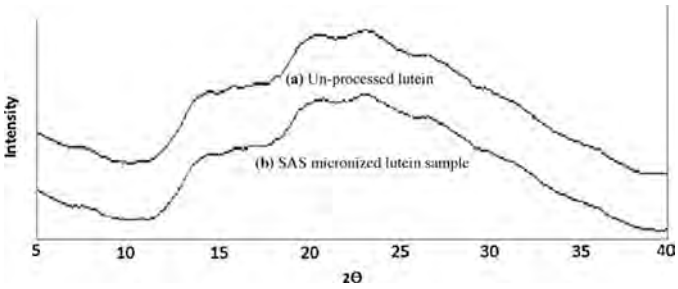

Fig. 8. XRD pattern of SAS micronized lutein samples; (a) un-processed lutein (b) SAS micronized lutein sample obtained at 50 °C, 8 MPa, 0.25 ml/min solution flow rate, 2.5 mg/ml lutein concentration, and 15 ml/min CO_2 flow rates.

3.2. Characterization of micronized lutein

Because the highest yield of non agglomerated micronized lutein particles were obtained for the sample number 7 (operated at pressure 8 MPa, 15 ml/min of SC-CO_2 flow rate and 2.5 mg/ml of lutein initial concentration), the particles were subjected to characterization study to examine the particle crystallinity and the changes in the chemical structures using XRD and FTIR analyses, respectively.

The XRD patterns of un-processed lutein and micronized lutein powder shown in Fig. 8 imply that lutein was completely amorphous in nature. The amorphous structure could be attributed to the fact that the supersaturation state was reached suddenly as a result in of the solubility decrease by the diffusion of SC-CO_2 into the solvent. Moreover, the FTIR spectroscopy results of the processed and un-processed lutein particles are shown in Fig. 9. The corresponding OH stretching vibration band between 3200 and 3600 cm^{-1} is a rather broad peak. The bands corresponding to stretching vibrations in the CH_2 groups are at 2852 cm^{-1} and 2922 cm^{-1}. The band assigned to the stretching vibrations of the C=C groups is at 1630 cm^{-1}. The bands corresponding to bending vibrations in the CH_3 groups are at 1447 cm^{-1} and 1376 cm^{-1}. The out of plane deformation of =C—H group (oop bend) is present

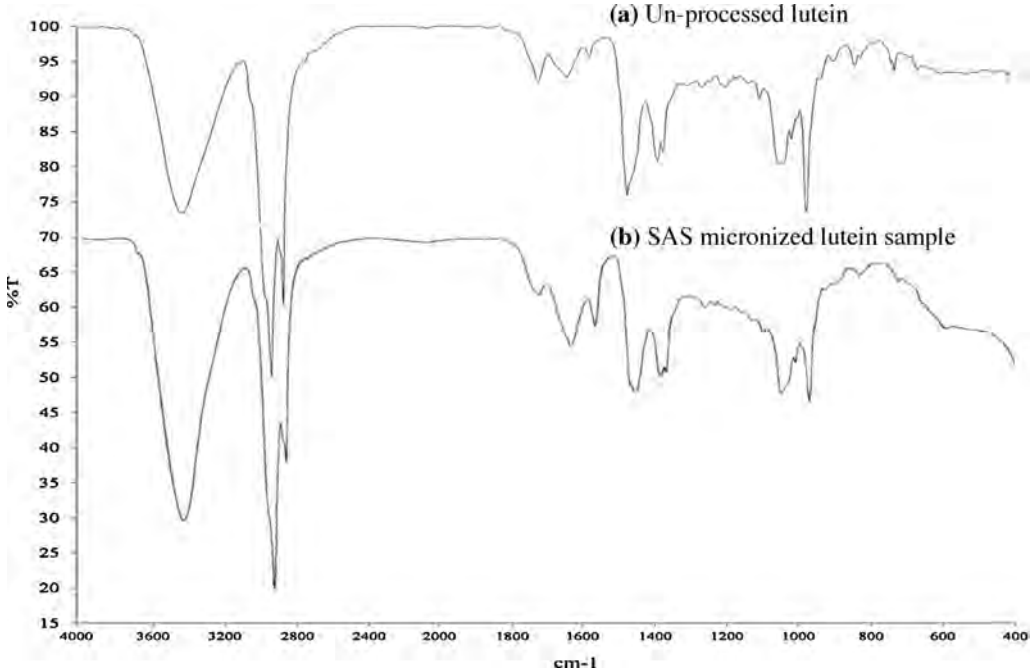

Fig. 9. FTIR Transmission of lutein samples; (a) un-processed lutein (b) SAS micronized lutein sample obtained at 50 °C, 8 MPa, 0.25 ml/min solution flow rate, 2.5 mg/ml lutein concentration, and 15 ml/min CO_2 flow rates.

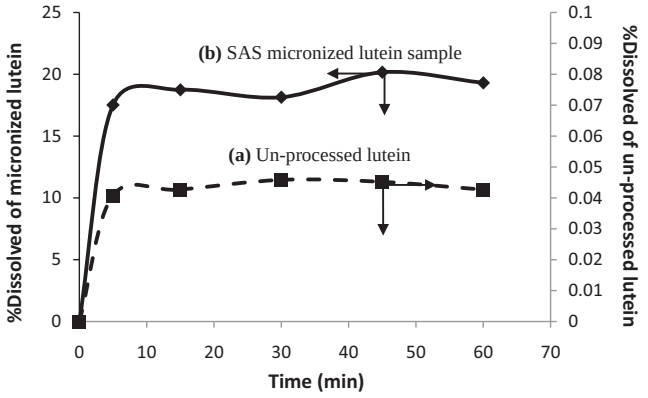

Fig. 10. Dissolution profile of (a) un-processed lutein and (b) SAS micronized lutein sample obtained at 50 °C, 8 MPa, 0.25 ml/min solution flow rate, 2.5 mg/ml lutein concentration, 15 ml/min CO_2 flow rates (dissolution conditions at 37 °C, rotating speed of 100 rpm, 15 mg of samples in 500 ml of medium solution (pH = 1.2)).

at 966 cm^{-1}. From the FTIR results, no chemical structural change in the micronized lutein particles occurred as a result of the SAS process.

3.3. Dissolution of micronized lutein particles

Dissolution study was conducted on the micronized lutein powder obtained for the sample number 7 (operated at pressure 8 MPa, 15 ml/min of SC-CO_2 flow rate and 2.5 mg/ml of lutein initial concentration) and the un-processed lutein. As shown in Fig. 10, the un-processed lutein was found to be hardly soluble in the aqueous solution, while the % dissolution of the micronized lutein particles sharply increased to about 17% within 5 min, then slowly increased, and reached 20% in 60 min. The improvement in % dissolution of the micronized lutein particles could be explained by the increased surface area as the MPS reduced from 202.3 μm to 2.2 μm (Table 1).

4. Conclusions

The supercritical anti-solvent (SAS) process could be employed for the micronization marigold lutein particles, using a mixture of hexane and ethyl acetate, the mobile phase in the earlier step of chromatography purification. Pressure was found to have significant effect on the particle morphology. The increase in lutein initial concentration from 1.5 mg/ml to 3.2 mg/ml and the increase in SC-CO_2 flow rate from 15 ml/min to 25 ml/min show no significant effect on the morphology of the micronized lutein particles. However, the reduction of lutein particle size from about 2 μm to 0.8 μm by increasing SC-CO_2 flow rate was observed. The suitable conditions for lutein micronization in this work were found to be at 8 MPa, 2.5 mg/ml initial concentration and 25 ml/min SC-CO_2 flow rate, giving non-agglomerated fine particles of 0.8 μm MPS. The micronized lutein particles were found to be amorphous and no significant changes in the chemical structure of lutein were observed after the SAS micronization process. Moreover, significant improvement in the dissolution micronized lutein particles was found compared to the un-processed lutein. In addition, in order to further gain better insights into the entire process, further investigations on phase equilibrium of the system and regime of liquid phase dispersion should be carried out.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.supflu.2013.03.033.

References

[1] G.W. Burton, Antioxidant action of carotenoids, J. Nutrition 119 (1989) 109–111.
[2] F. Granado, B. Olmedilla, I. Blanco, Nutritional and clinical relevance of lutein in human health, British J. Nutrition 90 (2003) 487–502.
[3] R.L. Ausich, D.J. Sanders, Process for the formation, isolation and purification of comestible xanthophylls crystals from plants, US Patent 5 648 564 (1997).
[4] F. Khachik, M. Beltsville, Process for isolation, purification, and recrystallization of lutein from saponified marigold oleoresin, US Patent 5 382 714 (1995).
[5] F. Khachik, M. Beltsville, Process for extraction, purification of lutein, zeaxanthin and rare carotenoids from marigold flowers and plants, US Patent 6 262 284 (2001).
[6] J. Vechpanich, A. Shotipruk, Recovery of free lutein from *Tagetes erecta*: determination of suitable saponification and crystallization conditions, Separation Science and Technology 46 (2011) 265–271.
[7] P. Boonnoun, T. Opaskonkun, P. Prasitchoke, A. Shotipruk, Purification of Free lutein from marigold flowers by liquid chromatography, Engineering Journal 16 (2012) 145–156.
[8] P. Varughese, J. Li, W. Wang, D. Winstead, Supercritical antisolvent processing of γ-indomethacin: effects of solvent, concentration, pressure and temperature on SAS processed indomethacin, Powder Technology 201 (2010) 64–69.
[9] C. Zhao, L. Wang, Y. Zu, C. Li, S. Liu, L. Yang, X. Zhao, B. Zu, Micronization of Ginkgo biloba extract using supercritical antisolvent process, Powder Technology 209 (2011) 73–80.
[10] M. Turk, G. Upper, P. Hils, Formation of composite drug–polymer particles by co-precipitation during the rapid expansion of supercritical fluids, J. Supercritical Fluids 39 (2006) 253–263.
[11] C.S. Su, M. Tang, Y.P. Chen, Recrystallization of pharmaceuticals using the batch supercritical anti-solvent process, Chemical Engineering and Processing 48 (2009) 92–100.
[12] Q. Can, J. Carlfors, C. Turner, Carotenoids particle formation by supercritical fluid technologies, Chinese J. Chemical Engineering 17 (2009) 344–349.
[13] Y.C. Cho, J.H. Cheng, S.L. Hsu, S.E. Hong, T.M. Lee, C.M.J. Chang, Supercritical carbon dioxide anti-solvent precipitation of anti-oxidative zeaxanthin highly recovered by elution chromatography from *Nannochloropsis oculata*, Separation and Purification Technology 78 (2011) 274–280.
[14] E. Reverchon, I. De Marco, Supercritical antisolvent precipitation of cephalosporins, Powder Technology 164 (2006) 139–146.
[15] Y. Wang, Y. Wang, J. Yang, R. Pfeffer, R. Dave, B. Michniak, The application of a supercritical antisolvent process for sustained drug delivery, Powder Technology 164 (2006) 94–102.
[16] E. Reverchon, I. De Marco, Supercritical antisolvent micronization of cefonicid: thermodynamic interpretation of results, J. Supercritical Fluids 31 (2004) 207–215.
[17] I. De Marco, E. Reverchon, Supercritical antisolvent micronization of cyclodextrins, Powder Technology 183 (2008) 239–246.
[18] M.S. Kim, S. Lee, J.-S. Park, J.S. Woo, S.-J. Hwang, Micronization of cilostazol using supercritical antisolvent (SAS) process: effect of process parameters, Powder Technology 177 (2007) 64–70.
[19] A. Gokhale, B. Khusid, R.N. Dave, R. Pfeffer, Effect of solvent strength and operating pressure on the formation of submicrometer polymer particles in supercritical microjets, J. Supercritical Fluids 43 (2007) 341–356.
[20] D.L. Obrzut, P.W. Bell, C.B. Roberts, S.R. Duke, Effect of process conditions on the spray characteristics of a PLA plus methylene chloride solution in the supercritical antisolvent precipitation process, J. Supercritical Fluids 42 (2007) 299–309.
[21] I. Tsivintzelis, D. Missopolinou, K. Kalogiannis, C. Panayiotou, Phase compositions and saturated densities for the binary systems of carbon dioxide with ethanol and dichloromethane, Fluid Phase Equilibria 224 (2004) 89–96.
[22] P. Boonnoun, H. Nerome, S. Machmudah, M. Goto, A. Shotipruk, Supercritical anti-solvent micronization of marigold-derived lutein dissolved in dichloromethane and ethanol, J. Supercritical Fluids 77 (2013) 103–109.
[23] S. Shibata, C. Ishihara, C. Matsumoto, Improved separation method for highly purified lutein from chlorella powder using jet mill and flash column chromatography on silica gel, J. Agricultural and Food Chemistry 52 (2004) 6283–6286.
[24] K. Mitri, R. Shegokar, S. Gohla, C. Anselmi, R.H. Muller, Lutein nanocrystals as antioxidant formulation for oral and dermal delivery, International J. Pharmaceutics 420 (2011) 141–146.

[25] F. Miguel, A. Martín, F. Mattea, M.J. Cocero, Precipitation of lutein and co-precipitation of lutein and poly-lactic acid with the supercritical anti-solvent process, Chemical Engineering and Processing 47 (2008) 1594–1602.
[26] A. Braeuer, R. Adami, S. Dowy, M. Rossmann, A. Leipertz, Observation of liquid solution volume expansion during particle precipitation in the supercritical CO_2 antisolvent process, J. Supercritical Fluids 56 (2011) 121–124.
[27] B.Y. Shekunov, J. Baldyga, P. York, Particle formation by mixing with supercritical antisolvent at high Reynolds numbers, Chemical Engineering Science 56 (2001) 2421–2433.
[28] E. Badens, O. Boutin, G. Charbit, Laminar jet dispersion and jet atomization in pressurized carbon dioxide, J. Supercritical Fluids 36 (2005) 81–90.

