(ร่าง) รายงานวิจัยฉบับสมบูรณ์

(ปกปิด)

โครงการ การใช้ประโยชน์จากน้ำมันรำข้าวและผลพลอยได้จากอุตสาหกรรมน้ำมันรำข้าวในอาหารเพื่อสุขภาพ

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สิงหาคม 2563

สัญญาเลขที่ DBG5280004

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คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

รายละเอียดโครงการ

สัญญาเลขที่	IUG5280004
ชื่อโครงการ (ไทย)	**การใช้ประโยชน์จากน้ำมันรำข้าวและผลพลอยได้จากอุตสาหกรรมน้ำมันรำข้าวเพื่อใช้ในอาหารเพื่อสุขภาพ**
ชื่อโครงการ (อังกฤษ)	**Use of Rice Bran Oil and By-Products from Rice Bran Oil Refining in Functional and Health Foods**
หัวหน้าโครงการ	รองศาสตราจารย์ ดร.ปาริฉัตร หงสประภาส
สังกัด	ภาควิชาวิทยาศาสตร์และเทคโนโลยีการอาหาร คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์
ผู้ประกอบการผู้ร่วมทุน	บริษัทน้ำมันรำข้าวสุรินทร์ จำกัด
งบประมาณ	1.5 ล้านบาท
ระยะเวลา	2 ปี

Abstract

This study explores the potential uses of deodorizer distillate (DD) and crude rice bran wax (RBW) from the physical refining process of edible rice bran oil (RBO) manufacturing as the sources of bioactive compounds and structuring ingredients for the formation of the water-soluble vesicle and oil-based three-dimensional network called oleogel, respectively.

The pilot-scale molecular distillation (MD) unit was used to concentrate the phytosterols, tocols, γ-oryzanol, monoglyceride and diglyceride in the unevaporated fraction (UMD) after the free fatty acids (FFAs) were evaporated out by the MD. The pilot-scale MD unit operated at 120, 140 or 160 °C, 0.1 Pa, and a flow rate of 10.14 - 10.66 kg/h could concentrate phytosterols from 1,540.8 mg in 100 g DD to 3,990.2 – 4,904.8 mg in 100 g UMDs. Although γ-oryzanol content was increased from 598.9 mg in 100 g DD to 870.0-1,018.1 mg when the temperature was raised to 160 °C, such high temperature decreased the tocol contents from 2,185.7 mg/100 g DD to 850.5 mg/100 g UMD, resulting in the reduction of antioxidant capacity of UMD measured as 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity.

The UMD obtained from the pilot-scale MD unit operated at 140 °C, 0.1 Pa, and a flow rate of 10.14 - 10.66 kg/h was used as a source of rice phytochemicals for the fabrication of vesicles with other surfactants. The water-soluble vesicles could be formed when polyoxyethylene sorbitan monooleate (Tween80) was used. The size of Tween 80/UMD vesicles ranged from 200 nm to 300 nm in phosphate-buffered saline (PBS) pH 7.0 suspensions could find potential use in shelf-stable drinks containing rice phytochemicals. The filtered sterile vesicle suspensions in the isotonic PBS, a model drink, were stable within the temperature range of 4 to 37 °C and maintained the size range of 200 – 300 nm for 96 h. Results indicated that the Tween 80/UMD vesicle was able to carry 814 μg phytosterol/mL, 453 μg tocols/mL, and 200 μg γ-oryzanol/mL as maximum load without causing phase separation of the oil phase in the PBS after preparation and storage at low temperatures. At high concentration of Tween80/UMD vesicle of 5 mg/mL (163 μg/mL phytosterol, 91 μg/mL tocols and 40 μg/mL γ-oryzanol), the vesicles reduced viability of Caco-2. Moreover, the vesicle showed potential immunomodulation properties in the THP-1 macrophages.

The crude RBW, a by-product from winterizing step, was explored for its use in the formation of an oleogel holding the liquid oil in the three-dimensional network at the temperature higher than the melting temperature of RBO. The tailored edible oleogel had increased storage modulus (G'), higher viscoelastic transition temperature, and a prolonged time for the oleogel to change from solid to liquid behavior. In a comparative study using crude RBW and ethylcellulose (EC) network as gelator holding liquid oil in the RBO oleogel, it was found that the RBO-EC and RBO-RBW-EC oleogels could prevent the sedimentation of salt and spices in the oil-based marinade sauce at 2 °C for two days and withstand the temperature at 90 °C during grilling. Nonetheless, the appearance and the texture of grilled pork marinated with RBO-RBW was more superior than those marinated with the sauce containing EC.

Keywords: bran; encapsulation; edible oil; oleogel; phytochemicals; rice; wax

บทคัดย่อ

งานวิจัยนี้ได้ศึกษาการใช้ของเหลวผลกลั่น (DD) จากกระบวนการกำจัดกลิ่น และการใช้ไขน้ำมันจากขั้นตอนการกำจัดไขที่ได้จากกระบวนการกลั่นน้ำมันรำข้าวแบบกายภาพ เป็นแหล่งวัตถุดิบที่จะนำมาทำเข้มข้นสารพฤกษเคมีด้วยเครื่องกลั่นโมเลกุลระดับโรงงานนำร่อง (MD) และเป็นแหล่งวัตถุดิบที่จะใช้เป็นส่วนผสมอาหารที่สามารถอุ้มน้ำมันไว้ในโครงข่ายสามมิติฐานน้ำมันที่เรียกโอลิโอเจล (oleogel) ตามลำดับ

ในการทำเข้มข้นไฟโตสเตอรอล โทคอลส์ แกมมาออไรซานอล โมโนกลีเซอไรด์และไดกลีเซอไรด์ด้วยเครื่อง MD ระดับโรงงานนำร่อง สารพฤกษเคมีเหล่านี้จะอยู่ในส่วนที่ไม่ระเหย (UMD) หลังจากที่กรดไขมันอิสระ (FFAs) ถูกกลั่นออกโดยเครื่อง MD เมื่อทำการกลั่นที่อุณหภูมิ 120, 140 หรือ 160 °ซ, 0.1 พาสคัล และอัตราการไหล 10.14 - 10.66 กก. ต่อชม. ซึ่งสามารถทำให้สารไฟโตสเตอรอลเข้มข้นจาก 1,540.8 มก. ใน 100 ก. ของของเหลวผลกลั่น (DD) สูงขึ้นเป็น 3,990.2 - 4,904.8 มก. ใน 100 ก. UMD แม้ว่าการเพิ่มอุณหภูมิการกลั่นเป็น 160 °ซ จะเพิ่มปริมาณแกมมาออไรซานอลจาก 598.9 มก. ใน 100 ก. DD เป็น 870.0-1,018.1 มก.ใน 100 ก. UMD แต่อุณหภูมิที่สูงเช่นนี้จะลดปริมาณโทคอลส์จาก 2,185.7 มก. ต่อ 100 ก. DD เป็น 850.5 มก. ต่อ 100 ก. UMD ส่งผลให้ความสามารถในการต้านอนุมูลอิสระของ UMD ลดลง

เมื่อนำ UMD ที่ได้จากการกลั่นโมเลกุลระดับโรงงานนำร่องที่อุณหภูมิ 140 °ซ 0.1 พาสคัล และอัตราการไหล 10.14 - 10.66 กก./ชม. และสารลดแรงตึงผิวหลายชนิดมาขึ้นรูปเวซิเคิลที่มีสารพฤกษเคมีจากน้ำมันรำข้าว พบว่าเวซิเคิลที่เตรียมโดยใช้ Tween80 (Tween 80/UMD) มีขนาดระหว่าง 200 นาโนเมตรถึง 300 นาโนเมตร ในน้ำไอโซโทนิกหรือน้ำเกลือฟอสเฟตบัฟเฟอร์ (PBS) ความเป็นกรด-ด่าง 7.0 ซึ่งเป็นแบบจำลองของเครื่องดื่มไอโซโทนิกที่มีความเสถียรในช่วงอุณหภูมิ 4 ถึง 37 °ซ เวซิเคิล Tween 80/UMD นี้สามารถนำส่งไฟโตสเตอรอล 814 ไมโครกรัมต่อมล. โทคอลส์ 453 ไมโครกรัมต่อ มล. และแกมมาออไรซานอล 200 ไมโครกรัมต่อ มล. โดยไม่ทำให้เกิดการแยกเฟสของเฟสน้ำมันในสารละลายไอโซโทนิคหลังการเตรียมและการเก็บรักษาที่อุณหภูมิต่ำ การใช้เวซิเคิล Tween80 /UMD ที่ให้ไฟโตสเตอรอล 163 ไมโครกรัมต่อ มล. โทคอลส์ 91 ไมโครกรัมต่อ มล. และ แกมมาออไรซานอล 40 ไมโครกรัม ต่อ มล. สามารถลดความมีชีวิตของเซลล์มะเร็งลำไส้ใหญ่ Caco-2 และส่งเสริมให้เซลล์มาโครฟาจ THP-1 แสดงคุณสมบัติสร้างภูมิคุ้มกัน

ไขน้ำมัน (RBW) ซึ่งเป็นผลพลอยได้จากขั้นตอนการกำจัดไข สามารถนำมาใช้ในการขึ้นรูปโอลิโอเจลที่อุ้มน้ำมันเหลวในโครงข่ายสามมิติ และคงรูปของแข็งที่อุณหภูมิสูงกว่าอุณหภูมิหลอมเหลวของน้ำมันรำข้าว เนื่องจากมีโมดุลัสกักเก็บ (G') และอุณหภูมิการลดความหนืดที่สูงขึ้น และใช้เวลานานขึ้นในการเปลี่ยนจากพฤติกรรมของของแข็งเป็นพฤติกรรมของของไหล ในการศึกษาเปรียบเทียบการใช้โครงข่ายของไขน้ำมันกับการใช้เอทิลเซลลูโลส (EC) เป็นสารทำให้เกิดเจลในการขึ้นรูปโอลิโอเจลน้ำมันรำข้าว พบว่าโอลิโอเจลที่เตรียมจากเอทีลเซลลูโลส และไขน้ำมันกับเอทีลเซลลูโลส สามารถป้องกันการตกตะกอนของเกลือและเครื่องเทศในซอสหมักฐานน้ำมันได้ระหว่างการหมักสเต็กหมูที่ 2 °ซ เป็นเวลาสองวัน และทนต่ออุณหภูมิที่ 90 °ซ ระหว่างการย่างได้ดีกว่าเต็กหมูหมักที่ใช้ซอสโอลิโอเจลที่มีไขน้ำมันเป็นสารทำให้เกิดเจล อย่างไรก็ตามการยอมรับทางประสาทสัมผัสของ สเต็กหมูย่างที่หมักด้วยซอสโอลิโอเจลน้ำมันรำข้าวที่ขึ้นรูปด้วยไขน้ำมันนั้น เหนือกว่าสเต็กหมูย่างที่หมักด้วยซอสโอลิโอเจลที่มี เอทีลเซลลูโลสทั้งสองสูตร