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Research Article

Simultaneous Production of Biodiesel and Free Lutein from *Chlorella vulgaris*

Biodiesel and valuable free lutein were demonstrated to be simultaneously produced from *Chlorella vulgaris* lipid extracts. The alkali catalyst used in the transesterification of triglycerides acted as a reactant in converting lutein fatty acid esters to free lutein. A maximum biodiesel yield of 33.6 % by weight of the algal lipids was obtained after a 4-h reaction with MeOH at the MeOH/biomass ratio of 16:1 using 6 % alkali catalyst. The excess of alkali and MeOH employed in the production of biodiesel ensured the complete saponification of all lutein fatty acid esters to free lutein, giving a maximum yield of 2.3 % by weight of the algal lipids. In addition, a process for the separation of the biodiesel and free lutein products from the reaction mixture is proposed. Finally, a preliminary economic assessment was conducted, the results of which suggest that the process for the simultaneous production of biodiesel and lutein from *C. vulgaris* may be economically feasible.

Keywords: Biodiesel, Lutein, Microalgae, Saponification, Transesterification

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1 Introduction

As an alternative to fossil fuels, biodiesel has now become widely accepted as a potential fuel that may help overcome the energy crisis faced by countries all over the world. However, currently it is still produced from oil crops, which poses some concerns regarding the invasion of the food crop area and the future availability of sufficient food for the growing world population.

Microalgae are another interesting source for biodiesel production due to their rapid growth rate, comparable lipid content and, above all, the need for only relatively small cultivation land areas [1]. Nevertheless, the production of biodiesel from microalgae has not yet been economically practiced on the industrial scale since the production cost is still much higher than that of the conventional fossil fuel. Besides, most systems for algal fuel production consume more energy than that contained in the algae [2]. Indeed, it has been suggested that the overall economic feasibility of biodiesel production from microalgae is largely dependent on the selection of a suitable microalgal strain. Desirable traits include not only an alga's rapid growth and high lipid content but also its environmental hardiness and the possibility of simultaneously generating valuable coproducts. Microalgal species that have recently been extensively studied as potential biodiesel feedstocks include *Chlamydomonas reinhardtii*, *Dunaliella salina*, *Botryococcus braunii*, and *Chlorella* sp. [3]. Due to the possible capacity of *Chlorella* species to produce high-value coproducts in particular, cultivation of these algal species to harvest the lipids [4–6] for biodiesel synthesis [7, 8] has gained a surge of interest in recent years. Besides their high lipid content (14–56 % by dry weight) [9], *Chlorella* is also one of the major sources of the naturally occurring carotenoid lutein (2–4 mg/g of dry biomass) [10, 11]. The compound has great capability of preventing cancers as well as eye and heart diseases [12]; its price is as high as USD 570–790 per kilogram (prices from Changsha Winner Bio-tech Co., Ltd., Changsha, China; Changsha Sunfull Bio-Tech Co. Ltd., Changsha, China; Xi'an Aladdin Biological Technology Co., Ltd., Xi'an, China). Indeed, *Chlorella* species have long been cultivated for the production of biomass (for protein) and high-value products [4, 5] and, as a consequence, the economical harvesting and processing methodologies for these products are already well established, regardless of biodiesel production [3]. Although a number of studies have recently been conducted on the production of biodiesel from *Chlorella* lipids, we believe our investigation into the simultaneous production of both biodiesel and free lutein from *Chlorella* microalgae to be the first study of its kind.

Specifically, we sought first to isolate algal lipids from *Chlorella vulgaris* which are rich in lutein fatty acid esters (LFE) and then to transesterify them using an excess of alkaline MeOH, yielding fatty acid methyl esters (FAME) (Fig. 1 a)

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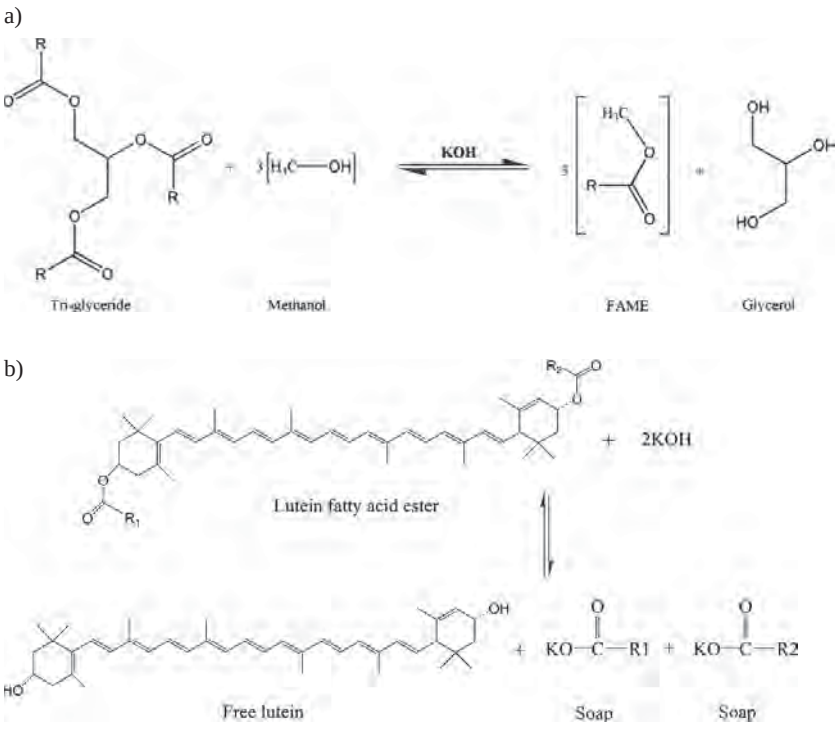

Figure 1. Reactions of (a) the transesterification of triglyceride, (b) the saponification of LFE.

known as biodiesel. These conditions were deemed suitable also for the saponification of LFE into free lutein (Fig. 1 b) [13], which then allows the production of biodiesel and free lutein as coproduct in a single step. Furthermore, the effects of key reaction parameters on the yields of biodiesel and free lutein were investigated, including the amount of catalyst, the biomass-to-alcohol ratio, and the reaction time. In addition, a possible process for the separation of the biodiesel and lutein products from the reaction mixture is proposed. Finally, a preliminary evaluation of the economic feasibility based on the experimental results is presented.

2 Materials and Methods

2.1 Biomass and Sample Preparation

C. vulgaris was cultivated in a bubble column photobioreactor with a diameter of 75 cm and a volume of 170 L (Department of Chemical Engineering, Chulalongkorn University) under outdoor conditions, i.e., 24–32 °C and a diurnal illumination cycle at the intensity of 0–100 klux. The cultivation medium, purchased from the Pathumthani Inland Fisheries Research and Development Center (Patumthani, Thailand), was composed of fertilizers including 10 mg L^{-1} triple super phosphate ($Ca(H_2PO_4)_2$), 120 mg L^{-1} ammonium phosphate ($(NH4)_3PO_4$), urea ($CO(NH_2)_2$), and lime. Aeration was applied at a rate of 10 L min^{-1}. The microalgae were harvested on day 4 at the end of log phase growth and dewatered by 8000-rpm continuous-flow centrifugation in a disc centrifuge (Alfa Laval DX203B-34; Spain). The resulting alga paste was subsequently lyophilized (FreeZone freeze-dry system (–50 °C); USA), and the dry *Chlorella* powder was then stored at 4 °C until use.

2.2 Feasibility Study on the Simultaneous Transesterification and Saponification of *C. vulgaris* Lipids

The experiment was carried out using a two-step (extraction-transesterification) method following the flow diagram shown in Fig. 2. Lyophilized *Chlorella* algae powder (3 g) was extracted for 4 h in a Soxhlet apparatus by a 2:1 v/v mixture (180 mL) of chloroform (RCI Labscan Ltd.) and MeOH (Mallinckrodt Chemicals Inc.). The solvent was then removed using a rotary evaporator and the weight of the remaining crude lipid was measured. The crude lipid was then charged into a glass vessel equipped with a condenser, along with a solution of KOH (6 % by dry weight of the biomass) (Wako, Japan) in MeOH, with a ratio to dry algae of 16:1. The system was then heated to 60 °C under agitation at 350 rpm with a magnetic stirrer, and was kept at this temperature for 4 h, after which it was cooled to room temperature. Chloroform and water were then added into the reaction mixture at a ratio of mixture/chloroform/water of 10:10:9, and the mixture was shaken vigorously. The mixture was then centrifuged (2000 rpm, 10 min) resulting in separation into two phases. MeOH and polar impurities such as glycerol dissolved in the water forming the upper phase, and the lower chloroform phase was retained for the analysis for the FAME and free lutein contents.

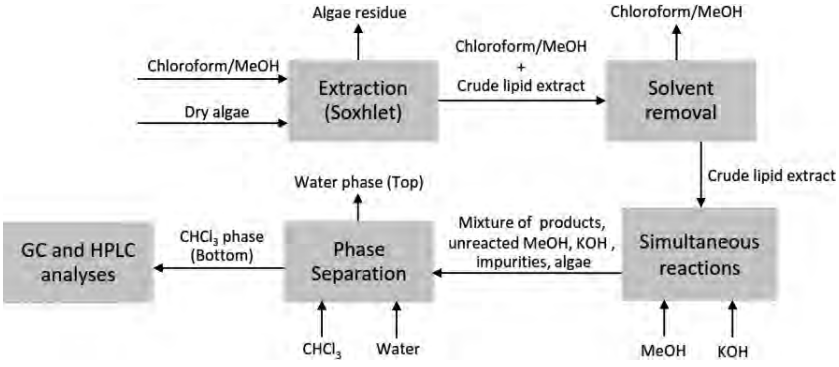

Figure 2. Diagram of the conventional two-step process of extraction and transesterification.

2.3 Effects of Varying Reaction Parameters on the Biodiesel and Free Lutein Yields

The procedure described in Sect. 2.2 was repeated in subsequent experimental runs, each one varying in transesterification duration (either 1, 2, 3, or 4 h), amount of KOH (in the range of 0.1–8.0 % by dry weight of the algae), or MeOH-to-biomass ratio (1:1, 8:1, 12:1, and 16:1 v/w).

2.4 Gas Chromatographic Analyses for FAME

The analyses for FAME were carried out using a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detector (FID). The sample injection volume was 0.1 µL and the column used was a capillary CP-FFAP CB column of 25 m length, 0.32 mm i.d., and 0.3 µm film thickness (Varian, CA, USA). The injector and detector temperatures were set at 270 and 300 °C, respectively. The elution temperature program featured an initial hold at 100 °C (5 min) prior to a linear ramp (10 °C min^{-1}) up to 250 °C. This final temperature was then held for 20 min, resulting in a total run time of 40 min. The samples were quantified using methyl heptadecanoate as internal standard.

2.5 High-Performance Liquid Chromatographic Analyses for Free Lutein

The high-performance liquid chromatography (HPLC) analyses for free lutein were carried out using the method reported by Roberta et al. [14] with modifications. Of sample, 20 µL was injected into a Lichrocart C-18 column equipped with a Diode Array Detector Module 335 (detection wavelength 450 nm). Chromatographic separation was obtained with a gradient system of acetonitrile/MeOH 9:1 (v/v) as solvent A (HPLC-grade acetonitrile; Sigma-Aldrich, Germany) and ethyl acetate as solvent B. Solvent B was run with a linear gradient at a flow rate of 1 mL min^{-1} from 0 to 100 % over 30 min.

3 Results and Discussion

3.1 Feasibility of the Simultaneous Production of Biodiesel and Free Lutein from *C. vulgaris* Lipids

The transesterification of a *C. vulgaris* crude lipid extract with MeOH at the MeOH/biomass ratio of 16:1 and 6 % KOH catalyst by algal weight resulted in a reaction product that contained FAME with a yield of 33.6 % by crude lipid weight (or 4.7 % by algal weight). This yield lies within the range of biodiesel yields reported by Montes D'Oca et al. [15] and Li et al. [16] for the transesterification of *C. pyrenoidosa* and *Nannochloropsis* lipids, respectively. In addition, the analysis of this sample revealed that the composition of the FAME produced in this study was similar to that reported by Lee et al. [6]. Specifically, methyl palmitate (37.7 wt %) and methyl linoleate (34.6 wt %) were found to be major components. Other than these, methyl linolenate (15.6 wt %), methyl oleate (9.7 wt %), and methyl stearate (2.4 wt %) were also detected.

Apart from the predominant FAME, the same sample was also analyzed by HPLC for the expected free lutein. The sample was found to contain free lutein with a yield of 2.0 % by weight of algal lipids or 3.0 mg free lutein/g of algae. Fig. 3 a shows the chromatogram of the crude extract, which reveals the peak of free lutein at the retention time of 9.863 min and a group of lutein esters at a later time. After the reaction (Fig. 3 b), a decrease in the peak intensity of LFE was observed while the peak intensity of free lutein (retention time of 9.956 min) had increased. This attests the occurrence of saponification of LFE during the production of algal biodiesel, and thus free lutein was coproduced.

3.2 Effects of Reaction Parameters on the Biodiesel and Free Lutein Yields

3.2.1 Effects of Reaction Time

The effects of the reaction time on the biodiesel and free lutein yields are shown in Fig. 4. The FAME yield increased with the reaction time from 1 to 4 h, while the free lutein yield increased similarly from 1 to 2 h but remained almost constant thereafter. This leveling off could simply be a consequence of

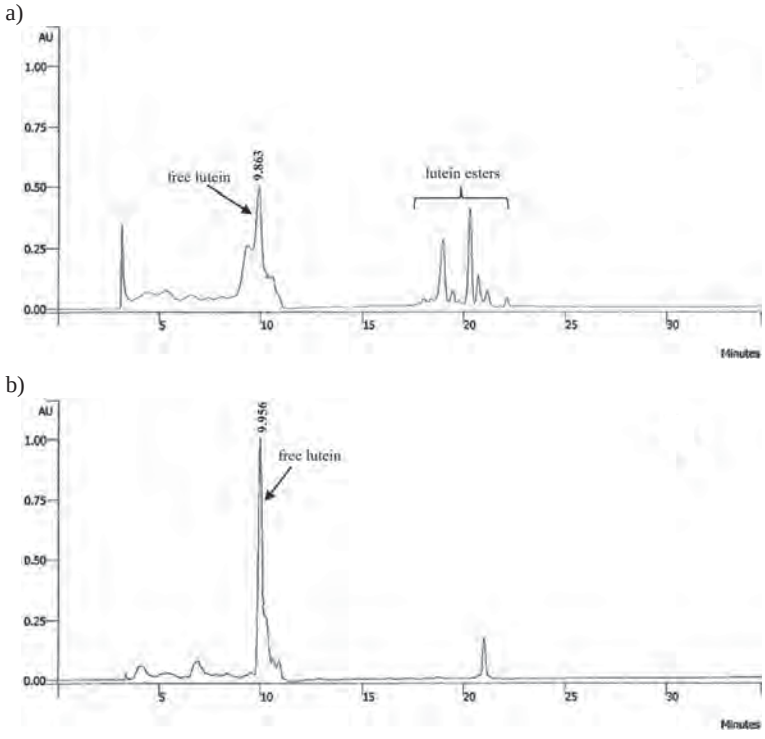

Figure 3. HPLC chromatograms of (a) the crude lipid extract, (b) the product after alkali reaction (6 % KOH, 16:1 MeOH/biomass, 4 h).