คำสำคัญ: รำ; การห่อหุ้ม; น้ำมันพืช; โอลิโอเจล; สารพฤกษเคมี; ข้าว; ไขน้ำมัน

บทสรุปผู้บริหาร

น้ำมันรำข้าวเป็นแหล่งที่สำคัญของโภชนเภสัชในกลุ่มของสารต้านอนุมูลอิสระ เช่น แกมมาออไรซานอล วิตะมินอีในรูปของโทโคฟีรอลและโทโคไตรอีนอล รวมทั้งไฟโตสเตอรอล ในกระบวนการผลิตน้ำมันรำข้าวผ่านกรรมวิธี (refined rice bran oil) ภายหลังการสกัดน้ำมันด้วยเฮกเซน จะมีผลพลอยได้ (by-product) จากขั้นตอนต่างๆ ในกระบวนการกลั่นน้ำมัน โดยเฉพาะจากขั้นตอนการกำจัดกลิ่นโดยการกลั่น (deodorization) และขั้นตอนการกำจัดไข (dewaxing) โดยการลดอุณหภูมิ (winterization) ผลพลอยได้ทั้งสองชนิดนี้โรงงานกลั่นน้ำมันรำข้าวจะจำหน่ายเป็นอาหารสัตว์ แม้จะมีสารออกฤทธิ์ทางชีวภาพที่สำคัญ คือ ไฟโตสเตอรอล (phytosterols) แกมมาออไรซานอล (γ-oryzanol) วิตามินอีหรือโทคอลส์ (tocols) และโพลิโคซานอล (policosanols) หลงเหลืออยู่ เนื่องจากขาดองค์ความรู้ในการพัฒนากระบวนการผลิต และการนำสารพฤกษเคมีดังกล่าวไปใช้ประโยชน์ในอุตสาหกรรมอาหารหรือเครื่องสำอางต่อเนื่อง

งานวิจัยนี้จึงได้ศึกษาผลของการกลั่นผลพลอยได้จากขั้นตอนการกำจัดกลิ่น ซึ่งมีกรดไขมันอิสระในสัดส่วนสูง โดยใช้เครื่อง Molecular Distillation หรือ Short-Path Distillation ในระดับโรงงานต้นแบบ เรียกโดยย่อว่าเครื่อง MD เพื่อทำให้สารพฤกษเคมีดังกล่าวมีความเข้มข้นเพิ่มขึ้น กำจัดกรดไขมัน และคงเหลือส่วนของโมโนกลีเซอไรด์และไดกลีเซอไรด์ที่มีกรดไขมันโอลีอิกเป็นองค์ประกอบหลัก สารพฤกษเคมีและสารลดแรงตึงผิวจากน้ำมันรำข้าวที่เข้มข้นขึ้น ได้ถูกนำมาขึ้นรูปให้อยู่ในรูปของเวซิเคิลขนาดไม่เกิน 300 นาโนเมตรที่มีความคงตัวในช่วงอุณหภูมิต่ำ เพื่อใช้ในผลิตภัณฑ์เครื่องดื่มเพื่อสุขภาพที่มีสัดส่วนของวิตะมินอี ไฟโตสเตอรอล และแกมมาออไรซานอลสูง ทำหน้าที่เป็นสารออกฤทธิ์ทางชีวภาพในเครื่องดื่ม โดยเฉพาะอย่างยิ่งการปรับเปลี่ยนระบบภูมิคุ้มกัน

นอกจากนั้นแล้วงานวิจัยนี้ยังได้ศึกษาแนวทางการใช้ประโยชน์ของไขน้ำมันจากกระบวนการกำจัดไข มาใช้ในการขึ้นรูปโอลิโอเจลน้ำมันรำข้าว (rice bran oil oleogel) เพื่อให้น้ำมันรำข้าวคงรูปที่อุณหภูมิห้องภายใต้สภาวะการผลิตที่เหมาะสม สามารถนำโอลิโอเจลน้ำมันรำข้าวนี้ไปใช้ประโยชน์ในอุตสาหกรรมอาหาร ซึ่งในงานวิจัยนี้ได้ทดสอบการนำโอลิโอเจลน้ำมันรำข้าวไปใช้ในซอสหมักฐานไขมันสำหรับผลิตภัณฑ์เนื้อสัตว์ เนื่องจากโอลิโอเจลน้ำมันรำข้าวสามารถพยุงผงเครื่องเทศและเกลือระหว่างการจัดจำหน่ายที่อุณหภูมิห้อง การหมักปรุงรสอาหารเนื้อสัตว์ที่อุณหภูมิต่ำ ซึ่งเป็นคุณลักษณะสำคัญของซอสหมักปรุงรสสเต็กในประเทศแถบยุโรป ปัจจุบันบริษัทน้ำมันรำข้าวสุรินทร์ จำกัด ซึ่งเข้าร่วมในโครงการวิจัยนี้ได้รับเลขทะเบียนจากสำนักงานคณะกรรมการอาหารและยาในการผลิตและจำหน่ายไขน้ำมันจากขั้นตอนการกำจัดไข โดยใช้องค์ความรู้จากงานวิจัยที่ได้ตีพิมพ์ประกอบการพิจารณาพัฒนาผลิตภัณฑ์ใหม่ๆ ของบริษัทเพื่อใช้ประโยชน์เชิงพาณิชย์ และสามารถขยายผลจากการศึกษาวิจัยนี้ในผลิตภัณฑ์อาหารประเภทอื่นต่อไปได้

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บทที่ 1 บทนำ

1.1 ความเป็นมาของโครงการ

น้ำมันรำข้าวเป็นแหล่งที่สำคัญของโภชนเภสัชในกลุ่มของสารต้านอนุมูลอิสระ เช่น แกมมาออไรซานอล วิตะมินอีในรูปของโทโคฟีรอลและโทโคไตรอีนอล รวมทั้งไฟโตสเตอรอล ประเทศไทยซึ่งเป็นผู้ส่งออกข้าวรายใหญ่ของโลกสามารถผลิตข้าวประมาณ 20-22 ล้านตันในแต่ละปี ซึ่งจะทำให้มีรำข้าวหลังการขัดสีประมาณ 1.12 ล้านตันต่อปีเข้าสู่อุตสาหกรรมอาหารสัตว์และอุตสาหกรรมน้ำมันรำข้าว

อุตสาหกรรมอาหารในปัจจุบันมุ่งเน้นการผลิตอาหารแปรรูปที่มีคุณค่าทางโภชนาการสูง หลีกเลี่ยงการมีส่วนประกอบของอาหารที่อาจมีผลเสียต่อสุขภาพ เช่น หลีกเลี่ยงกระบวนการผลิตอาหารไขมันสูง เลือกใช้สารต้านอนุมูลอิสระจากธรรมชาติแทนการใช้สารสังเคราะห์ เป็นต้น นอกจากนั้น ในปี 2562 ประเทศไทยได้ระงับการนำเข้าไขมันโครงสร้าง (structured fat) ที่ผ่านกระบวนการเติมไฮโดรเจนบางส่วน อุตสาหกรรมน้ำมันบริโภคจึงมีแนวคิดที่จะสกัดสารออกฤทธิ์ทางชีวภาพจากรำข้าวหรือผลพลอยได้จากกระบวนการกลั่นน้ำมันให้บริสุทธิ์ เพื่อเป็นการเพิ่มความหลากหลายของสินค้าที่จะทำกำไรได้นอกเหนือจากการผลิตและจำหน่ายน้ำมันรำข้าวบริโภค หรือการจำหน่ายรำข้าวหลังการสกัดน้ำมันและการจำหน่ายผลพลอยได้จากการกลั่นน้ำมันเพื่อเป็นอาหารสัตว์ รวมทั้งศึกษาความเป็นไปได้ในการผลิตน้ำมันรำข้าวที่มีความหลากหลายในการใช้บริโภคมากขึ้น เช่น การผลิตน้ำมันรำข้าวจุดหลอมเหลวสูง ซึ่งจะทำให้ผลิตภัรฑ์มีคุณลักษณะเป็นของแข็งที่อุณหภูมิห้อง ทำให้มีการขยายการใช้ประโยชน์จากน้ำมันรำข้าวให้กว้างขึ้น

ในกระบวนการผลิตน้ำมันรำข้าวผ่านกรรมวิธี (refined rice bran oil) ภายหลังการสกัดน้ำมันด้วยเฮกเซนจะมีผลพลอยได้ (by-product) จากขั้นตอนต่างๆ ในกระบวนการ โดยเฉพาะจากขั้นตอนการกำจัดกลิ่นโดยการกลั่น (deodorization) และขั้นตอนการกำจัดไข (dewaxing) โดยการลดอุณหภูมิ (winterization) ผลพลอยได้ทั้งสองชนิดนี้ ยังคงมีสารออกฤทธิ์ทางชีวภาพที่สำคัญ คือ ไฟโตสเตอรอล (phytosterols) แกมมาออไรซานอล (γ-oryzanol) วิตามินอีหรือโทคอลส์ (tocols) และโพลิโคซานอล (policosanols) ในสัดส่วนที่สูง ซึ่งควรนำมาใช้ประโยชน์ในอุตสาหกรรมอาหารหรือเครื่องสำอางต่อไป

1.2 วัตถุประสงค์

1. เพื่อศึกษากลไกในการควบคุมการเปลี่ยนเฟสของระบบอิมัลชัน ชนิด oil-in-water และ water-in-oil เพื่อใช้ในผลิตภัณฑ์อาหารเพื่อสุขภาพที่มีน้ำมันรำข้าวเป็นองค์ประกอบหลัก โดยประเมินความสามารถในการใช้ไฟโตสเตอรอล (phytosterols) แกมมาออไรซานอล (γ-oryzanol) และวิตามินอีหรือโทคอลส์ (tocols) ในการขึ้นรูปเวซิเคิล (vesicle) ขนาดไม่เกิน 300 นาโนเมตร เพื่อให้กระจายตัวในน้ำได้
2. เพื่อประเมินประสิทธิผลของระบบนำส่งสารโภชนเภสัชที่สำคัญจากน้ำมันรำข้าวในโมเดลเซลล์ของมนุษย์
3. เพื่อเสนอแนวทางการใช้โอลิโอเจลน้ำมันรำข้าว (rice bran oil) ซึ่งอยู่ในสถานะของของแข็งในการผลิตน้ำมันรำข้าวคงรูปที่อุณหภูมิห้องหรือโอลิโอเจลน้ำมันรำข้าว เพื่อให้มีคุณสมบัติของ zero-*trans* plastic fat เมื่อนำไปใช้ในอาหาร

1.3 แผนการดำเนินงาน

งานวิจัยนี้แบ่งการศึกษาออกเป็น 5 กิจกรรม ดังนี้

1 ศึกษาคุณสมบัติของของเหลวผลกลั่นจากกระบวนการกำจัดกลิ่น และสภาวะที่เหมาะสมในการใช้การกลั่นด้วย short-path distillation เพื่อทำให้สารพฤกษเคมีของของเหลวผลกลั่นมีความเข้มข้นเพิ่มขึ้น

2 ใช้สารพฤกษเคมีจากกระบวนการกลั่นด้วย short-path distillation ในการควบคุมคุณลักษณะของของไหลหรือของแข็งของน้ำมันรำข้าวที่อุณหภูมิห้อง

3 ใช้สารพฤกษเคมีจากกระบวนการกลั่นด้วย short-path distillation เป็นสารลดแรงตึงผิวร่วมในการขึ้นรูปเวซิเคิลที่ละลายน้ำและดูดซึมในโมเดลเซลล์ของมนุษย์

4 ศึกษาคุณสมบัติทางเคมีและกายภาพของไขน้ำมันจากกระบวนการกำจัดไข เพื่อใช้ขึ้นรูปโอลิโอเจลน้ำมันรำข้าวร่วมกับสารลดแรงตึงผิว เพื่อให้ได้โอลิโอเจลที่มีคุณลักษณะของของแข็งที่อุณหภูมิห้อง