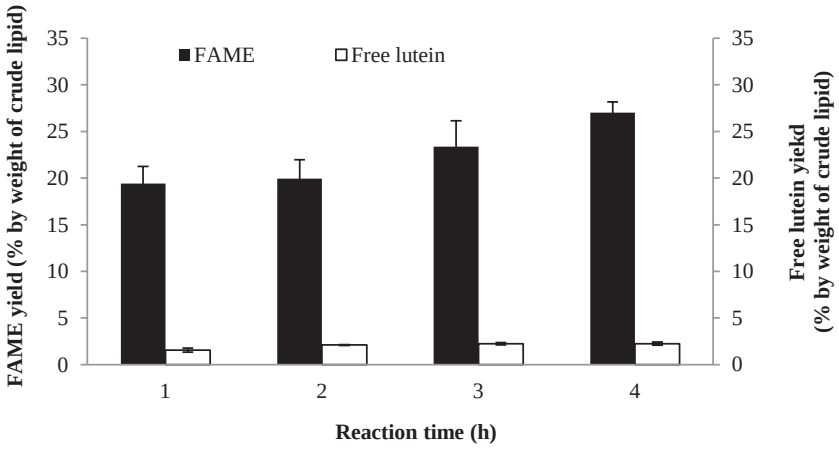

Figure 4. Effect of the reaction time on the biodiesel and free lutein yields (4 % KOH, 16:1 MeOH/biomass).

2 h being sufficient to release the entire free lutein content of the lipids in the sample [10].

3.2.2 Effects of the Alcohol-to-Biomass Ratio

Employing a fixed reaction time (4 h) and catalyst amount (4 %), the results shown in Fig. 5 reveal that an increase in the MeOH-to-biomass ratio from 8:1 to 16:1 resulted in only a slight increase in the biodiesel yield. This is probably because this surplus of MeOH was far greater than that in the theoretical ratio required for biodiesel conversion. Specifically, the theoretical molar ratio for the conversion of triglycerides is 3:1, which is equivalent to a MeOH-to-biomass (v/w) ratio of only 1:100. This was calculated based on the assumption that the total lipid content of dry algae is, on average, 14 wt % and that only 50 % of this lipid is suitable for biodiesel production. It should be noted that no biodiesel product was observed with the smallest ratio of MeOH/biomass of 1:1 used in this study, despite the fact that this ratio is considerably higher than the theoretical ratio (1:100). This result could be attributed to poor mixing in the highly viscous reaction system.

On the other hand, the free lutein yields obtained at any ratio of MeOH/biomass in this study did not differ significantly. In fact, the yield decreased slightly with increasing MeOH amount, probably due to KOH dilution by the excess MeOH slowing down the reaction.

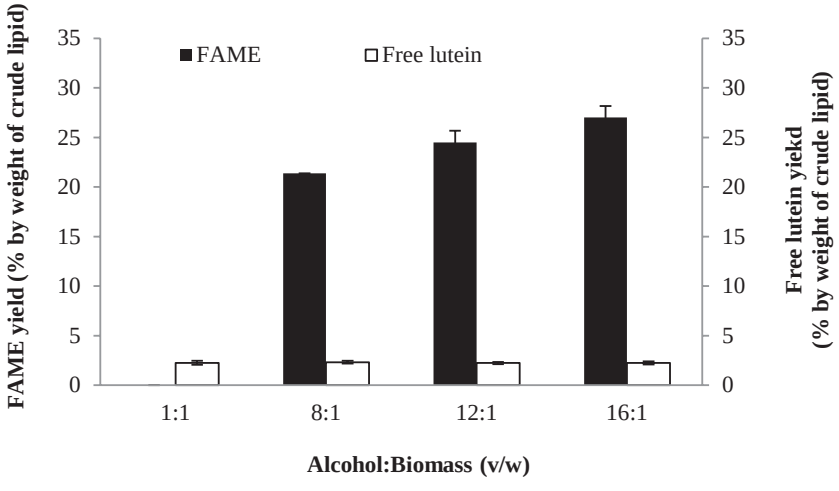

Figure 5. Effect of the MeOH/biomass ratio on the biodiesel and free lutein yields (4 % KOH, 4 h).

3.2.3 Effects of the Catalyst Quantity

The effects of varying amounts of KOH catalyst on the biodiesel yields were investigated by fixing the reaction time (4 h) and the alcohol-to-biomass ratio (16:1). Fig. 6 illustrates the increase in the biodiesel yield as the concentration of catalyst was raised from 0.1 to 6 %. However, the yield thereafter dropped on further introduction of catalyst (8 %). This decrease could arise from partial saponification of the FAME produced (forming water and amphipathic 'soap' salts), thereby competing with the desired transesterification reaction.

The amount of alkali catalyst also seems to have an effect on the free lutein yield. The use of 0.1 % KOH was found to yield very little free lutein, despite the fact that this quantity still provides a stoichiometric excess of KOH with the potential to completely transesterify all lutein esters in the algal lipid sample. One possible reason for the low free lutein yield is that part of the KOH instead preferentially participated (as catalyst) in the transesterification of triglycerides. Another reason may be based on interferences with the reaction caused by existing impurities in the *Chlorella* crude extract, such as free fatty acids (FFA) [17] and pigments such as β-carotene, chlorophyll, and astaxanthin [18]. As more KOH (1 %) was introduced, the free lutein yield increased from 0.9 to over 2.3 % by weight of algal lipids, after which further KOH (in the range of 2–8 %) did not appear to significantly affect the yield. Based on the results shown in Fig. 6, 1 % KOH is likely to be sufficient for the complete saponification of LFE from *Chlorella* biomass.

3.3 Proposed Product Separation Process

Although we demonstrated the possibility to simultaneously transesterify *Chlorella* lipid extracts and to saponify their LFE to produce biodiesel and free lutein, for achieving technical and economic feasibility of the entire process, the further downstream processing must also be taken into consideration. Various processes have been

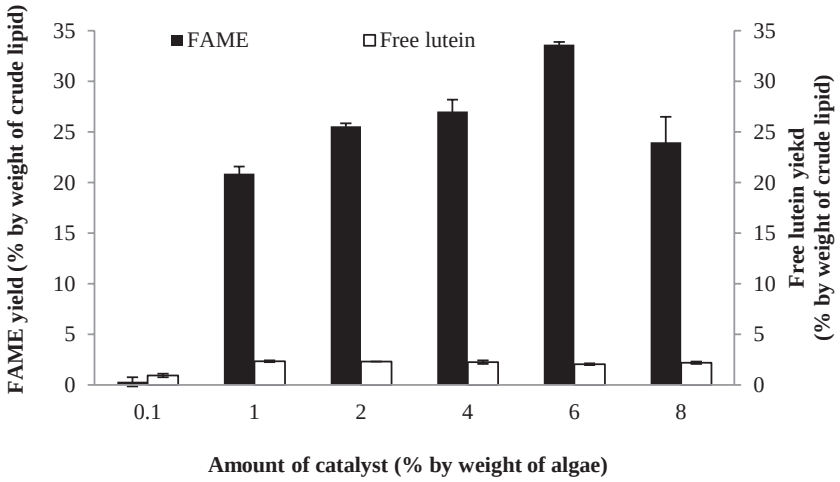

Figure 6. Effect of the catalyst amount on the biodiesel and free lutein yields (16:1 MeOH/biomass, 4 h).

proposed to isolate and purify lutein extracted from marigold and *C. vulgaris* after saponification, such as by recrystallization [13], chromatography [19], and partitioning in two-phase systems [20]. The last of these techniques was the simplest and the most economical method: 85–91 % of free lutein with 90–98 % purity could be recovered just by partitioning the extract of saponified *C. vulgaris* in various two-phase systems, followed by anti-solvent precipitation [20]. Given similar components in the saponified lutein extract and in the reaction mixture in our study (KOH solution in MeOH, free lutein, biodiesel, soap, glycerol, and unreacted glycerides), a process modified from that described in Li et al. [20] is proposed for the separation of biodiesel and free lutein from the reaction mixture (Fig. 7).

As shown in the diagram, the separation process begins with the addition of a proper organic solvent that dissolves biodiesel and free lutein (such as dichloromethane or chloroform) to the reaction mixture [20, 21]. Water is then added, which causes the system to form two phases. It should be noted that, unlike in a typical biodiesel production process, water should not be added directly to the reaction before the addition of the organic solvent. This is necessary to avoid the formation of a thick layer caused by the interaction between soap, water, and glycerides present in the reaction mixtures, which would complicate the separation process. The system of chloroform/mixture/water at a volume ratio of, e.g., 10:10:9 forms two phases: the top MeOH/water-rich phase containing KOH, soap and glycerol and the bottom chloroform-rich phase containing free lutein, biodiesel and unreacted glycerides. The bottom phase can then be collected and, after evaporation of the solvent, the mixture of free lutein, biodiesel, and unreacted glycerides is obtained. Isolation of the two main products (biodiesel and free lutein) can then be achieved in another two-phase system. Redissolving the mixture first in an 85 % aqueous EtOH solution followed by the addition of hexane at a hexane/aqueous EtOH solution ratio of 2:1 results in

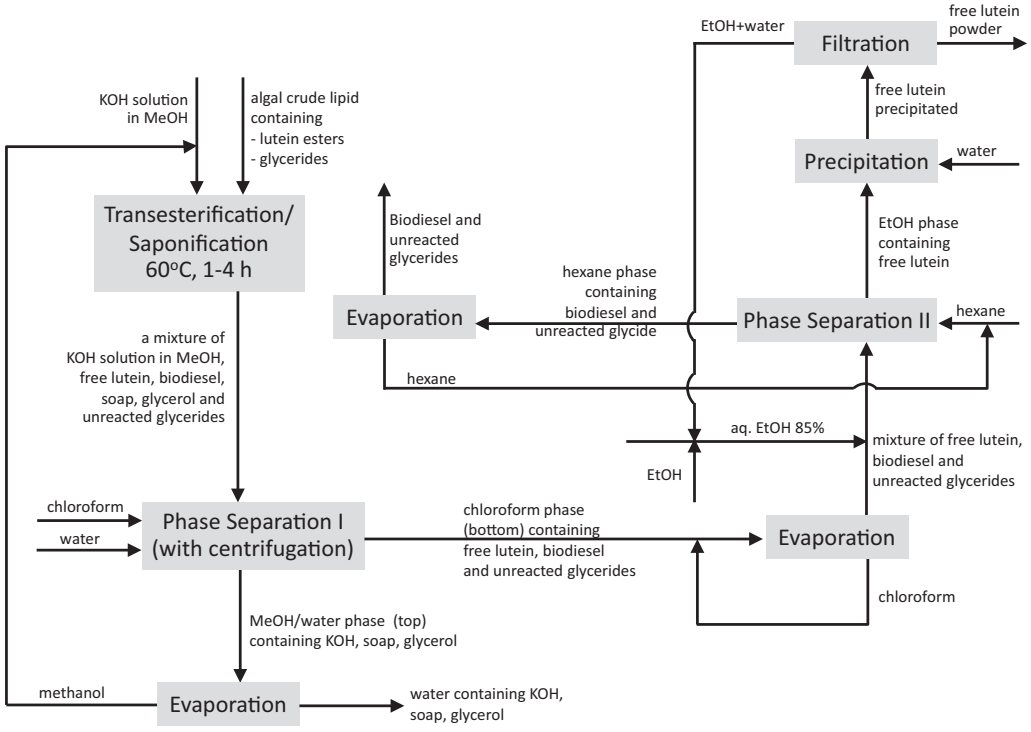

Figure 7. Process flow diagram of product separation.

phase separation into the hexane-rich phase containing biodiesel and unreacted glycerides and the EtOH/water-rich phase containing free lutein. Free lutein in the EtOH/water phase can be recovered by precipitation with further addition of water as an anti-solvent. Subsequently, a fine powder of free lutein can be obtained by filtration. Our preliminary investigation using a model mixture of free lutein and palm FAME to represent biodiesel indicated that partitioning of the mixture in a hexane/aqueous EtOH solution system followed by anti-solvent precipitation of free lutein as described above gave nearly 100 % recovery of the biodiesel and free lutein. Assuming that the solvents used in the process can be recycled, the additional cost to the process proposed in Fig. 7 compared with that of conventional biodiesel production would largely be the cost of the additional process equipment, such as for phase separation, precipitation and filtration of the free lutein, and for additional units needed for the evaporation and recovery of organic solvents (chloroform and hexane).

3.4 Preliminary Economic Feasibility Assessment

As a potential source of lipid for biodiesel production, microalgae have gained increased attention in recent years, and several studies on the economic feasibility of their biodiesel production process have been published [22–24]. In most of these studies, the production of algal biodiesel is reported to be uneconomical. Delrue et al. [24] reported the production cost of algae-derived biodiesel to be as high as USD 2.9–5.0 per kilogram, which was considerably higher than that of a current commercial biodiesel of only USD 1.63 per kilogram. (The production cost was the sum of the operating cost – i.e. utilities, labor and other costs at 0.9 % of the capital cost – and the fixed cost. The fixed cost was calculated from the depreciable capital cost, consisting of 55 % of the capital cost for the general maintenance, storage, engineering and spare parts costs, license fees, initial expenses at 2 % of the capital cost, and process start-up cost at 25 % of the operating cost. The calculation was based on 20-year annuities, an 8 % discount rate, and 7 % of the capital cost per year for maintenance cost, taxes, insurances, and business expenses.) Their sensitivity analysis indicated that the cost of production varies with the methods of cultivation (raceway or photobioreactor), dewatering/drying (centrifugation, belt filter press, solar drying or bed drying), and lipid extraction (*n*-hexane or dimethylether). Based on the 14 % lipid content in algal biomass determined in this study, the biodiesel yield of 20–34 % by weight of the crude lipids was calculated to be equivalent to 3–5 % by weight of the biomass. A similar calculation resulted in a yield of free lutein coproduct of approximately 0.15–0.35 % by weight of the biomass, which is equivalent to 0.03–0.12 kg of free lutein produced per 1 kg of algal biodiesel. Taking the price of free lutein to be between USD 570–790 per kilogram, our results suggest that approximately USD 17–95 worth of free lutein could potentially be produced with each kilogram of biodiesel. Despite the higher production cost due to additional equipment required for the separation process, the high price of free lutein nevertheless would justify the economic feasibility of the simultaneous production process described in this work.

4 Conclusions

The results of this work show that, apart from the main product biodiesel, free lutein can be obtained as a coproduct, by the simultaneous transesterification and saponification of *C. vulgaris* lipids. The maximum FAME yield (33.6 %) was achieved from a 4-h reaction with alcohol/biomass 16:1 v/w and 6 % alkali catalyst. Under these conditions, all LFE was deesterified to the more valuable free form. Separation of the biodiesel and free lutein from the reaction mixtures can be carried out by sequential partitioning in various liquid-liquid two-phase systems, followed by anti-solvent precipitation of the free lutein. Based on our preliminary evaluation, it can be concluded that the process for the simultaneous production of biodiesel and free lutein could be achieved technically and economically. Nevertheless, an investigation into process optimization, such as minimizing the soap formation that lowers the products yields and improving the product separation process, should be in the focus of future studies.

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References

[1] Y. Chisti, *Biotechnol. Adv.* **2007**, *25*, 294.
[2] J. Ferrell, V. Sarisky-Reed, *National Algal Biofeuels Technology Roadmap*, US Dept. Energy, Office of Energy Efficiency and Renewable Energy, Washington, DC **2010**.
[3] S. A. Scott, M. P. Davey, J. S. Dennis, I. Horst, C. J. Howe, D. J. Lea-Smith, A. G. Smith, *Curr. Opin. Biotechnol.* **2010**, *21*, 277.
[4] A. Converti, A. A. Casazza, E. Y. Ortiz, P. Perego, M. Del Borghi, *Chem. Eng. Process.* **2009**, *48*, 1146.
[5] X. M. Shi, X. W. Zhang, F. Chen, *Enzyme Microb. Technol.* **2000**, *27*, 312.
[6] J. Y. Lee, C. Yoo, S. Y. Jun, C. Y. Ahn, H. M. Oh, *Bioresour. Technol.* **2010**, *101*, 75.
[7] C. Prommuak, P. Pavasant, A. T. Quitain, M. Goto, A. Shotipruk, *Eng. J.* **2012**, *16*, 157.
[8] D. T. Tran, K. L. Yeh, C. L. Chen, J. S. Chang, *Bioresour. Technol.* **2012**, *108*, 119.
[9] L. Gouveia, A. C. Oliveira, *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 269.
[10] Z. Wu, S. Wu, X. Shi, *J. Food Process Eng.* **2007**, *30*, 174.
[11] D. Ruen-ngam, A. Shotipruk, P. Pavasant, S. Machmudah, M. Goto, *Chem. Eng. Technol.* **2012**, *35*, 255.
[12] H. B. Sowbhagya, S. R. Sampathu, N. Krishnamurthy, *Food Rev. Int.* **2004**, *20*, 33.
[13] J. Vechpanich, A. Shotipruk, *Sep. Sci. Technol.* **2011**, *46*, 265.

[14] P. Roberta, M. Marotti, S. Grandi, *Ind. Crops Prod.* **1998**, *8*, 45.
[15] M. G. Montes D'Oca, C. V. Viêgas, J. S. Lemões, E. K. Miyasaki, J. A. Morón-Villarreyes, E. G. Primel, P. C. Abreu, *Biomass Bioenergy* **2011**, *35*, 1533.
[16] Y. Li, S. Lian, D. Tong, R. Song, W. Yang, Y. Fan, R. Qing, C. Hua, *Appl. Energy* **2011**, *88*, 3313.
[17] S. Hatano, K. Kabata, M. Yoshimoto, H. Sadakane, *Plant Physiol.* **1982**, *70*, 1173.
[18] L. Gouveia, V. Veloso, A. Reis, H. Fernandes, J. Novais, J. Empis, *Bioresour. Technol.* **1996**, *57*, 157.
[19] H. B. Li, F. Chen, T. Y. Zhang, F. Q. Yang, G. Q. Xu, *J. Chromatogr. A* **2001**, *905*, 151.
[20] H. B. Li, Y. Jiang, F. Chen, *J. Agric. Food Chem.* **2002**, *50*, 1070.
[21] N. E. Craft, *J. Agric. Food Chem.* **1992**, *40*, 431.
[22] P. T. Pienkos, A. Darzins, *Biofuels Bioprod. Biorefin.* **2009**, *3*, 431.
[23] D. H. Lee, *Bioresour. Technol.* **2011**, *102*, 43.
[24] F. Delrue, P. A. Setier, C. Sahut, L. Cournac, A. Roubaud, G. Peltier, A. K. Froment, *Bioresour. Technol.* **2012**, *111*, 191.

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Microwave pretreatment of defatted rice bran for enhanced recovery of total phenolic compounds extracted by subcritical water

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HIGHLIGHTS

- Microwave pretreatment of defatted rice bran prior to SWE gave enhanced TP yields.
- Suitable pretreatment condition was 80 °C, 10 min and rice bran:water ratio of 1:2.
- SWE of microwave pretreated defatted rice bran gave up to 55% enhanced TP yield.
- Time required for SWE at 200 °C of pretreated bran was reduced from 30 to 10 min.
- Microwave pretreated rice bran gave extracts with increased antioxidant activity.

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ABSTRACT

Enhanced recovery of total phenolics (TP) from defatted rice bran (DRB) subjected to prior microwave pretreatment was achieved by subcritical water extraction (SWE). The effects of microwave pretreatment temperature (60–100 °C) and duration (0–30 min) were determined at raw material:water ratios (1:2 and 1:5) for SWE under fixed conditions. Optimal extraction was observed at 80 °C (for 10 min, at a ratio of 1:2). With pretreatment carried out under these conditions, a shorter extraction time of 10 min was required for SWE at 200 °C. Combining both optimized conditions, a TP yield of 190.4 ± 3.3 mg/g of DRB was achieved, some 55% more than was found to be extractable from un-pretreated samples. The antioxidant activity of the extract was also greater, as indicated by a corresponding decrease in IC_{50} from 38.8 ± 0.4 to 27.7 ± 0.5 μg/ml.

1. Introduction

Rice bran is a source of protein, oil, nutrients, energy and important antioxidants such as vitamin E (tocopherols and tocotrienols), gamma-oryzanol, and phenolic compounds. These substances suppress chronic diseases of the cardiovascular system, help quench free radicals and exhibit anti-cancer activity (Abdul-Hamid et al., 2007; Renuka Devi and Arumughan, 2007). As the sixth largest rice producer in the world, Thailand generates approximately 3 million tons of rice bran annually as a by-product of rice milling. About 75% of this rice bran is used as animal feeds, and only 15% is used in the process of edible oil extraction and production of other food. The defatted rice bran (DRB) by-product has a high content of non-allergenic proteins as well as phenolic acids which are beneficial to human health (Sereewatthanawut et al., 2008; Pourali et al., 2010). Therefore the recovery of these value-added compounds from DRB has long been the focus of various research (Renuka Devi and Arumughan, 2007; Sereewatthanawut et al., 2008; Pourali et al., 2009, 2010; Wiboonsirikul et al., 2007; Watchararuji et al., 2008; Hata et al., 2008; Adachi et al., 2009; Sunphorka et al., 2012).

Extraction of active components from rice bran can be carried out using organic solvents such as methanol, ethanol or acetone (Chiou et al., 2009). Other methods include hydrolysis with alkali or acid solution or enzymatic extraction. Alkali extraction results in rather low yield of products due to degradation of the desired compounds at high pH (Sereewatthanawut et al., 2008) and the process usually generates large quantities of waste water. Enzymatic extraction is a milder technique and is much more environmentally benign. The process is, however, time consuming and the high cost of enzymes is problematic for the feasibility of this technique on a large scale.

Subcritical water extraction (SWE) is an alternative technique which employs water at temperature between its boiling point (100 °C) and its critical temperature (374.15 °C), kept at a

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sufficiently high pressure to sustain the liquid state. Under these conditions, water has drastically altered properties such as a lowered relative dielectric constant and an increased K_w (ion product). When the temperature of water increases from ambient to 250 °C, its relative dielectric constant drops from 80 to 27, a value comparable to that of acetone at ambient temperature (Wiboonsirikul et al., 2007). The increased ionization of superheated water generates extra hydronium and hydroxide ions which catalytically accelerate hydrolysis reactions (Watchararuji et al., 2008).

A number of studies have been conducted on SWE of DRB for the recovery of valuable substances. Early work focused on the extraction of protein, amino acids and reduced sugars (Sereewatthanawut et al., 2008; Watchararuji et al., 2008; Hata et al., 2008), while subsequent studies revealed an abundance of phenolic compounds including caffeic, ferulic, gallic, gentisic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, vanillic acids and vanillin. Compared with conventional organic solvent extraction, SWE has been shown to be particularly favorable for recovering phenolic compounds since it is capable of releasing compounds that are known to extensively bind to carbohydrate lignin complexes in the cell walls (Pourali et al., 2010, 2009). Nevertheless, the need to pressurize the water means that SWE is often viewed as a relatively expensive technique. As a consequence, there is now great interest in both optimizing and economizing SWE, so that the full potential of this technique can be harnessed.

Various biological, physical and chemical methods of sample pretreatment have been applied in attempts to enhance the hydrolysis of lignocellulosic materials by increasing the surface area of these biopolymers (Hu and Wen, 2008; Liu and Cheng, 2010; Remond et al., 2010). However, each of these methods presents some drawbacks. For example, biological enzymatic methods require long treatment times, while physical treatments (e.g. chipping, milling, and grinding) are energy-intensive and are too costly to be scaled up. Physicochemical processes (e.g. steam explosion), on the other hand, require unfeasibly high pressures and temperatures, whereas chemical processes (e.g. dilute acid, alkali, using organic solvent, etc.) often involve toxic and environmentally damaging compounds (Ha et al., 2011).

As an alternative, microwave pre-heating offers the advantage of shorter required treatment times, accompanied by a consequent reduction in energy consumption (Gong et al., 2010; Liu et al., 2010). In this study, we attempted to enhance the recovery of TP by employing microwave pretreatment of DRB prior to SWE. To this end, we sought to evaluate the effects of pretreatment condition variables including temperature, time and the raw material-to-water ratio on the recovered yield of TP and to evaluate the antioxidant activity of the extracts obtained. Once the optimal pretreatment conditions were determined, the effects of varying the SWE conditions would then also be investigated. Finally, the structural morphologies of the DRB, the microwave pretreated rice bran and the solid residue obtained after SWE were all examined under a scanning electron microscope (SEM).

2. Methods

2.1. Materials and chemicals

DRB was obtained from Thai Edible Oil Co. Ltd., Ayuthaya, Thailand. The reference standards (gallic acid), Folin–Ciocalteau reagent, and ABTS were purchased from Sigma–Aldrich Chemicals (Missouri, USA). All water used had been distilled.

2.2. Microwave pretreatment

A microwave system from CEM Corp. (Matthews, NC, USA) was used in our experiments, consisting of 12 × 100 ml closed polyetheretherketone (PEEK) vessels covered with special TFM sleeves, a power sensor, a temperature sensor, and a temperature controller of MARS 5™. Pretreatment was carried out as follows: DRB (1 g) was placed in each vessel and mixed with 2 or 5 ml distilled water. The rice bran-to-water ratio of 1:5 was selected because it had previously been shown to be favorable for the SWE of DRB (Watchararuji et al., 2008). A lower water ratio of 1:2 was later employed to evaluate the possibility for reducing energy requirements during the microwave pretreatment. The vessels were closed and then arranged symmetrically in the microwave field. Irradiation pretreatment was then carried out for the specified temperature and time, after which bran samples were removed from the vessels. It should be noted that, for each run, the pretreatment time refers to the holding time at the desired pretreatment temperature, which in all cases took 5 min to be reached. Thus, a pretreatment time of 0 min implies that pretreatment was allowed to take place during the heating up process and was stopped as soon as the set temperature was reached.

After pretreatment, the reactor was submerged in a cooling water bath for 5 min, after which the liquid and solid contents of the reaction products were separated with a centrifuge and stored at 4 °C. The liquid was analyzed for the amount of TP released during the pretreatment, and the solid was subjected to SWE. In the first part of this study (determining the effect of pretreatment temperature and time on the extract's TP content), the extraction temperature remained fixed at 200 °C and the extraction time at 30 min. In the second part of the study, the pretreatment temperature and time were held constant at these optimized values to allow the subsequent determination of the best conditions for SWE. All experiments were conducted in duplicate.

2.3. SWE

SWE of DRB was conducted in an 8.8 mL SUS-316 stainless steel closed batch reactor (AKICO Co., Japan). In each run, microwave pretreated DRB (1 g) and distilled water (5 ml) were charged into the reactor. The reactor was then electrically heated to the desired temperature, 180–220 °C, which generally took 15–20 min. After a holding period of 10–30 min at the desired reaction temperature (referred to as the reaction time), the reactor was rapidly cooled to room temperature by submerging it in an ambient temperature water bath. The DRB residue was separated from the soluble liquid portion of the reaction products using a filter paper (Whatman No. 1). The liquid and solid contents in the reactor were collected and the remaining solid in the reactor was washed with distilled water (5 ml). These washings were then combined with the bulk of the aqueous extractant and samples from this mixture were then assayed for TP levels and antioxidant activity. It should be noted that the yields reported in this study indicate the sum of TP obtained from both the liquid portion obtained after microwave pretreatment and the combined soluble SWE extracts. IC_{50} values reported represent the average TP concentration of the two liquid portions.