5 เปรียบเทียบคุณสมบัติของโอลิโอเจลน้ำมันรำข้าวที่ขึ้นรูปด้วยไขน้ำมัน กับโอลิโอเจลน้ำมันรำข้าวที่ขึ้นรูปด้วยไบโอพอลิเมอร์เอทิลเซลลูโลส ในซอสหมักสเต็กสูตรต่างประเทศ ต่อคุณสมบัติของสเต็กหมูระหว่างกระบวนการหมักและการย่าง

บทที่ 2 ผลของกระบวนการกลั่นแยกกรดไขมันด้วยเครื่อง Molecular Distillationระดับโรงงานต้นแบบต่อคุณสมบัติทางเคมีและกายภาพของสารพฤกษเคมีเข้มข้น

Abstract

The potential uses of deodorizer distillate (DD) from the physical refining process of edible rice bran oil (RBO) manufacturing as the sources of bioactive compounds were investigated. The deodorizing step in the refining process evaporated a substantial amount of phytosterols and γ-oryzanol, which accumulated in the DD. This study used DD as a raw material fed to a pilot-scale molecular distillation unit (MD) to evaporate out the FFAs, resulting in an unevaporated fraction (UMD) that contained concentrated phytosterols, tocols, and γ-oryzanol. The pilot-scale MD unit operated at 120, 140 or 160 °C, 0.1 Pa, and a flow rate of 10.14 - 10.66 kg/h could concentrate phytosterols from 1,540.8 mg in 100 g DD to 3,990.2 – 4,904.8 mg in 100 g UMDs. Although γ-oryzanol content was increased from 598.9 mg in 100 g DD to 870.0-1,018.1 mg when the temperature was raised to 160 °C, such high temperature decreased the tocol contents from 2,185.7 mg/100 g DD to 850.5 mg/100 g UMD and antioxidant capacity of UMD measured as 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity. The ratio of FFA to monoacylglycerol (MAG) to diacylglycerol (DAG) to triacylglycerol (TAG) in the UMD obtained at 120 °C was 0.2:0.3:0.0:0.5, which was changed to 0.0:0.1:0.2:0.7 in the UMD obtained at 140 °C and 0.0:0.3:0.1:0.6 in the UMD obtained at 160 °C. Using different distillation temperatures during the MD process resulted in different compositions of rice phytochemicals and mono- and di-acyl glycerol (mainly oleic esterified) surfactants readily for use in further food processing.

2.1 Introduction

Rice bran oil (RBO) is well recognized as a healthy oil that contains a proper ratio of saturated, monounsaturated, and polyunsaturated fatty acid. Not only suitable fatty acid profile, but RBO is also known as the source of health-promoting phytochemicals, especially tocopherols, tocotrienols, phytosterols, and γ-oryzanol (Van Hoed et al. 2006). However, some of these phytochemicals are reduced in refined RBO during the refining process and become concentrated in the co-products, in particular gum, soapstock, deodorizer distillate (DD) and wax.

Our previous investigation revealed that the DD, a by-product obtained from the steam deodorization process, could be used as a source for the production of γ-oryzanol and phytosterols (Sawadikiat and Hongsprabhas 2014). Further distillation to separate free fatty acids (FFAs) could be employed in the attempt to concentrate those rice phytochemicals.

Molecular distillation (MD) or short-path distillation is a high-vacuum distillation process suitable for separation and purification of high-molecular-weight and thermally sensitive materials (Lei et al. 2005). The separation principle of MD is dependent on the difference in the molecular mean free path of materials (Lei et al. 2005). Generally, MD is characterized by low operating temperature (due to high vacuum), short exposure of the distilled liquid to elevated temperature, high vacuum, and a small distance between the evaporator and the condenser. Due to the high vacuum condition, oxidation that might occur in the presence of oxygen is reduced.

The separation efficiency of MD is dependent on many operational factors, i.e., evaporation temperature, feed flow rate, and operating pressure (Batistella et al. 2002; Liu et al. 2008; Martins et al. 2006; Posada et al. 2007). Therefore, the influences of evaporation temperature during the MD process on the retention of oil-soluble rice phytochemicals, namely tocols, phytosterols, and γ-oryzanols, in the unevaporated fraction (UMDs), were investigated in the current study.

2.2 Materials and methods

2.2.1 Materials

Deodorizer distillate (DD) from commercial production of physically refined rice bran oil was used as the raw material for the production of rice phytochemicals using a pilot-scale molecular distillation (MD) unit at Surin Bran Oil Co. (Surin, Thailand).

2.2.2 Characteristics of rice bran oil deodorizer distillate (DD)

Thermo-oxidative stability of DD

The thermo-oxidative stability of DD was characterized by a Mettler Toledo DSC821e differential scanning calorimeter (Schwerzenbach, Switzerland). Four to five mg of DD were weighed into a 40 µL aluminum sample pan, closed with a lid having a hole of 1 mm internal diameter drilled in the center. This hole allowed the sample to be in contact with an oxygen (O_2) or nitrogen (N_2) stream. A sealed aluminum empty pan was used as a reference. The sample and reference pans were heated at heating rates (β) of 2, 5, 10, 16, and 20 °C/min. For thermal stability evaluation, experiments were performed under a N_2 stream at 100 mL/min flow rate. The thermo-oxidative stability of DD was determined under an O_2 stream at a flow rate of 100 mL/min. When the run was completed, the onset temperature (T_o) of oxidation was determined as the intersection of the extrapolated baseline and the tangent line (leading edge) of the exothermic peak (Ostrowska-Ligeza et al. 2010). The characterization was performed in duplicate.

The Ozawa–Flynn–Wall method (OFW) was used to determine activation energy (E_a) (Ostrowska-Ligeza et al. 2010). Linear regression of log β versus $1/T_o$ was plotted using Eq. (1) to determine the slope A:

$$\log\beta = A(T_o) + B \qquad (1)$$

where β is the heating rate (°C/min), and T_o is the onset temperature of oxidation in Kelvin (K). The activation energy (E_a) was calculated using Eq. (2), where R is a gas constant:

$$E_a = -2.19\mathrm{R}\frac{\partial \log \beta}{\partial(1/To)} \quad (2)$$

The acid value of DD

The acid value of DD was determined according to the AOCS Official Method Cd 3d-63 (AOCS, 1997). Briefly, 2 mL of 1% phenolphthalein in isopropyl alcohol was added to 125 mL of solvent mixture (isopropyl alcohol: toluene at the ratio of 1:1 v/v) and neutralized to a faint pink using 0.1 N KOH. Samples were weighed into an Erlenmeyer flask; 125 mL of a solvent mixture containing phenolphthalein was added, followed by titration with 0.1 M KOH. The acid value was calculated using Eq. (3):

$$Acid\ value\ (mg\ KOH / \mathrm{g\ sample}) = \frac{(A-B) * Normality\ \mathrm{of\ KOH} * 56.1}{g\ \mathrm{sample}} \quad (3)$$

where A is mL of 0.1 N KOH titrated with the sample, and B is mL of 0.1 N KOH used in titrating a blank (solvent mixture).

Determination of tocopherols and tocotrienols in DD

The AOCS Recommended Practice Ce 7-87 (AOCS, 1997) was used to quantify tocopherol (α-T and γ-T) and tocotrienol (α-T3, β-T3, γ-T3, and δ-T3) contents. Briefly, 20 to 30 mg of samples were silylated by sylon BFT, i.e. 1 mL of pyridine and 2 mL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) mixture (99:1). Samples were heated at 50 °C for 10 min. The internal standard (heptadecanyl stearate) was added to silylated samples and mixed thoroughly. An aliquot (1 µL) of each sample was injected into a gas chromatographic apparatus and quantified for tocopherol and tocotrienol contents using the response factor (FC) equation shown in Eq. (4):

$$FC = \frac{A_{\mathrm{IS}} \times C_{\mathrm{Standard}}}{A_{\mathrm{Standard}} \times C_{\mathrm{IS}}} \quad (4)$$

where A_{IS} is the area of internal standard (heptadecanyl stearate), $A_{Standard}$ is the area of tocol standard, C_{IS} is mg of internal standard, and $C_{Standard}$ is mg of tocol standard.

Capillary gas chromatography was performed by a gas chromatograph equipped with a flame ionization detector (HP 6890 Series GC system; Agilent Technologies, Santa Clara, CA,

USA) and HP-5 capillary column (30 m × 0.32 mm × 0.25 μm; Agilent Technologies). Helium was used as a carrier gas at a flow rate of 2 mL/min. The oven temperature was programmed to increase from 140 to 300 °C at a rate of 10 °C/min, with a 6 min hold at 300 °C; the temperature was then increased from 300 to 320 °C at a rate of 5 °C/min and held for 10 min. The injector and detector were maintained at 240 and 345 °C, respectively.

Determination of γ-oryzanol in DD

The γ-oryzanol content in each sample was determined spectrophotometrically using the method described by Khatoon and Gopala Krishna (2004). Briefly, 10 mg of sample was weighed into a 10 mL volumetric flask, dissolved in hexane, and determined for γ-oryzanol content by a UV spectrophotometer (Genesys 10S UV-Vis; Thermo Fisher Scientific, Waltham, MA, USA) at 314 nm using 1 cm cell length. The γ-oryzanol content was calculated, as shown in Eq. (5):

$$\gamma - oryzanol\ (\mathrm{mg}/100g) \quad = \quad \frac{Absorbance \text{ at } 314 \text{ nm in hexane solution} * 10000}{g \text{ of sample} * 358.9} \tag{5}$$

Determination of phytosterols in DD

Rice phytosterol contents were determined according to the method described by Schwartz et al. (2008), using a flame ionization detector (HP 6890 Series GC system) and HP-5 capillary column (30 m × 0.32 mm × 0.25 μm; Agilent Technologies) as previously described by Sawadikiat and Hongsprabhas (2014).

Antioxidant capacity of DD

The total free radical scavenging capacity of DD, determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, was evaluated using a modified method described by Rossi et al. (2007) and Ghafoorunissa (2007). DPPH was dissolved in ethyl acetate at a concentration of 126.8 μM, and the dilution adjusted to obtain an absorbance at 515 nm of 0.6237 absorbance units (AU) (Infinite® M200 PRO microplate reader; Tecan Group Ltd., Männedorf, Switzerland). UMD samples were diluted with ethyl acetate to obtain approximately 200–3,000 μg/mL of UMD. A mixture of 100 μL of DPPH solution and 100 μL of diluted UMD sample, having a final

DPPH concentration of 63.4 μM, was incubated in the dark at 25 ± 0.1 °C for 30 min. The absorbance (Abs) of the mixture was measured at 515 nm. The % scavenging of the sample was determined, as shown in Eq. (6):

$$\%\ scavenging \quad = \quad \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100 \qquad (6)$$

where $Abs_{control}$ is the absorbance at 515 nm of DPPH solution with ethyl acetate (instead of the sample), and Abs_{sample} is the absorbance at 515 nm of DPPH solution containing the sample. The 50% inhibition concentration (IC_{50}, μg of sample/mL) was determined graphically by plotting a graph between % scavenging and sample concentration, and calculated as μg of sample per mL of solution required to obtain 50% of the maximum scavenging capacity. For comparison, α-tocopherol was evaluated under the same conditions.