2.4. Analytical methods

The TP content of aqueous extractant mixtures was determined by the Folin–Ciocalteau method, modified from that described in a previous study (Rodriguez-Meizoso et al., 2006) and calculated on the basis of a gallic acid calibration curve.

The antioxidant activity of the extracts was tested using the ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) method (Re et al., 1999). For the purpose of comparing the antioxidant activity of various extracts, the percent inhibition (PI) was measured (see Eq. (1)). To find this value, the concentrated extract was sequentially diluted with distilled water and each diluted

extract was added into $ABTS^{\cdot+}$ solution (mixture of 7 mM ABTS and 2.45 mM potassium persulfate having absorbance of 0.70 ± 0.02 at 734 nm) with a volume ratio of 1:10 (sample solution:ABTS solution). The solutions were mixed using a vortex and then incubated in the dark at room temperature for 10 min, after which the absorbance (734 nm) was measured using distilled water as a reference. The value of percent inhibition (PI) was then calculated for each reading using the following equation:

$$PI(\%) = [1 - (A_t/A_r)] \times 100 \quad (1)$$

where A_t and A_r are the absorbances of the test sample and of the $ABTS^{\cdot+}$ reference, respectively. Specifically, the concentration of antioxidant sample required to produce a 50% reduction in the radial $ABTS^{\cdot+}$ absorbance was determined. These values were plotted against sample concentration and linear regression of the data was made and used to determine the IC_{50} value.

The mophological structures of all rice bran samples were observed under a Supra 25 scanning electron microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) at an accelerating voltage of 15 kV.

3. Results and discussion

3.1. Determination of suitable microwave pretreatment conditions

3.1.1. Effects of microwave pretreatment temperature and time on TP yield

The effects of pretreatment temperature and duration on TP yields are shown in Fig. 1 for the sample whose raw material-to-water ratios were 1:2 and 1:5. All samples were subjected to SWE at 200 °C for 30 min. Compared with the TP yields obtained from the un-pretreated DRB (dashed lines), microwave pretreatment is seen to significantly enhance the TP content of the extracts. It is likely that microwave energy is delivered efficiently to materials through interaction with the electromagnetic field and offers a rapid transfer of energy to both the extraction solvent and the sample of interest (Hayat et al., 2009). This rapid energy transfer could cause structural and morphological changes to the plant tissues making them more accessible to the extraction solvent (Hu and Wen, 2008).

For both raw material-to-water ratios, the TP yield significantly increased with pretreatment time, showing the greatest yield of TP for 10 min of pretreatment for both water ratios studied (Fig. 1). As the pretreatment time was prolonged further, the TP yields thereafter fell steadily. Structural morphological changes of the rice bran sample caused by long exposure to microwave over a certain limit could make it susceptible to harsh SWE conditions, resulting in the degradation of TP at long pretreatment time.

Considering the effect of pretreatment temperature, for the sample whose raw material-to-water ratios was 1:2, Fig. 1(a) indicates that the TP yields of the extracts increased slightly as the pretreatment temperature was increased from 60 to 80 °C. The highest TP yield of 169.3 ± 1.9 mg/g of DRB was obtained for the pretreated sample at 80 °C, a 38% increase from the 122.8 ± 1.8 mg/g obtained without any pretreatment. TP yields were seen to decrease considerably, however, when the temperature was raised to 100 °C. This drop in TP yield could, again possibly be due to TP decomposition at the high extraction temperature (200 °C) rather than the pretreatment itself, although the higher pretreatment temperature could also have contributed to the extent of degradation. The results for pretreatment at raw material-to-water ratio of 1:5, shown in Fig. 1(b), were slightly different, in that the TP yield continued to improve as the temperature was increased from 60 all the way to 100 °C.

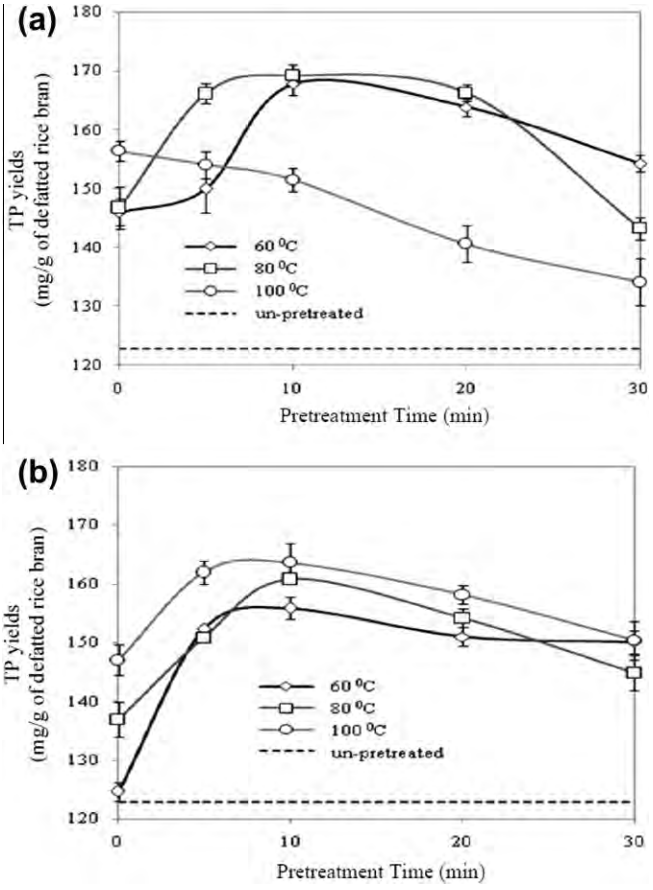

Fig. 1. Effects of microwave pretreatment temperature and time on TP yields obtained from SWE at 200 °C for 30 min, raw material-to-water ratio (a) 1:2, (b) 1:5.

3.1.2. Effects of pretreatment temperature and time on antioxidant activity

The antioxidant activity of the extracts, was evaluated with an $ABTS^{\cdot+}$ scavenging assay. The IC_{50} values of the extracts at various pretreatment temperatures and times (Fig. 2) are the average of values of the liquid samples obtained after microwave pretreatment and after SWE for raw material-to-water ratios of 1:2 (Fig. 2(a)) and 1:5 (Fig. 2(b)). The IC_{50} values of the extracts obtained from SWE of the pretreated sample at 60 °C were not significantly different from those from un-pretreated samples. On the other hand, the IC_{50} values of the extracts of the DRB pretreated at higher temperature (80 and 100 °C) were lower in most cases, indicating stronger antioxidant activities. At high pretreatment temperature, it is possible that hydrolysis occurred to such an extent that antioxidative compounds such as phenolics could be released more easily. Considering the effect of pretreatment time on the IC_{50} values of extracts, there seems to be some interdependency between the pretreatment time and temperature and the antioxidant activity. For instance, at the highest pretreatment temperature of 100 °C, the IC_{50} values of the extract increased as the pretreatment time was extended to 30 min, producing the extract the lowest antioxidant activity. This decrease in the antioxidant activity was probably caused by the prolonged exposure to microwave irradiation at high pretreatment temperature, causing some oxidative compounds in the plant tissue to become susceptible to degradation during SWE.

It should be noted that there was no clear relation between the TP yields and the antioxidant activities of the extracts. For example, although the highest antioxidant activity (IC_{50} = 31.3 ± 0.5 μg/ml) was obtained at 100 °C for 10 min with 1:2 raw

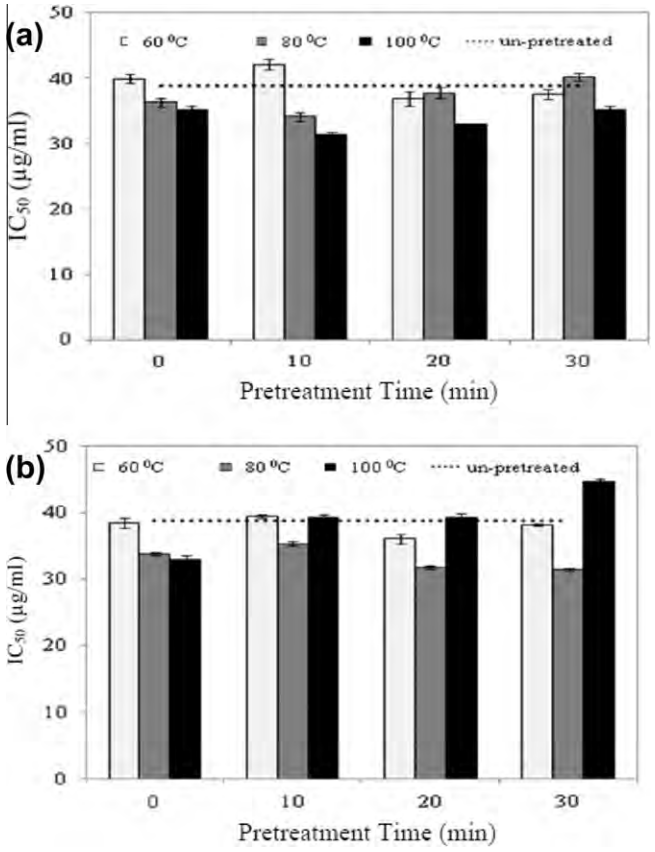

Fig. 2. Effects of pretreatment temperature and time on antioxidant activity for extracts obtained from SWE at 200 °C for 30 min, raw material-to-water ratio (a) 1:2, (b) 1:5.

material-to-water ratio, the pretreatment condition at 80 °C for 10 min and raw material-to-water of 1:2 gave only a slightly lower antioxidant activity (IC_{50} = 34.1 ± 0.6 µg/ml) but considerably higher TP yields (169.3 ± 1.9 versus 151.5 ± 2.0 mg/g of DRB). This discrepancy could arise from the presence of antioxidative compounds other than phenolics in the extracts. Nevertheless, due to its higher TP yields, the latter condition was chosen as the preferred pretreatment condition, and was henceforth employed throughout subsequent optimization of SWE conditions.

3.2. Determination of suitable SWE condition

When determining the effects of pretreatment conditions previously, the fixed condition of SWE at 200 °C and 30 min was used based our previous study which adopted it as the most suitable condition for extraction of un-pretreated DRB (Sereewatthanawut et al., 2008). Nevertheless, we deemed as necessary a complete reevaluation of suitable SWE conditions for our new pretreated samples.

3.2.1. Effects of temperature and time of SWE

Fig. 3 shows the effects of temperature and time on the TP yields of the subcritical water extracts of DRB (microwave pretreated at 80 °C for 10 min). The results indicate that the TP yields slightly increase as the temperature is raised from 180 to 200 °C. The increase in TP yield at elevated temperature is due to the enhanced hydrolysis activity caused by the raised water ionization constant (K_w) (Watchararuji et al., 2008). The maximum TP yield of 190.41 ± 3.34 mg/g of DRB was obtained at 200 °C, some 55% greater than that obtained with the un-pretreated rice bran. However, the TP yield sharply decreased as the temperature was increased further (to 220 °C), possibly due to the thermal degradation of the compounds at this extreme temperature. At all hydrolysis temperatures, TP yield was found to fall as the extraction time was extended from 10 to 30 min, again due to the degradation that probably arises from prolonged heating.

3.2.2. Effects of SWE temperature and time on antioxidant activity

At 200 and 220 °C, the antioxidant activity of the extracts obtained after SW hydrolysis time of 10 min (Fig. 4) corresponds well to the TP yield (Fig. 3). It therefore seems the antioxidant activity here appears to be mainly attributable to the phenolics content of the extract. Nevertheless, the antioxidant activity of DRB extract obtained at 180 °C was greater at longer extraction time, despite the significant expected TP decomposition during these longer experimental runs. Thus, there could here again be other contributions to the antioxidant activity of the extract from other non-phenolic oxidative compounds in rice bran. Although long exposure to high temperature is known to cause decomposition of antioxidative compounds, the antioxidant activities of the extracts did not considerably vary with extraction times in the range studied.

Apart from having high content of TP, the extract resulting from SWE at 200 °C for 10 min has a significantly lower IC_{50} (27.7 ± 0.5 µg/ml) than those obtained under most of the alternative conditions. Thus this condition was selected to be the optimum SWE condition. Compared with un-pretreated sample from previous research (Sereewatthanawut et al., 2008), in which the extracts having the highest antioxidant activities were those obtained at 200 °C after 30 min of SWE or 220 °C after 20 min, it can be concluded that microwave pretreatment of DRB could enhance the TP recovery and the extract antioxidant activity, while considerably shortening the SWE time and thereby saving energy.

3.3. Evaluation of sample morphology

The spherical bodies seen in the SEM pictures for the un-pretreated DRB (Supplementary Fig. S1a) and the DRB pretreated with microwave (Supplementary Fig. S1b) are globular protein. These protein globules are seen to have become slightly smaller in microwave pretreated sample as some protein has been dissolved into water during the pretreatment process.

The globular protein structures were no longer observed in the SW hydrolyzed samples (Supplementary Fig. S1c–d), indicating that they have been removed as a result of SWE. Interestingly, the surface of the DRB SW extracted samples appears to be more porous than the original DRB, implying that water had indeed penetrated into the rice bran structure during whilst dissolving some of the solid content of the bran. The residue of the microwave pretreated rice bran after SWE (Supplementary Fig. S1c), however,

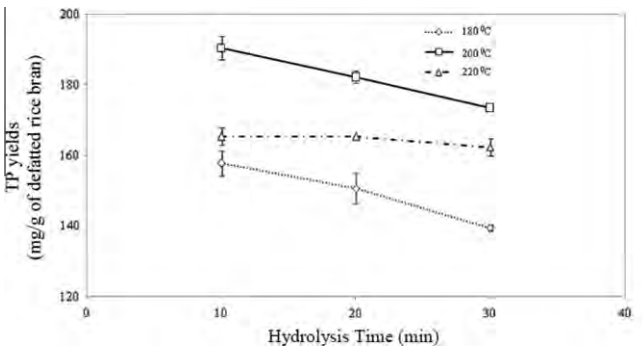

Fig. 3. Effects of SWE temperature and time on TP yield (Pretreatment condition: 80 °C, 10 min, 1:2 raw material-to-water ratio).