2.2 3 Effect of distillation temperature on chemical characteristics of the unevaporated fraction (UMD)

A pilot-scale MD unit modified at Surin Bran Oil Co. (Surin, Thailand) was operated at distillation temperatures of 120, 140, and 160 °C and a pressure of 0.1 Pa. The unevaporated fraction, designated as UMD, was analyzed for acid value, γ-oryzanol, tocotrienols, tocopherols, and phytosterols, as well as DPPH antioxidant capacity, using the methods described above. All samples were kept at 4 °C in amber glass bottles before analyses.

The saponifiable matter in the UMDs obtained after molecular distillation at different temperatures was determined by high-performance thin-layer chromatography (HPTLC) (Camag, Berlin, Germany). First, a silica plate was pre-developed in hexane and diethyl ether using a ratio of 1:1 (v/v). The plate was activated at 110 °C to remove impurities. The standards and UMD samples were spotted near the bottom of the plate using a glass micro-syringe. The plate was first developed at a distance of 4.5 cm from the origin. The solvent system consisted of a mixture of methyl acetate: isopropanol: chloroform: methanol: 0.25% (w/v) KCl in a ratio of 25: 25: 25: 10: 9 by volume. The plate was dried over NaOH in a desiccator for 30 min. The second development of the plate was performed at a distance of 9.5 cm in a mixture of hexane: diethyl ether: glacial acetic acid in a ratio of 80: 20: 2 (v/v).

Separate lipid classes were detected by spraying with 3% (w/v) cupric acetate in 8% (w/v) phosphoric acid, followed by charring at 160 °C for 20 min to visualize the bands.

2.2.4 Effect of distillation temperature on thermal properties of the unevaporated fraction (UMD)

Ten mg samples of UMDs obtained at different operating temperatures were weighed into individual stainless steel pans and analyzed by differential scanning calorimetry (DSC) (Pyris 1; PerkinElmer, Norwalk, CT, USA). The DSC program was set for the following cycle: heating from 25 °C to 90 °C and holding at 90 °C for 3 min, cooling from 90 °C to −60 °C at 10 °C/min and holding at −60 °C for 3 min, and heating from −60 °C to 90 °C at 10 °C/min. The onset temperature (To) and the end temperature of melting (Te) were determined.

2.2.5 Physical characteristics of rice bran oil (RBO) supplemented with the unevaporated fraction (UMD) and commercial mono-, diacylglycerol (MDG)

Thermal properties

One g samples of RBO, RBO supplemented with 1% UMD obtained after distillation at 140 °C, RBO supplemented with 1% commercial MDG, and RBO supplemented with 1% UMD and 1% commercial MDG were heated at 70 °C. Fifteen mg samples were weighed into stainless steel pans and analyzed by DSC. The DSC program was set for the following cycle: heating from 25 °C to 60 °C and holding at 60 °C for 3 min, cooling from 60 °C to −60 °C at 10 °C/min and holding at −60 °C for 3 min, and heating from −60 °C to 60 °C at 10 °C/min. The onset temperature (To) and the end temperature of melting (Te) were determined. Solid fat content (SFC) was calculated by dividing the partial area under the melting curve by the total area from −60 to 60 °C and multiplying by 100.

Apparent viscosity

RBO and RBO-AMF – in the absence or presence of 1% UMD obtained after distillation at 140 °C, or 1% commercial MDG – were melted at 70 °C and cooled down to 25 °C. The apparent viscosity of each sample was measured using a Brookfield viscometer equipped with

a UL adapter (DV-III programmable rheometer; Brookfield Engineering, Middleborough, MA, USA) at 60 rpm and 25 °C.

2.2.6 Statistical analysis

One batch of DD was distilled at different temperatures using a pilot-scale MD unit in two separate trials. Results were subjected to analysis of variance (ANOVA) with confidence interval set at 95% ($P < 0.05$) using the statistical software program SPSS for Windows version 12 (SPSS Inc., Chicago, IL, USA). Differences among means were differentiated using Duncan's multiple range test at $P < 0.05$.

2.3 Results and discussion

2.3.1 Chemical characteristics of unevaporated fractions (UMDs) after molecular distillation of deodorizer distillate (DD)

The DSC thermogram in Figure 1 illustrates that in the absence of oxygen (under a N_2 stream), DD obtained from the physical refining process of rice bran oil was thermostable between 25–300 °C. However, when O_2 was present, the oxidation of DD started at 121.2 °C at a heating rate of 2 °C/min.

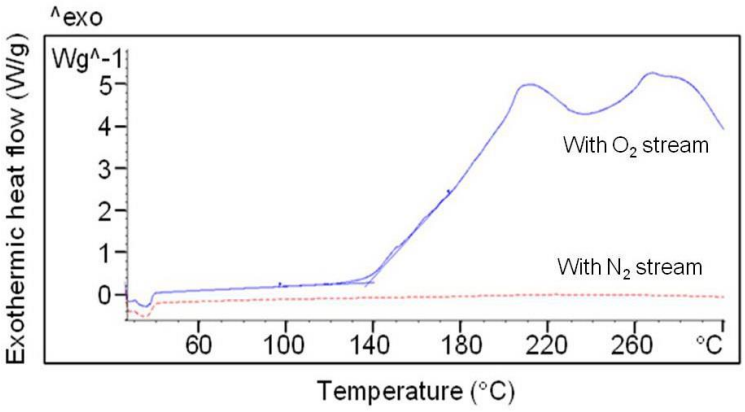

Figure 1 DSC thermograms of deodorizer distillate (DD) at a heating rate of 10 °C/min under oxygen (solid line) and nitrogen (dotted line) streams at a flow rate of 100 mL/min.

Table 1 indicates that raising the heating rate increased the onset temperature (T_o) of oxidation. The calculated activation energy (E_a) of DD was 111.3 kJ/mol, which was higher than those of sunflower oil, soybean oil, and corn oil (Adhvaryu et al. 2000). The E_a of rice bran oil DD reported in the present study was within the same range as olive oil (Ostrowska-Ligeza et al. 2010). The reason is probably that both oils contain around 2% polyunsaturated fatty acids and around 85% monounsaturated fatty acids, while sunflower oil, soybean oil, and corn oil contain around 46–61% polyunsaturated fatty acids (Adhvaryu et al. 2000; Ostrowska-Ligeza et al. 2010).

Table 1 Effect of heating rate on onset temperature of oxidation (T_o) and activation energy (E_a) of deodorizer distillate (DD).

Heating rate (°C/min)	T_o (°C)
2	$121.2^{e} \pm 0.2$
5	$128.8^{d} \pm 0.4$
10	$137.1^{c} \pm 0.4$
16	$142.2^{b} \pm 0.8$
20	$149.0^{a} \pm 0.5$
E_a (kJ/mol), calculated from Arrhenius equation	111.2 ± 2.1

Means ± s.d. followed by different superscripts are significantly different ($P < 0.05$).

Table 2 indicates that DD was a rich source of oil-soluble rice phytochemicals. The γ-oryzanol content in DD was 598.9 mg/100 g. The DD also contained high contents of tocotrienols, tocopherols, and phytosterols. The antioxidant capacity, expressed as the IC_{50}, was 2,301.6 μg/mL. However, the acid value was very high, i.e., 125.4 mg KOH/g (Table 2), which is unsuitable for food use.

Table 2 Chemical characteristics of rice bran oil deodorizer distillate (DD) obtained from physical refining process

Chemical constituents	Mean ± s.d.
Acid value (mg KOH/ g)	125.4 ± 1.0
γ-Oryzanol (mg/100 g)	598.9 ± 0.5
Tocotrienol contents (mg/100 g)	
α-Tocotrienol	77.7 ± 1.4
β-Tocotrienol	1,132.6 ± 16.1
γ-Tocotrienol	139.2 ± 3.2
δ-Tocotrienol	612.9 ± 15.5
Tocopherol contents (mg/100 g)	
α-Tocopherol	166.1 ± 1.2
γ -Tocopherol	57.2 ± 0.4
Phytosterol contents (mg/100 g)	
β-Sitosterol	970.8 ± 77.9
Campesterol	259.2 ± 24.3
Stigmasterol	310.8 ± 17.4
DPPH radical scavenging capacity (IC_{50}; μg/mL)	2,301.6 ± 45.3

Nonetheless, most FFAs were evaporated after the MD process, resulting in UMDs having low acid values (Table 3). The acid values of UMDs were reduced dramatically at distillation temperatures at and above 140 °C and reached the minimum values of less than 2.1 mg KOH/g. After FFAs were removed, some oil-soluble rice phytochemicals were concentrated in the UMD fractions. Table 3 indicates that γ-oryzanol content was the highest in UMD obtained at a distillation temperature of 140 °C ($P<0.05$).

Table 3 Effect of distillation temperature during molecular distillation on chemical contents of unevaporated fraction after molecular distillation (UMD)

Chemical constituents	Distillation Temperature		
	120 °C	140 °C	160 °C
Acid value (mg KOH/ g)	42.9^{a} ± 2.6	2.1^{b} ± 0.5	1.5^{b} ± 0.1
γ-Oryzanol (mg/100 g)	870.0^{b}±25.1	1070.3^{a}±4.1	1018.1^{a}±28.1
Tocotrienol contents (mg/100 g)			
α-Tocotrienol	150.5^{a}±5.2	151.6^{a}±6.7	81.8^{b}±6.4
β-Tocotrienol	1804.0^{a}±42.2	1206.4^{b}±179.0	471.6^{c}±6.3
γ-Tocotrienol	205.7^{b}±14.9	257.7^{a}±18.3	83.4^{c}±7.4
δ-Tocotrienol	955.1^{a}±20.3	429.8^{b}±72.8	152.7^{c}±21.2
Tocopherol contents (mg/100 g)			
α-Tocopherol	265.5^{b}±11.9	295.8^{a}±19.4	61.0^{c}±3.9
γ -Tocopherol	108.9^{a}±0.4	76.0^{b}±5.8	not detected
Phytosterol contents (mg/100 g)			
β-Sitosterol	2635.0^{b}±134.6	2778.7^{b}±190.4	3160.7^{a}±37.5
Campesterol	763.4^{b}±48.5	748.0^{b}±54.5	860.8^{a}±17.6
Stigmasterol	591.8^{b}±44.1	816.2^{a}±60.1	883.3^{a}±23.5
DPPH radical scavenging capacity (IC_{50}; µg/mL)	1053.9^{b} ±61.7	899.0^{c}±30.7	1385.8^{a}±1.1

Means in the same row, followed by different superscripts are significantly different (P<0.05).

Tocotrienols were the main tocols in DD and all UMD samples. Tables 2 and 3 indicate that β-tocotrienol was the most abundant isomer, followed by δ-tocotrienol, γ-tocotrienol, and α-tocotrienol, respectively. Both tocopherols and tocotrienols were effectively concentrated at a low distillation temperature of 120 °C. As distillation temperature increased to 160 °C, all tocol concentrations in UMDs were dramatically decreased (P<0.05). The chromatogram in Figure 2 indicates that a high distillation temperature of 160 °C reduced some unsaponifiable matter, i.e., tocols having a retention time between 12–20 min, shown as a lower number of peaks in UMD obtained after distillation at 160 °C.

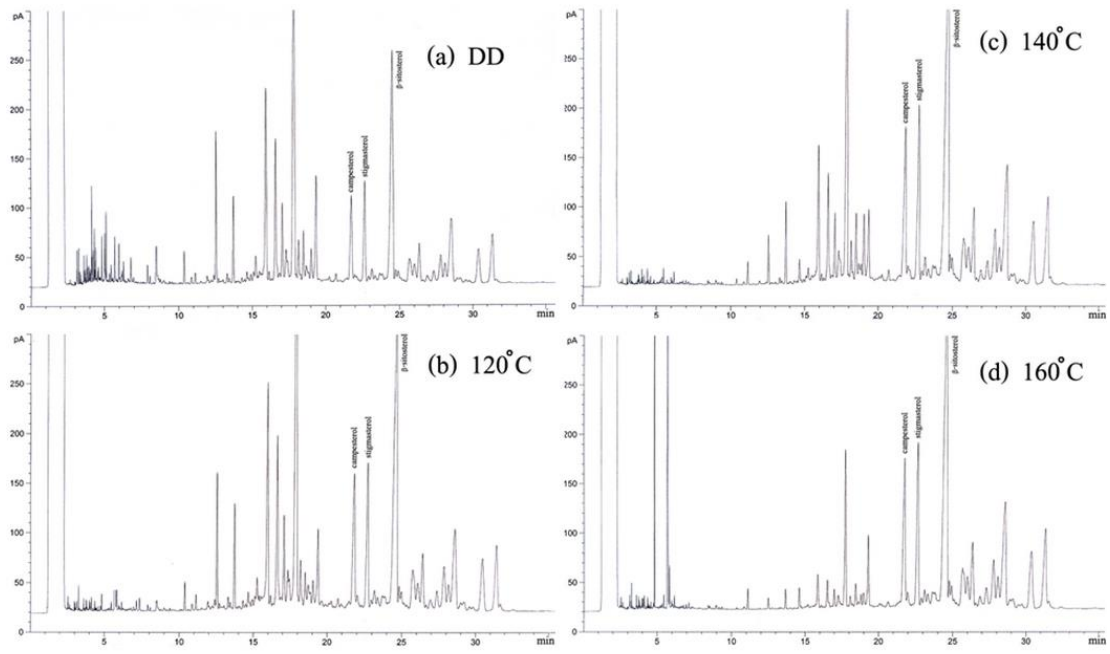

Figure 2 Effect of distillation temperature on phytochemical profiles of (a) deodorizer distillate, and unevaporated fractions obtained after molecular distillation at (b) 120 °C, (c) 140 °C and (d) 160 °C.

Among all four isomers of tocotrienol, more of the δ isomer was lost during MD. More α isomer, however, was retained at a high distillation temperature than the others. The variation in the reduction of different isomers may be due to the differences in thermal stability and boiling point of each isomer. The boiling point of each isomer at 760 mmHg was predicted using ACD/PhysChem Suite software from ACD/Labs (Toronto, Canada); the α-tocotrienol isomer had a boiling point of 542 °C, while δ-tocotrienol had a lower boiling point of 517 °C (The Royal Society of Chemistry, 2013). The difference in the boiling point could be due to the different structure of the chromanol head group, of which α-tocotrienol has three methyl-substituted groups, whereas δ-tocotrienol has only one methyl-substituted group. The increase in methyl-substituted groups on the chromanol head group likely increased intermolecular forces. Consequently, the α-tocotrienol isomer has a higher boiling point compared with the δ-tocotrienol isomer and was retained to a great extent when MD was operated at 140 °C and 160 °C.

The MD process in the present study was performed under high vacuum pressure (i.e., 0.1 Pa). As a result, oxidation of tocols was likely minimized due to low O_2 content. Verleyen

et al. (2001) reported that the headspace pressure and O_2 concentration above the α-tocopherol in triolein hardly influenced the degradation of α-tocopherol at a high temperature of around 180–260 °C and reduced pressure of 400–4,000 Pa. Therefore, it is most likely that the loss of tocols (Table 3) was mainly due to evaporation rather than thermo-oxidative degradation since the high vacuum pressure was used in the pilot-scale MD unit investigated in this study. This was due to the thermal stability of DD against the high temperature, and a very low pressure (0.1 Pa) used during the MD process investigated in the current study. Phytosterols and γ-oryzanol, however, had higher boiling points than the tocols, and thus more were concentrated at higher distillation temperature.

Low contents of tocols when the DD was distilled at 160 °C, and low γ-oryzanol contents when the distillation was carried out at 120 °C, significantly reduced the DPPH radical scavenging capacity ($P<0.05$). The values of IC_{50} of UMD obtained after distillation at 160 and 140 °C were different (Table 3). A distillation temperature of 140 °C was then used in a further investigation as the source of phytosterols, tocols, and γ-oryzanols to be encapsulated due to the high content of γ-oryzanol and reasonably high tocols.

The constituents from UMDs obtained at different distillation temperatures contained TAGs the most, followed by DAGs or MAGs, depending on distillation temperature. The HPTLC chromatograms showed only triolein, diolein, and monoolein as compared to the standards (Figure 3).

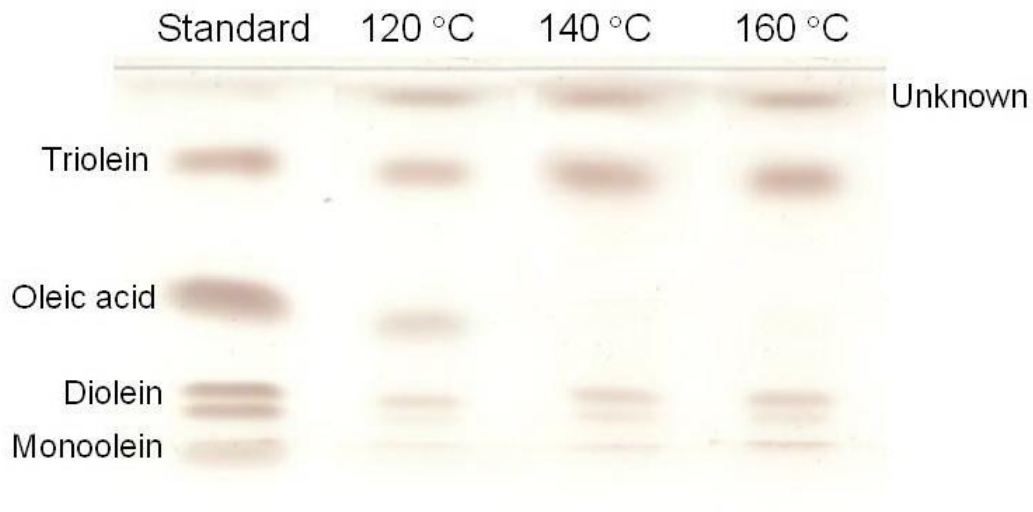

Figure 3 Effect of distillation temperature during molecular distillation on the profile of non-polar compounds of the unevaporated fractions (UMD), determined by high-performance thin-layer chromatography.

Densitometric analysis revealed differences in the ratios of non-polar fractions of FFA: MAG: DAG: TAG in UMDs obtained after distillation at different temperatures ($P<0.05$; Table 4). The increase in the distillation temperature from 120°C to 140°C markedly reduced FFA content. TAGs remained at a level of more than 50% in all UMDs, regardless of distillation temperature. The increase in temperature from 140°C to 160°C during the MD process, however, did not significantly change the γ-oryzanol content.

Table 4 Effect of distillation temperature during molecular distillation on the mass ratios of non-polar fractions and γ-oryzanol content in the unevaporated fraction (UMD).

Constituents	Distillation temperature during molecular distillation		
	120 °C	140 °C	160 °C
Mass ratios of non-polar fractions			
Free fatty acid	$0.20^{a} \pm 0.04$	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
Monoacylglycerol	$0.30^{a} \pm 0.05$	$0.10^{b} \pm 0.00$	$0.30^{a} \pm 0.01$
Diacylglycerol	$0.00^{c} \pm 0.01$	$0.20^{a} \pm 0.01$	$0.10^{b} \pm 0.02$
Triacylglycerol	$0.50^{c} \pm 0.00$	$0.70^{a} \pm 0.01$	$0.60^{b} \pm 0.01$

Mean values in the same row, followed by different superscripts are significantly different ($P<0.05$).

2.3.2 Physical characteristics of the unevaporated fractions (UMDs) obtained after molecular distillation

Figure 4 illustrates the thermograms of UMDs obtained after the molecular distillation (MD) process at different temperatures, i.e., 120, 140, and 160°C, at 0.1 Pa. The UMDs obtained at distillation temperatures of 140 and 160°C started melting at (-)46 °C. However, the UMD obtained at a distillation temperature of 120 °C started melting at (-)29 °C. All UMDs completely melted at 38°C. Similar thermograms of UMDs obtained after distillation at 140 and 160°C suggested that both UMDs had similar constituents, but they were different from those of the UMD obtained after the MD process at 120°C. Unlike the UMDs obtained from distillation at high temperatures, the UMD obtained after distillation at 120°C showed an endothermic peak between around (-)29 and (-)9°C.

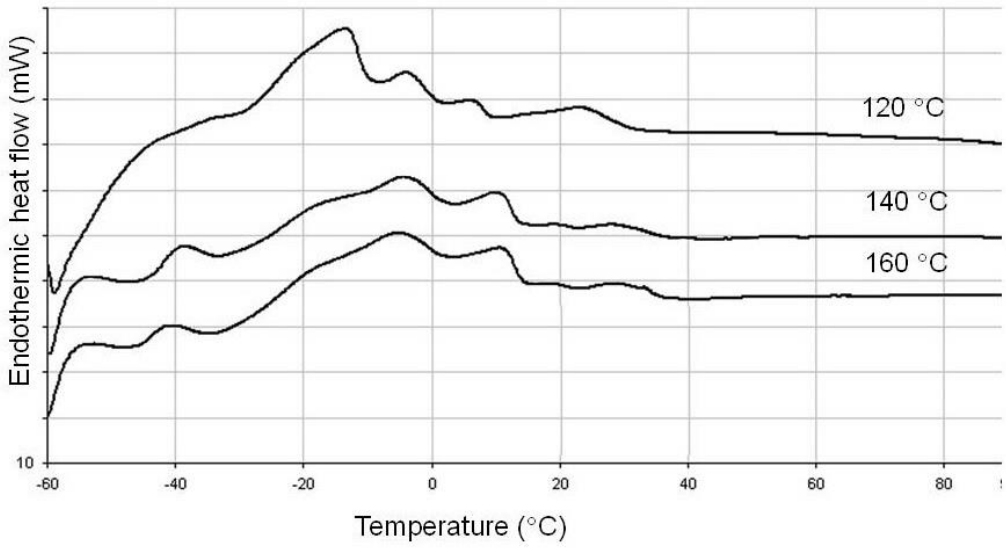

Figure 4 Effect of distillation temperature during molecular distillation on melting profiles of the unevaporated fraction (UMD).

The endothermic peak was most likely residual FFAs present in the UMD; HPTLC chromatograms confirmed this as a band of oleic acid, which was found only in the UMD obtained after distillation at 120°C (Figure 3). The UMD obtained after distillation at 140 °C (UMD_{140}) was then used in further investigations as the source of mono- and diacylglycerols from rice bran source on physical characteristics of RBO blends since the UMD_{140} contained no FFA, had high γ-oryzanol content, MAG, and DAG.

2.3.3 Effect of surfactants on thermal properties and apparent viscosity of RBO and RBO-AMF blends

The commercial MDG contained 62.39% palmitic acid, according to manufacturer datasheet. The commercial MDG had a high content of saturated fatty acid, while the majority of the MAG and DAG in the UMD was monounsaturated fatty acid of oleoyl esters. Thermograms of RBO containing UMD and/or commercial MDG are shown in Figure 5. The addition of UMD and/or commercial MDG did not significantly change the melting characteristics of RBO, although they were different in terms of chemical composition and melting point. The melting point range of UMD was between (-)46.2 to 37.8°C (Table 2), while

the melting point range of commercial MDG was between 45-65°C (results not shown). The melting temperature of RBO was within a range of (-)30 to 5°C.