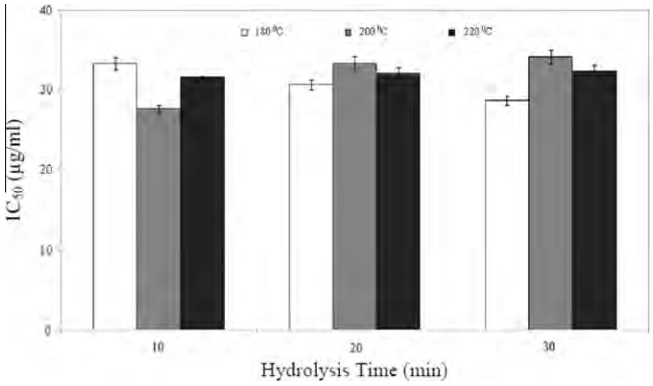

Fig. 4. Effects of SWE temperature and time on antioxidant activity (Pretreatment condition: 80 °C, 10 min, 1:2 raw material-to-water ratio).

seemed to possess higher crystalline structures of the insoluble rice bran fibers, compared with the un-pretreated DRB undergoing SWE (Supplementary Fig. S1d). This suggests that through structural changes of the sample, the microwave pretreatment might have made the sample more accessible to SW during extraction. Gong et al. (2010) have suggested that the structural changes occur as the microwave energy is absorbed and subsequently converted into heat. During this process, they claim, the moisture begins to evaporate, thereby generating pressure within the cell wall, which leads to cell rupture, and consequent loose and irregular surfaces. Although in our study, the structural changes were not observed clearly for microwave pretreated samples (Supplementary Fig. S1a and b), the SEM images of the pretreated sample after SWE showed that microwave irradiation played a role in the DRB surface modification, and therefore enhanced the hydrolysis reaction. Similar results have also been observed by other authors Hu and Wen, 2008 who studied physical structure changes of switchgrasses pretreated by microwaves.

4. Conclusions

Microwave pretreatment has been shown to increase the yields of TP and the antioxidant activity of the extracts obtained by SWE. The optimal pretreatment conditions were found to be 10 min irradiation at 80 °C with a 1:2 raw material-to-water weight ratio. Moreover, the reduction in time required for SWE suggested the potential application of microwaves as a means for pretreatment of plant samples for SWE in a larger scale. A detailed identification of all extraction products and investigations probing further potential energy reduction during microwave pretreatment (e.g. by using lower water ratios) would both be promising focuses of future study.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.08.053.

References

Abdul-Hamid, A., Raja Sulaiman, R.R., Osman, A., Saari, N., 2007. Preliminary study of the chemical composition of rice milling fractions stabilized by microwave heating. J. Food Compos. Anal. 20, 627–637.
Adachi, Sh., Neoh, T.L., Kobayashi, T., 2009. Subcritical water treatment of defatted rice bran to produce functional food materials. J. Biosci. Bioeng. 108, S141.
Chiou, T.Y., Neoh, T.L., Kobayashi, T., Adachi, S.H., 2009. Properties of extract obtained from defatted rice bran by extraction with aqueous ethanol under subcritical conditions. International Conference on Supercritical Fluid, Japan, pp. 2–324.
Gong, G., Liu, D., Huang, Y., 2010. Microwave-assisted organic acid pretreatment for enzymatic hydrolysis of rice straw. Biosystems. Eng. 107, 67–73.
Ha, S.H., Mai, N.L., An, Gw., Koo, Y.M., 2011. Microwave-assisted pretreatment of cellulose in ionic liquid for accelerated enzymatic hydrolysis. Bioresour. Technol. 102, 1214–1219.
Hata, S., Wiboonsirikul, J., Maeda, A., Kimura, Y., Adachi, Sh., 2008. Extraction of defatted rice bran by subcritical water treatment. Biochem. Eng. J. 40, 44–53.
Hayat, K., Hussain, S., Abbas, Sh., Farooq, U., Ding, B., Xia, Sh., Jia, Ch., Zhang, X., Xia, W., 2009. Optimized microwave-assisted extraction of phenolic acids from citrus mandarin peels and evaluation of antioxidant activity in vitro. Sep. Purif. Technol. 70, 63–70.
Hu, Z., Wen, Zh., 2008. Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment. Biochem. Eng. J. 38, 369–378.
Liu, Ch., Cheng, X., 2010. Improved hydrogen production via thermophilic fermentation of corn stover by microwave-assisted acid pretreatment. Int. J. Hydrogen Energy 35, 8945–8952.
Liu, J., Takada, R., Karita, Sh., Watanabe, T., Honda, Y., 2010. Microwave-assisted pretreatment of recalcitrant softwood in aqueous glycerol. Bioresour. Technol. 101, 9355–9360.
Pourali, O., Asghari, F.S., Yoshida, H., 2009. Sub-critical water treatment of rice bran to produce valuable materials. Food Chem. 115, 1–7.
Pourali, O., Asghari, F.S., Yoshida, H., 2010. Production of phenolic compounds from rice bran biomass under subcritical water conditions. J. Chem. Eng. 160, 259–266.
Re, R., Pellegrini, N., Protegenete, A.S., Pannala, A., Yang, M., Rice-Evans, C.A., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med. 26, 1231–1237.
Remond, C., Aubry, N., Cronier, D., Noel, S., Martel, F., Roge, B., Rakotoarivonina, H., Debeire, P., Chabbert, B., 2010. Combination of ammonia and xylanase pretreatments: impact on enzymatic xylan and cellulose recovery from wheat straw. Bioresour. Technol. 101, 6712–6717.
Renuka Devi, R., Arumughan, C., 2007. Phytochemical characterization of defatted rice bran and optimization of a process for their extraction and enrichment. Bioresour. Technol. 98, 3037–3043.
Rodrıguez-Meizoso, I., Marin, F.R., Herrero, M., Senorans, F.J., Reglero, G., Cifuentes, A., Ibanez, E., 2006. Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization. J. Pharmaceut. Biomed. Anal. 4, 1560–1565.
Sereewatthanawut, I., Prapinthip, S., Watchiraruji, K., Goto, M., Sasaki, M., Shotipruk, A., 2008. Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. Bioresour. Technol. 99, 555–561.
Sunphorka, S., Chavasiri, W., Oshima, Y., Ngamprasertsith, S., 2012. Kinetic studies on rice bran protein hydrolysis in subcritical water. J. Supercrit. Fluids 65, 54–60.
Watchararuji, K., Goto, M., Sasaki, M., Shotipruk, A., 2008. Value-added subcritical water hydrolysate from rice bran and soybean meal. Bioresour. Technol. 99, 6207–6213.
Wiboonsirikul, J., Hata, Sh., Tsuno, T., Kimura, Y., Adachi, Sh., 2007. Production of functional substances from black rice bran by its treatment in subcritical water. LWT Food Sci. Technol. 40, 1732–1740.

Subcritical Water Extraction of Resveratrol from Barks of *Shorea Roxburghii* G. Don.

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Running Head: Subcritical water extraction of resveratrol

Abstract

This study investigated subcritical water extraction of *trans*-resveratrol and its glycosylated form, *trans*-piceid, from barks of *Shorea roxburghii G. Don.* The effects of extraction temperature (100-190°C) and water flow rate (2-4 ml/min) at the pressure of 10 MPa were determined on the amounts of compounds extracted. The amount of *trans*-resveratrol and *trans*-piceid in the extracts increased with increasing temperature and flow rate, although the increase in flow rate from 3 to 4 ml/min had no significant effect on the extraction yield. Thus the flow rate of 3 ml/min was taken to be the most suitable. At suitable condition (190°C, 3 ml/min), the amount of *trans*-resveratrol and *trans*-piceid were 23.18 and 350.28 μg/g dry weight, respectively. With equal extraction duration, the amount of *trans*-resveratrol and *trans*-piceid compound obtained from extraction using 190°C subcritical water were higher than those from extractions with ambient water and 80% ethanol.

Key words: subcritical water extraction; *trans*-piceid; *trans*-resveratrol; *Shorea Roxburghii* G. Don.

1. Introduction

Shorea roxburghii G. Don. (*S. roxburghii*) is a plant in the Dipterocarpaceae family. It is found to be distributed in mixed deciduous forests or in evergreen forests of Asia, Africa, and South America (1). In Thailand, the plant is commonly known as Pha-Yom. All parts of Pha-Yom including barks, flowers, leaves, roots and seeds have been used for medicinal purposes as they contain several phenolic compounds particularly resveratrol oligomers (2). Resveratrol is found as a free form or glycosylated forms (known as piceid) (3), both free and bound resveratrol exists in *cis*- or *trans*- isomeric forms. *trans*-Resveratrol (TR) is known to possess various biological activity such as anticancer activity, antifungal activity, anti-inflammatory, anti-aging activity (4-5). In nature, resveratrol is found mostly in the form of *trans*-piceid (TP) (6-8) which has been shown to inhibit platelet aggregation (9) and lower serum cholesterol levels (10). Nevertheless, the bioavailability of the TP is much lower, compared with TR (11). In some studies, TP in the extract was converted into the TR via hydrolysis with β-Glucosidase enzyme to increase the quantities of biologically active TR (12). Nevertheless, TP has been reported to be absorbed more efficiently in human intestine than TR, due to the conjugation with glucose, which increases the hydrophilicity of the molecule (13). Moreover, TP can naturally be hydrolyzed to release TR in the digestive tract by glucosidase produced by human intestinal bacteria (14).

For extraction of TR and TP, various solvents have been investigated to determine the suitability for the compound recovery from various plants. Organic solvents such as 100% acetone, 80% ethanol, 100% ethyl acetate and 90% methanol have been applied for extraction of resveratrol from grape berry skins (15) and supercritical carbon dioxide extraction (SC-CO_2) has also been applied to extract resveratrol from dried grape, including grape pomace, seed, stem and skin (16). SC-CO_2 was shown not to be suitable for extraction of polar analytes such as TR and TP without use of organic solvent modifier. Alternatively, subcritical

water extraction (SWE) has become an interesting technique for extraction of herbal plants in recent years. In this process, hot water at temperatures between boiling and critical point under sufficient pressure to maintain the liquid state is used as an extraction solvent. SWE has been shown to be a promising alternative for the conventional method of extraction of polar and slightly non-polar compounds. Besides being an environmentally friendly technique, other advantages of SWE include its simplicity, shorter extraction time, low cost of the extracting agent. Examples of phytochemicals that have been extracted from plants by using SWE were lactone from kava root (17), total phenolic contents from *Chaga Mushroom* (18), terpinene-4-ol and (E)-1-(3,4-dimethoxyphenyl) butadiene from *Zingiber cassumunar* (19) and nutraceutical compounds from *Citrus Pomaces* (20).

In this study, the suitable condition for SWE of TR and TP from *S. roxburghii* barks was determined. The effects of water flow rate and extraction temperature on the amount of TR and TP contents were considered and the results were compared with those of the extracts obtained by other extraction methods such as Soxhlet and stirred vessel water, ethanol and 80% ethanol extraction.

2. Material and methods

2.1 Materials

The barks of *S. roxburghii* used in this study were from the plants grown locally in northeast of Thailand. TR and TP were obtained from Sigma Chemical Co. (St Louis, Mo, USA.). Water used in the experiments was distilled and deionized water. AR grade acetic acid, HPLC grade ethanol and acetonitrile, were purchased from Merck (Darmstadt, FR Germany).

2.2 Sample preparation

The barks of *S. roxburghii* were dried in an oven at 50°C about 1 day until it reached a constant weight. The dried sample was crushed into fine powder using moulinex blender. The powdered samples were sieved through a mesh of size 500 micrometer. The sample (0.2-0.5 mm) was then stored at 4°C in a domestic refrigerator until use.

2.3 SWE

SWE experiments were carried out to determine the effects of extraction temperature (100-190 C) and extraction flow rates (2-4 ml/min) on the amount of TR and TP exracted. The experiments were performed using an apparatus shown in Fig. 1. The extraction system consisted of two HPLC pumps (PU 980, JASCO, Japan), a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS, Germany), an extraction vessel (10 ml, Thar Design, USA), a pressure gauge, and a back pressure regulator value (AKICO, Japan), a heating cable and a self-made temperature controller. All connections were made with stainless steel capillaries (1/16 inch outside diameter).

Distilled water was pumped, first, through a degassing equipment to remove dissolved oxygen. The degassed water was then passed through the tubing which was wrapped with a heating cable used to preheat the water before it entered the oven. The water was further preheated through a preheating coil, made from 3 m stainless steel tubing, installed inside the oven before it entered the extraction vessel, which was preloaded with 0.5 g of sample. The back pressure regulator placed at the outlet of the extraction system was used to maintain the system pressure to ensure that the water was in liquid state at all temperatures employed in the experiment. Before starting the extraction, all connections were checked for possible leakage. To prevent the line behind the extraction vessel from clogging, the second pump was then turned on to deliver degassed water at constant flow rate of 1 ml/min to wash off any

residual product that may precipitate within the line exiting the extractor. The extract was cooled in a coil immersed in a water bath to prevent possible product degradation, before being collected in fractions in sample collecting vials. The samples were taken at every 30 min interval during the first 2 h, and then every 60 min onwards. The extracts were then evaporated by a vacuum evaporator (R-210/R-215 Rotavapor®, Buchi Co. Ltd, Switzerland) to reduce the water volume to about 10 ml. Each of the extract was thoroughly passed through a syringe filter, in which a 13 mm nylon filter (with a 0.45 µm pore size diameter) was placed inside a polypropylene housing. The filtered samples were stored at 4°C until being analyzed by HPLC.

2.4 Extractions with water and ethanol at ambient pressure

Water and ethanol extractions were carried out both in a stirred vessel and in a Soxhlet apparatus to compare the results with SWE. In a stirred vessel, 0.5 g of ground *S. roxburghii* barks was extracted with 200 ml of water or ethanol for 1 hour. For extraction in Soxhlet apparatus, 0.5 g of ground *S. roxburghii* barks was placed into a thimble and was extracted with 200 ml of distilled water or 80% of ethanol for 2 hours. The extraction time of 1 and 2 hours were used here as they were found to give the highest yields for both methods, respectively. Each extract was then evaporated under vacuum to about 10 ml. The concentrated extract was stored at 4°C until analysis.