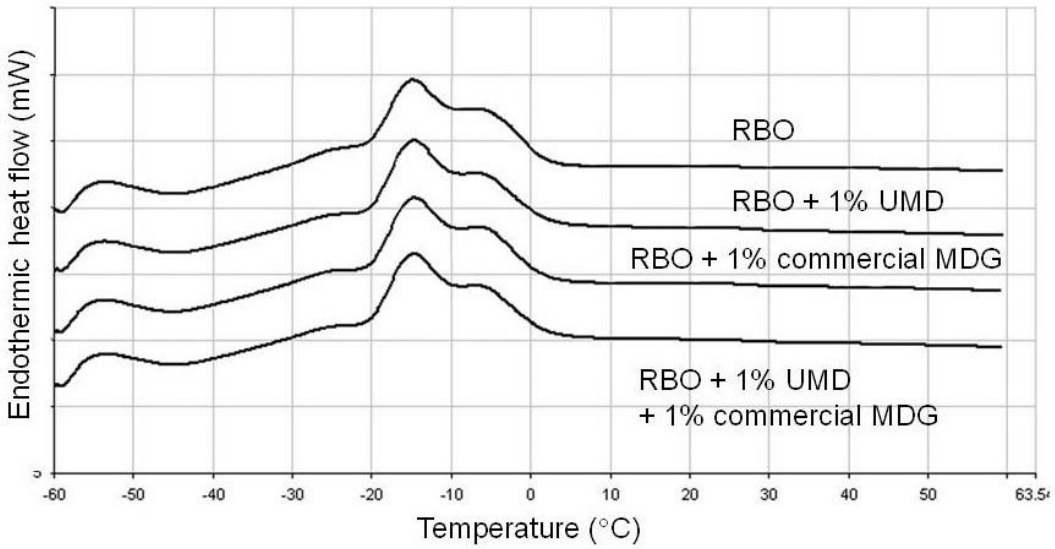

Figure 5 Effect of surfactant addition on the melting profiles of rice bran oil (RBO). The surfactants used were the unevaporated fraction after molecular distillation at 140 °C (UMD) and a commercial mono- and diacylglycerol (MDG).

From practical standpoints, physically refined RBO was prone to clouding when stored at low temperatures (15 and 25°C) as in the refrigerator or in the temperate zone, observed as the solid fat content (SFC) at low temperature (Table 5). Although the melting profiles of RBO supplemented with UMD and commercial MDG were not significantly different, the addition of UMD and MDG slightly increased SFC of RBO at 15°C ($P<0.05$). The presence of MDG slightly increased the SFCs of RBO at 25°C ($P<0.05$), indicating that commercial surfactant could induce nucleation of solid fat crystal shown as clouding defect in the liquid oil. However, such crystallization of solid fat was too low to form a fat crystal network between 15-35°C.

Table 5 Effect of surfactant addition on the onset of melting (To) and solid fat content (SFC) of rice bran oil (RBO). Surfactants used were the unevaporated fraction after molecular distillation at 140 °C (UMD) and commercial mono- and diacylglycerol (MDG).

Treatments	RBO	RBO +1% UMD	RBO +1% commercial MDG	RBO + 1% UMD + 1% commercial MDG
To (°C)	$-30.6^{a} \pm 1.0$	$-28.5^{a} \pm 1.2$	$-28.6^{a} \pm 1.1$	$-27.7^{a} \pm 1.6$
Te (°C)	$4.9^{a} \pm 0.7$	$4.9^{a} \pm 0.3$	$5.4^{a} \pm 0.6$	$5.0^{a} \pm 0.4$
SFC at 15 °C	$0.7^{c} \pm 0.0$	$0.8^{b} \pm 0.1$	$0.8^{ab} \pm 0.1$	$0.9^{a} \pm 0.1$
SFC at 25 °C	$0.4^{b} \pm 0.0$	$0.5^{ab} \pm 0.0$	$0.6^{a} \pm 0.0$	$0.6^{a} \pm 0.1$
SFC at 35 °C	$0.1^{a} \pm 0.0$	$0.1^{a} \pm 0.0$	$0.1^{a} \pm 0.01$	$0.1^{a} \pm 0.0$

Mean values (± standard deviation) in the same row, followed by different superscripts are significantly different ($P<0.05$).

The addition of UMD did not affect the apparent viscosity of RBO and mixed RBO-AMF at the ratio of 75:25. However, the addition of commercial MDG increased the apparent viscosity of RBO and RBO-AMF slightly ($P<0.05$; Table 6), which suggested that the solid fat particles s induced by commercial MDG, despite minute amount, could induce nucleation that resisted the liquid flow of RBO observed as a slight increase in viscosity of RBO-MDG blend. However, the presence of UMD caused a minute amount of SFC to be retained at 25°C (Table 5), it did not affect the viscosity of RBO at SFC similar to that of MDG (Table 4).

Table 6 Effect of surfactant addition on apparent viscosity at 25 °C of rice bran oil (RBO) and rice bran oil–anhydrous milk fat (RBO-AMF) blended at a ratio of RBO to AMF of 75:25. Surfactants used were the unevaporated fraction after molecular distillation (UMD) obtained at 140 °C and commercial mono- and diacylglycerol (MDG).

Treatments	Apparent viscosity (mPa·s)
RBO	$79.2^{b} \pm 3.5$
RBO + 1% UMD	$75.9^{b} \pm 1.7$
RBO + 1% commercial MDG	$88.6^{a} \pm 3.2$
RBO-AMF	$75.9^{b} \pm 9.4$
RBO-AMF + 1% UMD	$73.6^{b} \pm 2.3$
RBO-AMF + 1% commercial MDG	$85.6^{a} \pm 2.8$

Mean values (± standard deviation) followed by different superscripts are significantly different ($P < 0.05$).

This study indicated that deodorizer distillate (DD) from the physical refining process of rice bran oil was a rich source of tocotrienols, tocopherols, phytosterols, and γ-oryzanol. DD was quite thermo-oxidatively stable for the MD process under the operating conditions used in the current study. Nevertheless, the distillation temperature during the MD process was a crucial processing parameter influencing the retention of rice phytochemicals in the unevaporated fraction in UMDs. The UMD, which contained high contents of γ-oryzanol, tocols, and phytosterols, as well as readily present rice surfactants, could further be used in further food processing.

บทที่ 3 การใช้สารพฤกษเคมีจากกระบวนการกลั่นด้วยเครื่อง Molecular Distillation เป็นสารลดแรงตึงผิวร่วมในการขึ้นรูปเวซิเคิลที่ละลายน้ำ

Abstract

The UMD obtained from the pilot-scale MD unit operated at 140 °C, 0.1 Pa, and a flow rate of 10.14 - 10.66 kg/h was used as a source of rice phytochemicals and co-surfactants during vesicle preparation using different surfactants, namely polyoxyethylene sorbitan monooleate (Tween80), soy lecithin, or sucrose palmitate. It was found that the vesicles formed by soy lecithin and sucrose palmitate were polydispersed and varied in size. However, the size of Tween 80/UMD vesicles ranged from 200 nm to 300 nm in phosphate-buffered saline (PBS) pH 7.0 suspensions, which could be used as a carrier for rice phytochemicals when used in the aqueous phase. The filtered sterile vesicle suspensions in PBS, a model drink, were stable within the temperature range of 4 to 37 °C and maintained the size range of 200 – 300 nm for 96 h. Results indicated that the Tween 80/UMD vesicle was able to carry 814 μg phytosterol/mL, 453 μg tocols/mL, and 200 μg γ-oryzanol/mL as maximum load without causing phase separation of the oil phase in the PBS after preparation and storage at low temperatures. The suspensions containing Tween 80/UMD vesicles could be uptaken by Caco-2 and THP-1 cells. At high concentration of 163 μg/mL phytosterol, 91 μg/mL tocols and 40 μg/mL γ-oryzanol, the vesicles reduced viability of Caco-2. Moreover, the vesicle showed potential immunomodulation properties in THP-1 macrophages.

3.1 Introduction

Rice bran oil is well known as healthy oil since it is composed of many nutraceuticals that possess many health benefits (Orthoefer, 2005). Many factors influence the immune system development, including the diets and nutritional status of the individual. Among nutrients, lipids have a crucial role in the immune system (Sierra *et al.*, 2005). The unsaponifiable components of RBO are, in part, responsible for the health effect of RBO. Sierra and colleague (2005) showed that RBO modulated the immune system by enhancing B-lymphocyte proliferation and T-helper 1-type (T_H1-type) cytokine such as Interleukin-2 (IL-2) or

Tumor Necrosis Factors- α (TNF-α) in mice. They also suggested that RBO may have antiallergenic properties due to the reduction of the T-helper 2 (TH2) cytokine Interleukin-4 (IL-4) and immunoglobulin E (IgE) levels in mice. Moreover, they also pointed out that γ-oryzanol may partly modulate the immune system.

Vitamin E is another RBO component that plays a crucial role in the normal function of immune cells. Yamada *et al.* (2002) suggested that tocotrienol and α-tocopherol modulate lipid metabolism and immune functions in aged Sprague-Dawley rats. Supplementation of vitamin E above currently recommended levels has been shown to improve immune functions in the aged rats, which include delayed-type hypersensitivity skin response and antibody production in response to vaccination (Meydani *et al.*, 2005). Vitamin E improved immune functions by mediating through the increased production of IL-2, leading to the enhanced proliferation of T cells, and through reduced production of prostaglandin E_2, a T-cell suppressive factor, as a result of a decreased peroxynitrite formation. The vitamin E-induced enhancement of immune functions in the aged animals was associated with significant improvement in resistance to influenza infection in aged mice and a reduced risk of acquiring upper respiratory infections in nursing home residents (Meydani *et al.*, 2005). Moreover, tocotrienol has been shown to contribute to the immunomodulation, antibody production, and resistance to the implanted tumor (Nesaretnam *et al.*, 2006).

Phytosterols component in RBO is well known as natural hypocholesterolemic agents. However, not only the cholesterol-lowering effect, phytosterol also showed other benefits such as protective effect in cancer and cardiovascular disease and immunological effects (Desai *et al.*, 2009). According to Calpe-Berdiel *et al.* (2007), phytosterols modulated the T-helper immune response *in vivo*, in part independently of their hypocholesterolemic effect in a setting of acute, aseptic inflammation in a mouse model. Moreover, β-sitosterol, a significant member of phytosterol, is effectively modulating the secretion of pro/anti-inflammatory cytokines and showed a beneficial effect in multiple sclerosis management without side effect related to statin therapy (Desai *et al.*, 2009).

However, the insolubility of these rice phytochemicals in the aqueous phase could lead to their limited use in aqueous food systems. In the present study, we proposed that the UMDs could be used in the fabrication of small vesicles that can disperse in water due to their indigenous phytosterols, MAG and DAG in the UMDs (Nukit et al. 2014; Sawadikiat and Hongsprabhas 2014). Amphiphilic molecules usually fabricate a closed spherical structure of vesicle in a bilayer membrane (Mollet and Grubenmann 2001). A well-known vesicle fabricated by phospholipids is called a liposome. Usually, the fabrication of a vesicle also requires cholesterol to stabilize the vesicular structure (Vemuri and Rhodes 1995), which would be replaced by rice phytosterols in the current study.

We hypothesized that the rice phytochemicals concentrated in the UMDs could act as co-surfactant and vesicle stabilizer. To our knowledge, the use of the unevaporated fraction (UMDs) – obtained after molecular distillation of DD from the physical refining process of rice bran oil – which contains a mixture of rice phytochemicals and surfactants, has received little attention and thus merits further investigation. The objectives of the present study were to explore the effects of UMDs in the fabrication of vesicles that were stable in the aqueous phase and explore if the human cell model could passively absorb such small-sized vesicles. The insights obtained could be used to enhance the utilization of co-products from rice bran oil refinery in the production of water-dispersible rice nutraceuticals.

3.2 Materials and methods

3.2.1 Materials

Deodorizer distillate (DD) from commercial production of physically refined rice bran oil was used as the raw material for the production of rice phytochemicals by a pilot-scale molecular distillation (MD) unit at Surin Bran Oil Co. (Surin, Thailand) in the unevaporated fraction (UMD) using process conditions as previously described. De-oiled soy lecithin (Solae, St. Louis, MO, USA) and sucrose palmitate (HLB 16) (P1670; Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan) were kindly provided by Rama Production Co., Ltd., Thailand, and Caltech Corp., Ltd., Thailand, respectively. Polyoxyethylene sorbitan monooleate (Tween 80) was purchased from Sigma-Aldrich. Other chemical reagents were of analytical grade.

Heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) cell passage number between 30-54 (American Type Culture Collection, Rockville, MD, USA) and the human monocytic leukemia cell line (THP-1, American Type Culture Collection, Rockville, MD, USA) were used in this study.

3.2.2 Effect of UMD and commercial surfactants on vesicle fabrication

Vesicles were prepared using the Bangham method, described by Takahashi et al. (2007). Commercial surfactants – soy lecithin, Tween 80, or sucrose palmitate (0.05 to 0.16 g) – and UMD samples (0.04–0.15 g) obtained from MD operated at 140 °C were dissolved in 8 mL chloroform in a screw-cap test tube and mixed thoroughly. The solvent was evaporated to dryness under a N_2 stream. The residual solvent was further dried overnight in a hood. Then 8 mL of phosphate-buffered saline (PBS) pH 7.3 was added to the thin film of surfactant/UMD and heated at 55–60 °C for 10 min. The test tube was then shaken vigorously using a vortex mixer for 5 min. The solid concentration of surfactant/UMD vesicles, having different ratios of surfactant to UMD of 1:0, 1:0.25, 1:1, 1:2, 1:3, 1:4 and 1:5 in the suspension, was 2.5% (w/v) in PBS.

Determination of vesicle size distribution in PBS

The size distribution of surfactant vesicles and surfactant/UMD vesicles in PBS was analyzed by a Zetasizer Nano-ZS (Zen 3600; Malvern Instruments Ltd., Worcestershire, UK). Only treatments capable of UMD holding capacity (no observable phase separation between the aqueous phase and oil phase) were used.

Storage stability of vesicles in PBS

Surfactant/UMD vesicle suspensions in PBS were aseptically filtered to sterile the suspensions using a 0.22 μm nitrocellulose membrane (MF-Millipore™ membrane filter; Millipore, Billerica, MA, USA). The sterile suspensions were kept at 4–5 °C or 37 °C for 0, 24, 48, 72, and 96 h before determination of size distribution using a Zeta Nano-ZS.

3.2.3 Biofunctional properties of Tween80/UMD vesicle in Caco-2 cell monolayer and THP-1 macrophages

Formation of Tween80-based vesicles containing different oil-soluble phytochemicals

The tween80-based vesicle was prepared by using the Bangham method as previously described, with or without the UMD. Extra virgin olive oil was also used as a lipid phase in the absence of UMD. β-Sitosterol and dioleoyl glycerol surfactants were tested for their ability to stabilize the vesicle and as a co-surfactant, respectively. Tween80-based vesicle composition in each treatment is presented in Table 7. The suspensions were prepared by dissolving the chemicals and UMD in 8 mL chloroform in a screw-cap test tube and mixed thoroughly. The solvent was evaporated to dryness under the N_2 stream. The residual solvent was further dried overnight in a hood. Then the thin film of surfactant-UMD was added with 8 mL of phosphate-buffered saline (PBS) pH 7.3 and heated at 55-60 °C for 10 min. The test tube was shaken vigorously using a vortex mixer for 5 min. The suspensions were tested for size distribution by using Zetasizer Nano-ZS (Zen 3600, Malvern Instruments Ltd., Worcestershire, UK) since the oil medium was changed from rice bran oil-based to virgin olive oil-based in the formulation without UMD. The experiments were carried out at Wageningen University and Research, The Netherlands.

Table 7 Formulation of Tween80-based vesicles

Treatment	Tween80 (surfactant) (mg)	β-Sitosterol (stabilizer) (mg)	Dioleoylglycerol (co-surfactant) (mg)	Olive oil (oil phase) (mg)	UMD (mg)
A	50	-	-	113.5	-
B	50		-	-	150
C	50	6.5	-	113.5	-
D	50	-	30	113.5	-
E	50	6.5	30	113.5	-

Stability of Tween80/UMD vesicle during in vitro gastrointestinal digestion

The prepared Tween80/UMD vesicles were exposed to various *in vitro* digestion steps, as previously described by Vreeburg *et al.* (2012). Briefly, 10 g of Tween80/UMD vesicle suspension (25 mg/mL) was mixed with 140 mM NaCl 5 mM KCL 10 mL in 50 mL tube and adjusted pH to 2 with 1 M HCl. A 0.667 mL of 40 g/L porcine pepsin in 0.1 M HCl was added to the suspension and incubated for 30 min at 37 °C. After incubation, 1 M $NaHCO_3$ was used to raise the pH to 5.8, then 0.95 mL of 40 g/L pancreatin in 0.1 M $NaHCO_3$, 40 g/L lipase in 0.1 M $NaHCO_3$ and 0.5 mL of bile salt were added to the suspension and pH was adjusted to 6.5 by 1 M $NaHCO_3$. The tube headspace was flushed with N_2 gas and incubated at 37°C for 30 min. The digestion was stopped by adjusting the pH to 7.5 using 1 M $NaHCO_3$. The digested sample was further analyzed for their size distribution by a Zetasizer Nano-ZS to estimate the stability of vesicles against the physiological conditions of the GI tract.

Influence of Tween80/UMD vesicle on the viability of Caco-2 monolayer

The colorimetric MTT metabolic activity assay was used to determine the viability of Caco-2 cells monolayer after they were exposed to Tween80/UMD vesicles. Briefly, Caco-2 cells were seeded at a density of 1.95 X 10^5 cells per insert in a 24 transwell plates format and grew to confluence for 21 d. The culture medium was replaced every other day. The transepithelial electrical resistance (TEER) value was measured by using MilliCell-ERS Ω-meter to assess the integrity of Caco-2 monolayers. Caco-2 monolayers with a TEER value above 200 Ω/cm^2 were used for viability tests. The 21-days old Caco-2 monolayers were exposed to Tween80/UMD vesicle at concentrations 0.1, 1.0, and 5.0 mg/mL for 3 h at 37 °C in 5% CO_2 humidified incubator. After incubation, medium in basolateral and apical chambers was removed, and the cells were washed with fresh PBS, added with 50 µL of 0.25% trypsin-EDTA into the transwell apical chamber and incubated for 10 min at 37°C to detach cells from transwell membrane. The 100 µL of DMEM medium was added into the apical chambers to inhibit trypsin activity.

The detached cell suspension was transferred to 96-wells V bottom plate, centrifuged at 500 relative centrifugation force (rcf) for 5 min. The supernatant was removed by using a multi-channel micropipette. The sediment cells were resuspended in 100 µL of MTT solution containing 0.5 mg/mL MTT in DMEM and 10% FBS and incubated for 2 h at 37°C in 5% CO_2 humidified incubator. The plate was centrifuged at 500 rcf for 5 min, and the MTT solution

was discarded using a multi-channel micropipette. A mixture of 50 μL of DMSO: ethanol (1:1) was added into each well, and the plate was mildly shaken for 5 min. The absorbance was measured at 570 nm using a microplate reader (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland). All experiments were performed in duplicate. Viability of Caco-2 cells in PBS in a similar amount as the treatment groups was used as a control.

Influence of Tween80/UMD vesicle on the viability of THP-1 macrophage

The human monocytic leukemia cell line (THP-1, American Type Culture Collection, Rockville, Md.) was grown in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (P/S). The THP-1 monocytes were differentiated into macrophages by the addition of phorbol 12-myristate 13 acetates (PMA, Sigma). In brief, 0.5 ml (5 x 10^5 cells) or 82 μL (8.2 x 10^4 cells) of cell suspension containing PMA (final concentration 100 ng/ mL of cell suspension) were seeded into 24 wells and 96 wells cell culture plate, respectively, and incubated in an incubator humidified with 5% CO_2 at 37 °C for 48 hrs. After incubation, undifferentiated monocyte and PMA were discarded, and differentiated macrophages were washed twice with RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (P/S) and rest for 24 hrs before the experiment.

The colorimetric MTT metabolic activity assay was used to determine the viability of THP-1 macrophage after exposure to Tween80/UMD vesicles. The differentiated macrophages were treated with 100 uL of the sample, i.e., PBS (control) and Tween80/UMD vesicles at concentrations 0.01, 0.1, and 1.0 mg/ml into the designed well and incubated for 24 hrs. After incubation, the stimulating mediums were removed, and 100 μL of MTT solution (0.5 mg/ml) were added to each well and incubated for 2 hrs. MTT solutions were discards and 30 μL DMSO: ethanol (1:1 v/v) were added and mildly shake the plate for 5 minutes and read absorbance at 570 nm.

Effect of oil-soluble phytochemicals in Tween80-based vesicles on THP-1 gene expression

Various types of Tween80-vesicles were prepared by using the Bangham method as described previously. Tween80-vesicle containing UMD was designated as a Tween80/UMD vesicle (UV). The 8 mL of vesicle suspensions contained Tween80 (50 mg), dioleoylglycerol

(30 mg), trioleoylglycerol (113.5 mg) and β-sitosterol (6.5 mg) was designated as Tween80/ dioleoylglycerol/trioleoylglycerol/ β-sitosterol vesicle (EV). Tween80/dioleoylglycerol/ trioleoylglycerol/β-sitosterol vesicle containing either α-tocopherol (0.44 mg/8 mL) or γ-oryzanol (1.6 mg/8 mL) was designated as Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/tocopherol vesicle (TV), and Tween80/dioleoylglycerol/trioleoylglycerol/ β-sitosterol/γ-oryzanol vesicle (OV), respectively. The treatments were designed to elucidate the constituent in the UMD was most likely responsible for the immune responses of THP-1 macrophase.

The differentiated THP-1 macrophages were stimulated for 24 h with Tween80-vesicles at concentrations of 0.01, 0.1, and 1 mg/mL. Stimulated macrophages were harvested, and immunomodulatory activities were investigated by measuring gene expression. The expression of genes encoding for pro-inflammatory cytokines, i.e., IL-1β, IL-8 and TNF-α, were evaluated.