2.5 HPLC analysis of *S. roxburghii* extract

The analysis of TR and TP were determined using High Performance Liquid Chromatography (HPLC) following the modified method of Rudolf et al. (21). The HPLC were performed with a C_{18} Inertsil ODS-3 column (250 x 4.6 mm ID, 5 µm particle size), equipped with a photodiode array (PDA) detector. The PDA absorbance was monitored at

307 nm. The mobile phase consists of solvent A (0.1% acetic acid in water) and solvent B (100% acetonitrile, HPLC grade). The gradient system started with 5% solvent B at 0 min and was changed to 37% solvent B at 23 min, then followed by an increase of solvent B to 72% over 5 min, with the total run time of 30 min. All the sample extract injection volume was 20 µL. The flow rate was set at 1.5 ml/min and the column temperature was maintained at 25°C throughout the test. The concentrations of TR and TP in the sample were determined using standard calibration curves, constructed from a plot of peak areas versus concentrations for a series of standard solutions whose concentrations range from 0.1-10 µg/ml for TR and 1-25 µg/ml for TP, respectively. The chromatogram of the extract is shown in Fig. 2, which suggested that the retention times of TP and TR were 19.6 and 24.8 min, respectively.

3. Results and Discussion

3.1 Effect of extraction temperature

The effects of extraction temperature on the amounts of TR and TP were investigated in the range of 100-190°C. A constant water flow rate of 2 ml/min was used and the extraction was carried out at a constant pressure of 10 MPa. Fig. 3 a and b respectively show the amount of TR and TP extracted at extraction duration from 0 to 360 min. The amount of TR in the *S. roxburghi* bark extracts (0.68-13.01 µg/g dry weight) was found to be considerably smaller than that of TP (61.37-301.70 µg/g dry weight). The extraction yield of both TR and TP increased as temperature increased up to 190°C. Despite that the high temperature could cause compound degradation, SWE, in many cases (especially for phenolic compounds extraction), was reported to give higher yield. This was possibly due to the increase in the ionization constant of water at high temperatures, which enhances the hydrolysis of larger and complex lignin structures in the cell wall into the smaller aromatic compounds including phenolic compounds (22-23). In addition, the increase in extraction yield with temperature is

due to the fact that the polarity of water decreases with temperature, resulting in increased solubility of TP in water. It is noted that dielectric constant (ε) of subcritical water at 190°C is relatively close to that of 80% ethanol at 25°C (24), the concentration of ethanol which was reported to give the highest yield for TR and TP extraction (25-26).

3.2 Effect of water flow rates

The effect of water flow rate on the amount of TR and TP extracted was investigated by varying the flow rate from 2 to 4 ml/min at a fixed extraction temperature of 190°C and a pressure of 10 MPa. The results are presented in Fig. 4 which shows that the TR and TP extracted increased with increasing flow rate. The profiles in Fig. 4 for various flow rates can be divided into two regions, i.e. (i) the initially high extraction rate region between 0-30 min in which the concentration gradient of the extracted solutes across the surface of the *S. roxburghii* sample was high, and (ii) the subsequent lower extraction rate region. For TR, the initial extraction rates increased with increasing flow rate. This can be explained by the fact that the mass transfer rate depends principally on two main factors, mass transfer coefficient (k) and the concentration different driving force (ΔC) according the following equation (27)

$$N = k\Delta C = k\left(C_s - C_o\right) \quad (1)$$

Where N is the molar flux of component, mole/area.time, k is the mass transfer coefficients, m/s, C_s is the concentration of the solute in the solid plant sample, mol/m^3 and C_o is the solute concentration in bulk solution, mol/m^3. In a laminar flow region which is the case in this work here, the quantity 'k' generally varies with water flow rate.

For TP extraction on the other hand, the extraction rate was found to have different behavior. In this case, the initial rate did not seem to vary with the variation in 'k' which

suggested that the initial extraction process was not limited by mass transfer, but perhaps to a more significant level by the driving force or in this case, the concentration difference (ΔC). This is possible as during the initial period, a large amount of TP still stayed inside the solid matrix, resulting in large difference between the concentrations in the solid matrix and in the bulk fluid. The extraction at the later time of 30-360 min showed much clearer difference in the mass transfer rates at various flow rates, which indicates that the mass transfer coefficient, k, started to play a more important role. As time passed, the solid phase concentration started to fall which rendered the concentration gradient small, and this is when the rate of extracted TP became limited by the mass transfer, and the rate increased with the increasing flow rate. It is noted from the figure that the amount of TP extracted still increased after 360 min. This was probably due to the effect of enhanced extraction with SW. Nevertheless, with the data up to 360 min, general trends on the effects of different variables could be drawn. In practice, the proper flow rate must be chosen such that it would result in shorter extraction time and higher concentration of the extract. Since TP was present in a much larger amount compared with TR, the determination of the most suitable flow rate was based on the TP results. Since the extraction rate of TP did not significantly increased with increasing flow rate from of 3 to 4 ml/min, the flow of 3 ml/min was taken to be the most suitable.

3.3 Comparison of SWE and other extraction methods

In the present study, comparisons were made between the SWE and the other extraction methods such as conventional water and ethanol extractions using a stirred vessel and Soxhlet apparatus. The results are summarized in Table 1. Extraction in a stirred vessel at 70°C and Soxhlet extraction at 78°C indicated that ethanol and 80% ethanol were better solvents for TP and TR than water. These results were similar to previous study which demonstrated that 80% ethanol was the best solvent for TP and TR extraction from Grape Berry Skin (15).

Nevertheless, the extraction yield can be increased for water extraction at higher temperature as can be seen by the results obtained with Soxhlet extraction which took place at 100°C. SWE at 100°C however gave lower amount of TP compared with Soxhlet extraction with water methods carried out at the same temperature. This was possibly because in the system used for SWE did not allow loading of the barks sample after the preset extraction temperature was reached, instead the sample must be loaded into the vessel from the beginning. The sample therefore was subjected to heating which took approximately 45 min before actual extraction started, thus leading to the compound degradation. SWE at 190°C however resulted in higher amounts of TP and TR compared with the other extraction methods. This is probably because the increase in TP and TR solubility and the degree of hydrolysis caused by high amount of water ions produced at this condition could exceed the negative effect from degradation.

Conclusions

The results in this study demonstrated that *S.roxburghii* bark is a good source of TR and TP and could be extracted with subcritical water, which is a suitable and environmentally benign solvent. The amount of TR and TP in the extracts increased with increasing temperature and flow rate. The maximum amounts of TR and TP were 23.18 and 350.28 μg/g DW, respectively, obtained at the extraction temperature of 190°C and the water flow rate of 3 ml/min. The extracts obtained by 190°C subcritical water contained higher amount of TR and TP than those obtained by ambient water, ethanol and 80% ethanol using either Soxhlet apparatus or a stirred vessel.

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References

1. Sasaki, S. (2008) Physiological characteristics of tropical rain forest tree species: a basis for the development of silvicultural technology. *Proc. Jpn. Acad. Ser. B-Phys Biol. Sci*., 84(2): 31-57.
2. Ito, T.; Furusawa, M.; Iliya, I.; Tanaka, T.; Nakaya, K.I.; Sawa, R.; Kubota, Y.; Takahashi, Y.; Riswand, S.; Iinumab, M. (2005) Rotational isomerism of a resveratrol tetramer, shoreaketone, in shorea uliginosa. *Tetrahedron Lett.*, 46(17): 3111–3114.
3. Patcharamun, W.; Sichaem, J.; Siripong, P.; Khumkratok, S.; Jong-aramruang, J.; Tip-pyang, S. (2011) A new dimeric resveratrol from the roots of shorea roxburghii. *Fitoterapia*, 82(3): 489-492.
4. Ito, T.; Tanaka, T.; Nakaya, K.; Iinuma, M.; Takahashi, Y.; Naganawa, H.; Ohyama, M.; Nakanishi, Y.; Bastow, K.F.; Lee, K.H. (2001) A novel bridged stilbenoid trimer and four highly condensed stilbenoid oligomers in vatica rassak. *Tetrahedron Lett*., 57(34): 7309-7321.
5. Pace-Asciak, C.R.; Hahn, S.; Diamandis, E.P.; Soleas, G.; Goldberg, D.M. (1995) The red wine phenolic trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: Implication for protection against coronary heart disease. *Clin. Chim. Acta.*, 235(2): 207-219.

6. Zhou, J.J.; Zhang, H.J.; Yang, P.J.; Li, H.N. (2002) Determination of resveratrol glucoside and resveratrol in radix and rhizome of polygonum cuspidatum yielded in Hanzhong Region. *Chinese Traditional and Herbal Drugs,* 33: 414-416.

7. Jerkovic, V.; Brohan, M.; Monnart, E.; Nguyen, F.; Nizet, S.; Collin, S. (2010) Stilbenic profile of Cocoa liquors from different origins determined by RP-HPLC-APCI (+)-MS/MS. Detection of a new resveratrol hexoside. *J. Agric. Food Chem.,* 58(11): 7067-7074.

8. Benova, B.; Adam, M.; Pavlikova, P.; Fischer, J. (2010) Supercritical fluid extraction of piceid, resveratrol and emodin from japanese knotweed. *J. Supercrit. Fluids,* 51(3): 325-330.

9. Orsini, F.; Pelizzoni, F.; Verotta, L.; Aburjai, T. (1997) Isolation, synthesis, and antiplatelet aggregation activity of resveratrol 3-O-D-glucopyranoside and related compounds. *J. Nat. Prod.,* 60: 1082-1087.

10. Du, J.; Sun, LN.; Xing, W.W.; Huang, B.K.; Jia, M.; Wu, J.Z.; (2009) Liquid lowering effects of polydatin from polygonum cuspidatum in hyperlipidemic hamsters. *Phytomedicine*, 16: 652-658.

11. Meng, X.F.; Maliakal, P.; Lu, H.; Lee, M.J.; Yang, C.S. (2004) Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. *J. Agr. Food Chem.,* 52(4): 935-942.

12. Todaro, A.; Palmeri, R.; Barbagallo, R.N.; Pifferi, P.G.; Spagna, G. (2008) Increase of trans-resveratrol in typical Sicilian wine using β-glucosidase from various sources. *Food Chem.,* 107(4): 1570–1575.

13. Paganga, G.; Rice-Evans, C.A. (1997) The identification of flavonoids as glycosides in human plasma. *FEBS Lett.*, 401(1): 78-82.

14. Niu, P.Q.; Guo, C.Y. (2006) Investigative development of pharmacology of resveratrol. *Herald Med*., 6: 524-525.

15. Ana, I.R.P.; Rosa, M.L.R.; Cristina, A.L.; Torre-Boronat, M.C. (2001) Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J. Agr. Food Chem*., 49(1): 210-215.

16. Casas, L.; Mantell, C.; Rodriguez, M.; Martinez de la Ossa, E.J.; Roldan, A.; Ory, I.; Caro, I.; Blandino, A. (2010) Extraction of resveratrol from the pomace of palomino fino grapes by supercritical carbon dioxide. *J. Food Eng.*, 96(2): 304–308.

17. Kubátová, A.; Miller, D.J.; Hawthorne, S.B. (2001) Comparison of subcritical water and organic solvents for extracting kava lactones from kava root. *J. Chromatogr. A*, 923(1-2): 187-194.

18. Seo, H.K.; Lee, S.C. (2010) Antioxidant activity of subcritical water extracts from chaga mushroom (Inonotus obliquus). *Separ. Sci. Technol.*, 45(2): 198-203.

19. Chienthavorn, O.; Poonsukcharoen, T.; Pathrakorrn, T. (2011) Pressurized liquid and superheated water extraction of active constituents from zingiber cassumunar roxb. rhizome. *Separ. Sci. Technol.*, 46(4): 616-624.

20. Kim, J.W.; Nagaoka, T.; Ishida, Y.; Hasegawa, T.; Kitagawa, K.; Lee, S.C. (2009) Subcritical water extraction of nutraceutical compounds from *citrus pomaces. Separ. Sci. Technol.*, 44(11): 2598-2608.

21. Rudolf, J.L.; Resurreccion, A.V.A.; Saalia, F.K.; Phillips, R.D. (2005) Development of a reverse-phase high-performance liquid chromatography method for analyzing *trans*-resveratrol in peanut kernels. *Food Chem.*, 89(4): 623–638.

22. Budrat, P.; Shotipruk, A. (2009) Enhanced recovery of phenolic compounds from bitter melon (*Momordica charantia*) by subcritical water extraction. *Sep. Purif. Technol*., 66(1): 125–129.

23. Wahyudiono; Sasaki, M.; Goto, M. (2008) Recovery of phenolic compounds through the decomposition of lignin in near and supercritical water. *Chem. Eng. Process.*, 47(9-10): 1609-1619.

24. Lide, D.R. (2005) *CRC Handbook of Chemistry and Physics*, 1stEd.; CRC Press: Boca Raton, Florida, USA.

25. Cho, Y.J.; Hong, J.Y.; Chun, H.S.; Lee, S.K.; Min, H.Y. (2006) Ultrasonication-assisted extraction of resveratrol from grapes. *J. Food Eng*., 77(3): 725–730.

26. Romero-Perez, A.I.; Lamuela-Raventos, R.M.; Andres-Lacueva, C.; Torre-Boronat, M.C. (2001) Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J. Agr. Food Chem.*, 49(1): 210–215.

27. Bird, B.; Stewart, W.E.; Lightfoot, E.N. (2002) *Transport Phenomena*, 2ndEd.; John Wiley & Sons, Inc.: New York, USA.

Figure Caption

Figure 1 Diagram of experimental setup of SWE

Figure 2 HPLC chromatograms of (a) standard compounds: TP (1) and TR (2)
(b) *S. roxburghii* extract

Figure 3 Effect of extraction temperature on the amounts of (a) TR and (b) TP at fixed flow rate of 2 ml/min and pressure 10 MPa.

Figure 4 Effect of water flow rate on the amount of extracted TP and TR for SWE at 190°C and pressure 10 MPa.

Table Caption

Table 1 Comparison of TP and TR for other extraction methods

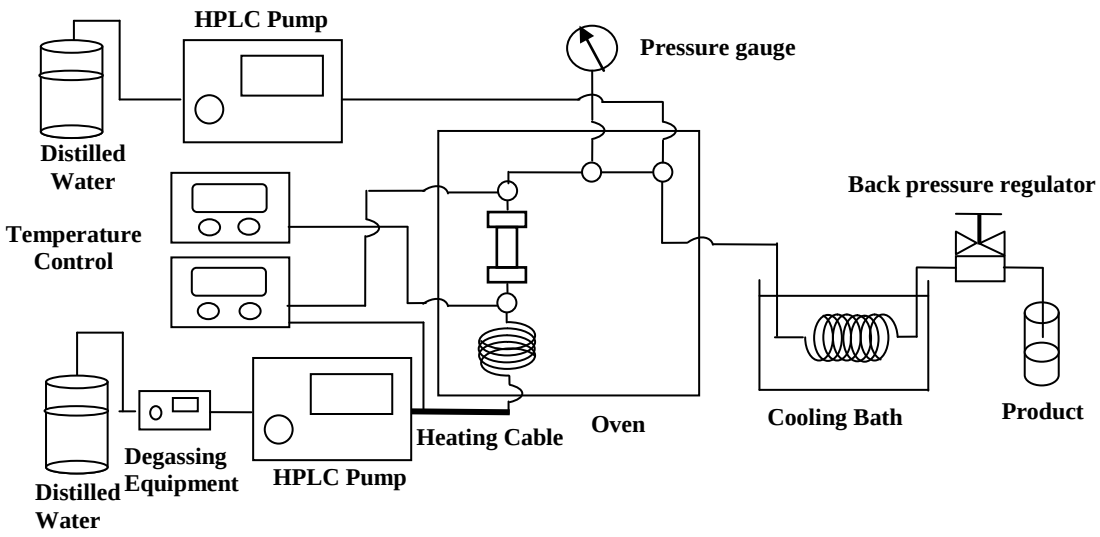
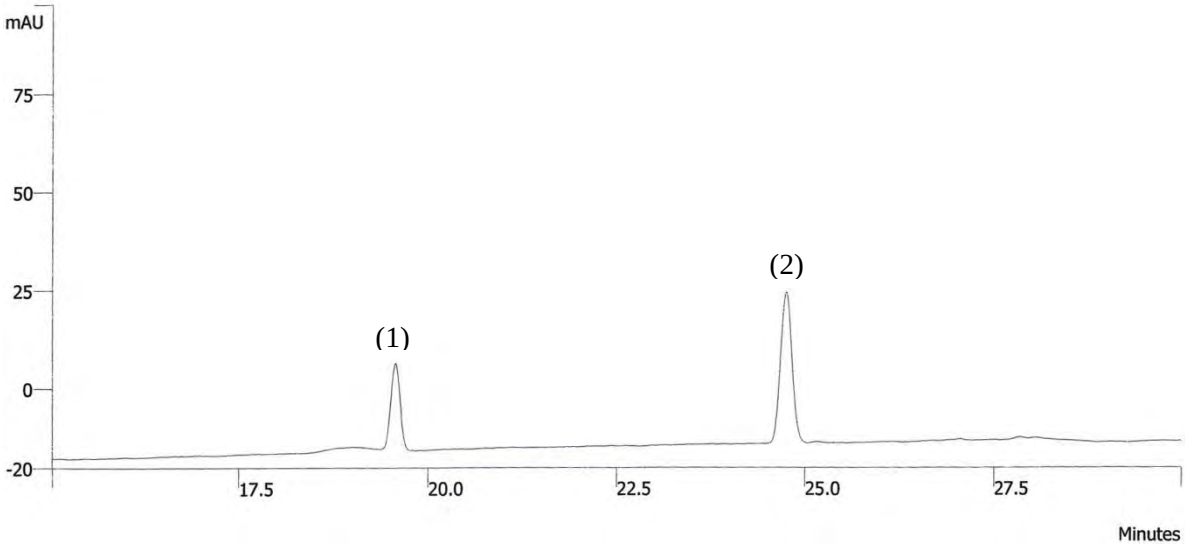

HPLC Pump
Pressure gauge
Distilled Water
Back pressure regulator
Temperature Control
Oven
Cooling Bath
Product
Heating Cable
Degassing Equipment
HPLC Pump
Distilled Water

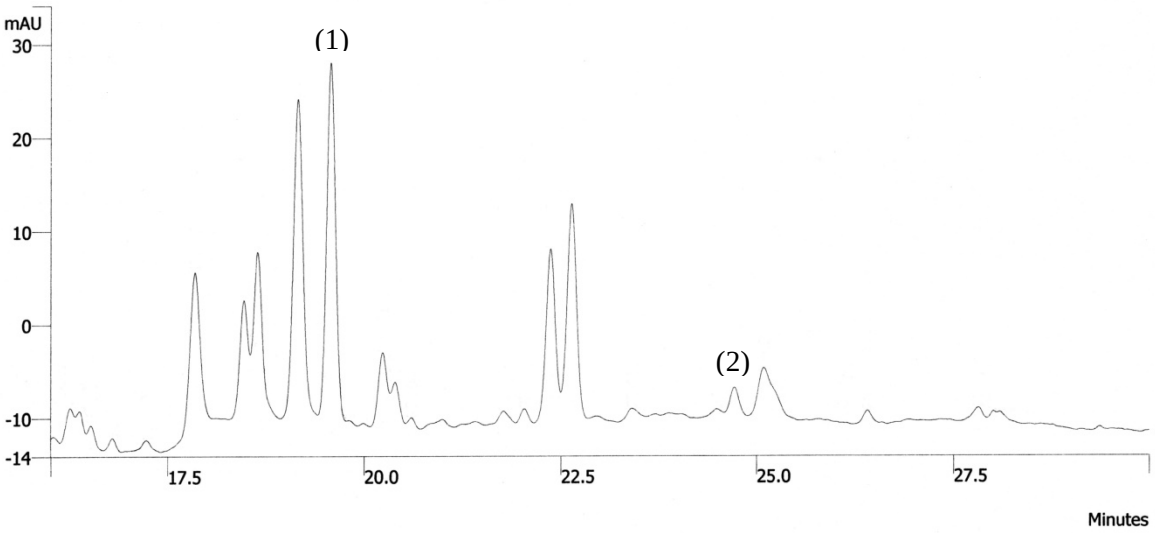
Figure 1

1



2 (a)

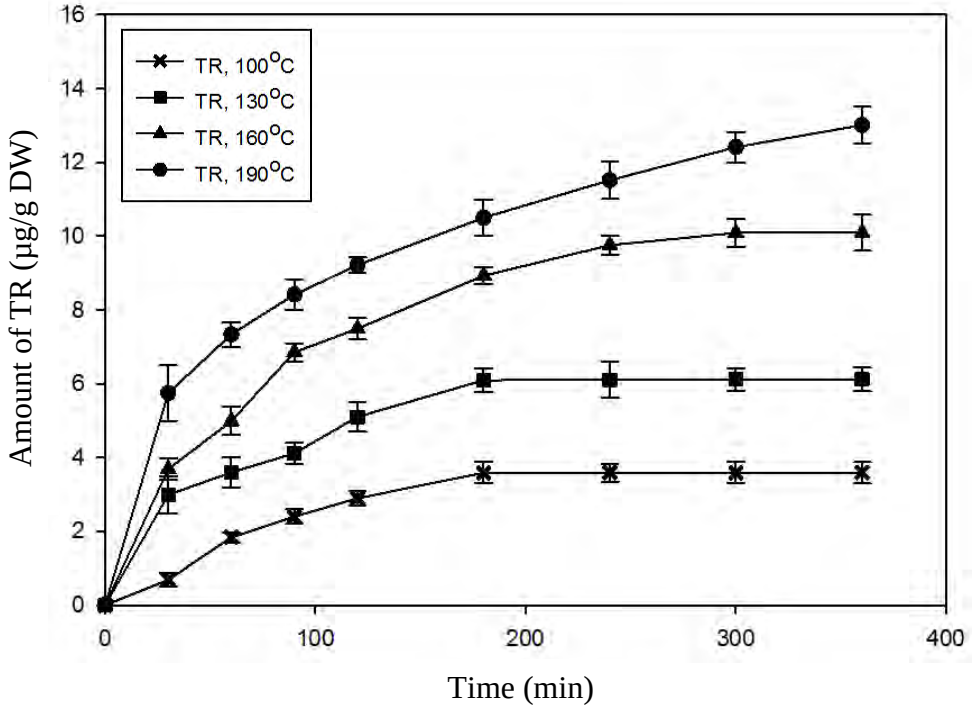
3



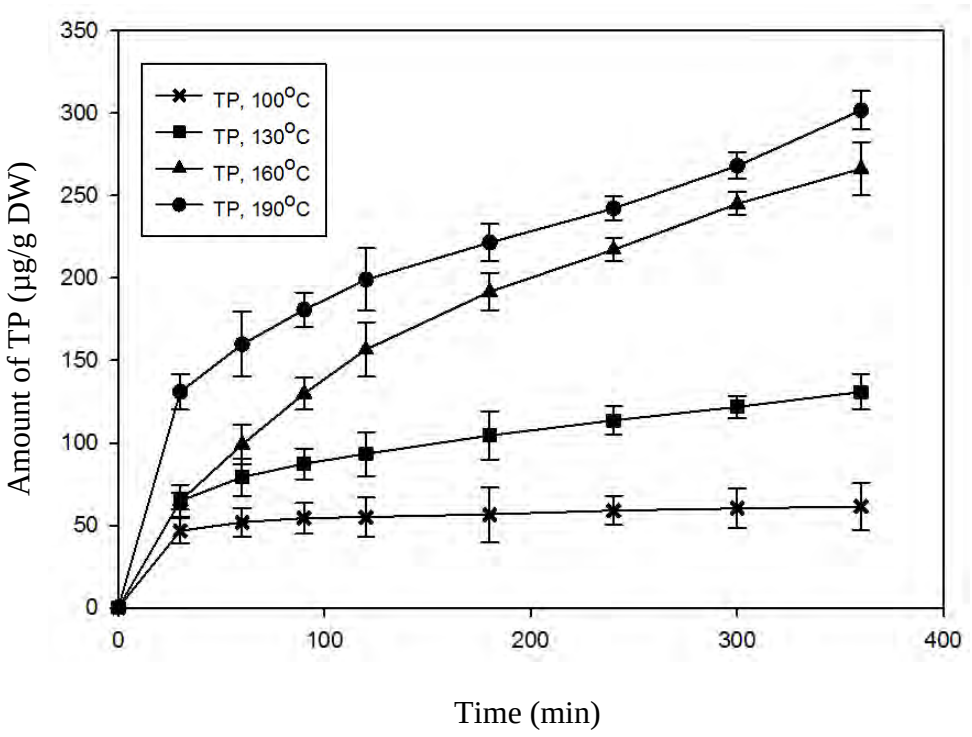
4 (b)

5 **Figure 2**

6


TR, 100°C
TR, 130°C
TR, 160°C
TR, 190°C
Amount of TR (µg/g DW)
Time (min)
0
2
4
6
8
10
12
14
16
0
100
200
300
400

(a)


TP, 100°C
TP, 130°C
TP, 160°C
TP, 190°C
Amount of TP (µg/g DW)
Time (min)
0
50
100
150
200
250
300
350
0
100
200
300
400

(b)

Figure 3

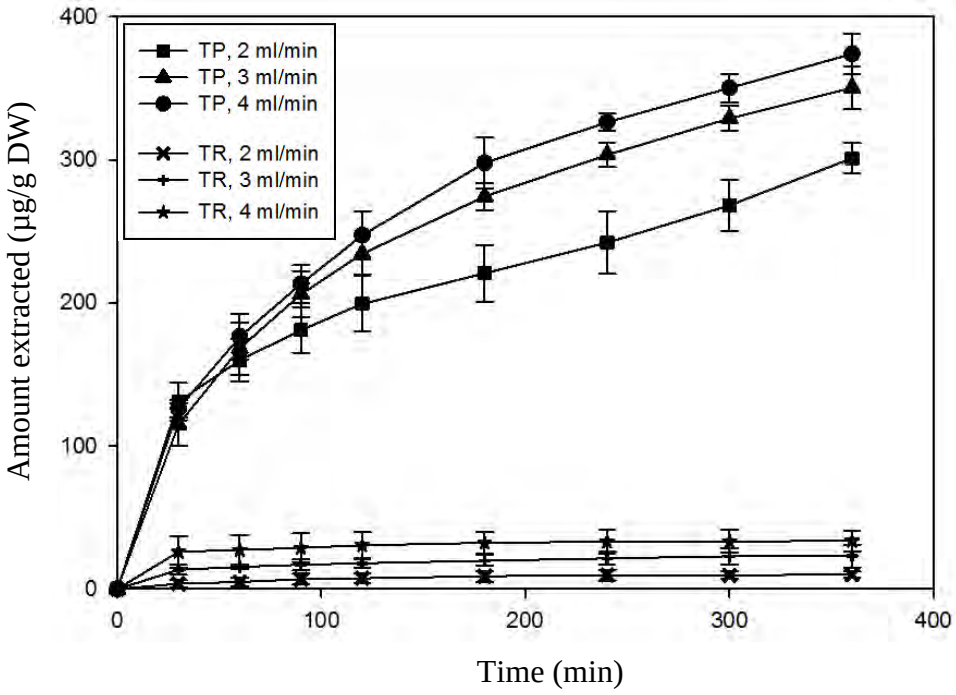

TP, 2 ml/min
TP, 3 ml/min
TP, 4 ml/min
TR, 2 ml/min
TR, 3 ml/min
TR, 4 ml/min
Amount extracted (µg/g DW)
Time (min)
0
100
200
300
400

Figure 4

Table 1

Extraction Methods	Temperature (°C)	Time (min)	TP (μg/g DW)	TR (μg/g DW)
Extraction with ethanol stirred vessel	70	60	68.37±1.02	1.91±1.10
Extraction with water in stirred vessel	70	60	36.96±1.52	0.88±0.30
Soxhlet extraction with 80%ethanol	78	60	71.12±1.00	2.13±0.05
Soxhlet extraction with 80%ethanol	78	120	92.50±0.50	3.23±0.93
Soxhlet extraction with water	100	60	60.02±0.86	2.09±0.30
Soxhlet extraction with water	100	120	74.87±3.00	3.91±0.90
SWE	100	30	51.71±2.50	3.13±0.60
SWE	100	120	54.90±3.00	4.07±0.21
SWE	190	30	130.88±2.06	6.93±0.40
SWE	190	120	198.97±2.07	8.20±0.26