The gene expression by real-time qPCR was conducted by isolation of total RNA by using the RNeasy mini kit (Qiagen, USA) with RNase-free DNase (Qiagen, USA) treatment for 15 min. The 1% agarose gel was used for checking the purity of the RNA sample, and Nanodrop was used to calculate the concentration of RNA in the sample. The complementary DNA (cDNA) was synthesized from isolated RNA sample with an iScript cDNA synthesis kit (Bio-Rad, USA). cDNA 200 ng was mixed with 10 μL of IQ™ SYBR Green Supermix (Bio-Rad, USA) and primer pair in 20 μL reaction volume. The mixture was preheated at 95 °C for 90 s, followed by PCR for 40 cycles, denaturing at 95 °C for 10 s, annealing at 58 °C for 10 s, elongation at 72 °C for 15 s, and finally elongation at 72 °C for 2 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen for normalization. The values expressed as fold change relative to the value at time point zero, calculated as ΔΔCt as described in Eq. 7.

$$\Delta\Delta Ct = 2^{-(\Delta Ct\ GAPDH - \Delta Ct\ sample)} \quad (7)$$

3.2.4 Statistical analysis

Results were subjected to analysis of variance (ANOVA) with confidence interval set at 95% ($P < 0.05$) using the statistical software program SPSS for Windows version 12 (SPSS Inc., Chicago, IL, USA). Differences among means were differentiated using Duncan's multiple range test at $P < 0.05$.

3.3 Results and discussion

3.3.1 Effect of commercial surfactants on the formation of vesicles containing UMD

Vesicles containing UMD in the presence of a commercial surfactant, i.e., soy lecithin, Tween 80, and sucrose palmitate, were fabricated. In the absence of UMD, soy lecithin vesicles showed a bimodal size distribution, with a high % intensity of the particles having sizes around 170 nm and 970 nm (Figure 6a). Incorporation of UMD into soy lecithin/UMD vesicles using lecithin to UMD ratio of 1:0.25 (w/w) resulted in bimodal size distribution with smaller particle sizes of 60 nm and 380 nm. Further increasing the UMD ratios in lecithin/UMD vesicles to 1:1, 1:2, and 1:3 resulted in polydispersed colloidal suspensions, which indicated the instability of soy lecithin/UMD vesicles in PBS.

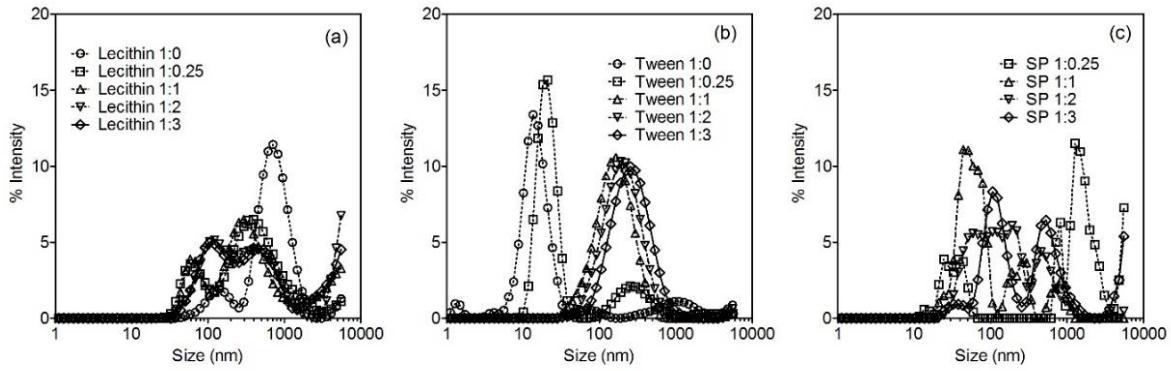

Figure 6 Particle size distributions of surfactant/UMD vesicles prepared using different ratios of surfactant to UMD: (a) soy lecithin/UMD vesicles, (b) Tween 80/UMD vesicles, and (c) sucrose palmitate (SP)/UMD vesicles.

The size distribution of vesicles fabricated using Tween 80 in PBS showed bimodal distribution (Figure 6b). Most Tween 80 vesicles had a size around 20 nm, whereas a much lower population had a diameter of around 1,000 nm. The incorporation of UMD into Tween 80/UMD vesicles at a Tween 80 to UMD ratio of 1:0.25 (w/w) resulted in two major groups of vesicles. The first group with a high % intensity had a size of about 20 nm. The second group of vesicles, with lower % intensity, had a size range between 200 to 300 nm. When the UMD ratio was increased to 1:1, 1:2, and 1:3 (w/w), the Tween 80/UMD vesicles showed monomodal size distribution, with the majority having sizes of around 200 to 300 nm, and no lipid separation was observed, suggesting that the fabricated Tween 80/UMD vesicles could hold a high content of UMD, indicating the potential for encapsulating rice phytochemicals concentrated from DD by the MD process. Increasing the ratio of UMD to 1:4 and 1:5, however, resulted in the aggregation of Tween 80/UMD vesicles at a size of around 3000–4000 nm (results not shown), which separated from the aqueous phase after 3 h of preparation. In this study, Tween 80/UMD vesicles had an average diameter higher than that of micellar Tween 80 of 35 Å (Amani et al. 2011).

Sucrose palmitate (SP), however, gelled in PBS at the concentration used in the current study. Therefore, the size distribution of vesicles in PBS could not be determined. Nonetheless, incorporation of UMD into SP/UMD vesicles resulted in suspensions instead of gel. However, the SP/UMD vesicles showed polydispersed distribution, ranging from submicron to micrometer size (Figure 6c).

The more exceptional ability of Tween 80, compared with soy lecithin and sucrose palmitate, in aiding the formation of surfactant/UMD vesicles may be due to the structure of the surfactant and the composition of the UMD itself. The UMD was composed of triacylglycerol, DAG, and MAG, with an oleoyl chain as the major esterified fatty acid (Nukit et al. 2014). Therefore, the hydrophobic tail of the oleoyl chain in Tween 80 molecules and the oleoyl chain in the UMD reported in the current study may self-assemble into stable vesicles.

Despite the highly negatively charged groups of phosphatidylcholine and phosphatidylethanolamine, which generally favor liposome or vesicle formation by soy lecithin, the major acyl groups of commercial soy lecithin are usually linoleoyl (65.9 %) and oleoyl (10.6%) chains (Magil et al. 1981). The conjugated double bond of linoleoyl and oleoyl chains may not favor the formation of a thermodynamically stable bilayer of vesicles. Similar results were observed in SP/UMD vesicles, of which the esterified palmitate was the major acyl chain.

After filtered sterilization, the size distribution of Tween 80/UMD vesicles in PBS showed the monomodal distribution of around 200 nm, with a narrow size range. The filtered sterile suspensions were quite stable at a low temperature of 4–5 °C and 37 °C for 0, 24, 48, 72, and 96 h (Figure 4). After storage, the average vesicle diameter remained around 200 nm (Figure 7). The Tween 80/UMD vesicles, however, had a wider size range compared to that before storage at 0 h, possibly due to vesicle flocculation. Nevertheless, Tween 80/UMD vesicles in PBS showed potential use in the formulation of vesicle suspensions that were stable over a temperature range from chilled storage to body temperature.

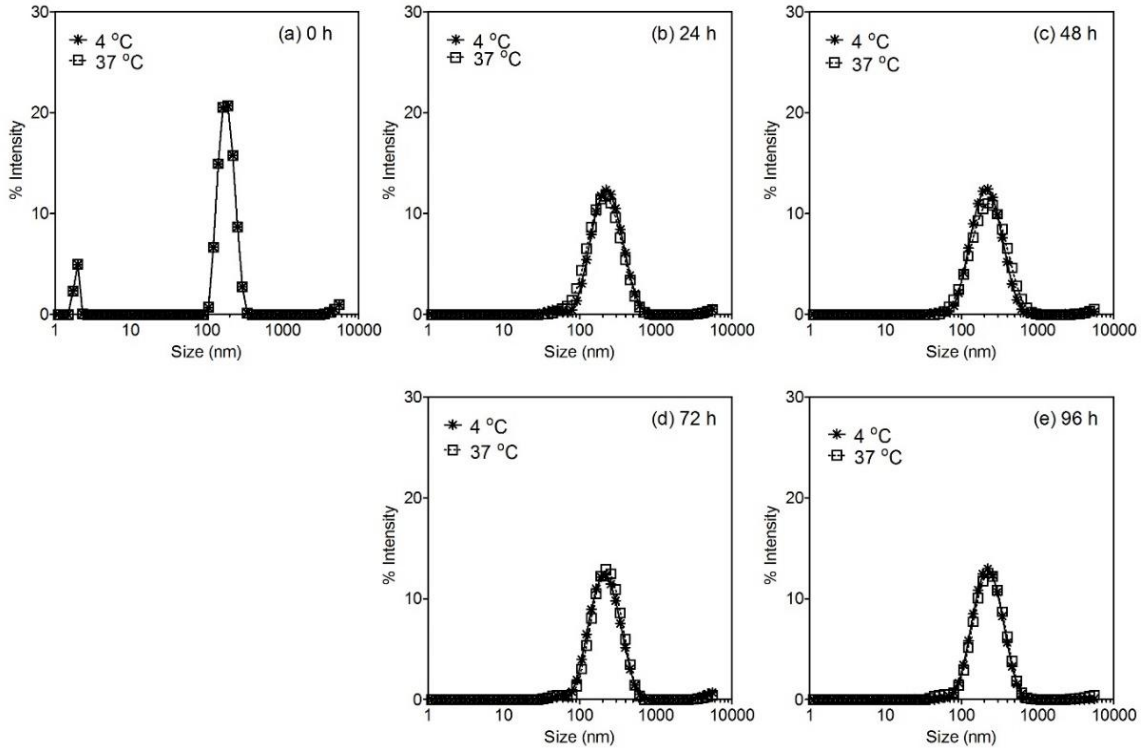

Figure 7 Effect of storage time and temperature on the particle size distribution of Tween 80/UMD vesicles stored at different temperatures.

3.3.2 Effect of phytosterol and diacylglycerol formulation on the stability of vesicle

Figure 8a illustrates that Tween80 alone could not stabilize Tween80/olive oil vesicle. The vesicles in PBS were polydispersed and had a size range of 20 nm to above 6 μm. On the other hand, UMD, which contained residual triacylglycerol, monoacylglycerol, diacylglycerol (Nukit *et al.*, 2014), as well as mixed phytosterols such as β-sitosterol, campesterol, stigmasterol, as well as tocols and γ-oryzanol (Sawadikiat et al. 2015), was able to maintain the average size of the vesicle ranged from 30 to 1,000 nm, with the average size of 200-300 nm (Figure 8b).

The vesicles containing Tween80, olive oil, and β-sitosterol (Figure 8c), and those containing Tween80, olive oil, and dioleoylglycerol (Figure 8d), showed polydispersed suspensions. Only the vesicles containing Tween80, olive oil, dioleoylglycerol, and β-sitosterol (Figure 8e) maintained the size range of 50-1500 nm with the highest % intensity at 400 nm.

The result confirmed that both dioleoylglycerol and β-sitosterol were essential in the formation of Tween80-based vesicles. However, the size of the vesicles shown in Figure 8b (Tween80/UMD vesicles) was smaller than vesicles shown in Figure 8e. Both vesicle types contained Tween80, olive oil, dioleoylglycerol, and β-sitosterol. The Tween80/UMD vesicles contained additional surfactants like monooleoylglycerol in the UMD (Nukit et al., 2014). This result suggested that the monoacylglycerol in UMD could also act as co-surfactant along with dioleoylglycerol and be responsible for the fabrication of vesicles having a size range of 200-300 nm. Such a small size could help enhance the cellular uptake of the vesicles and phytochemicals through passive diffusion.

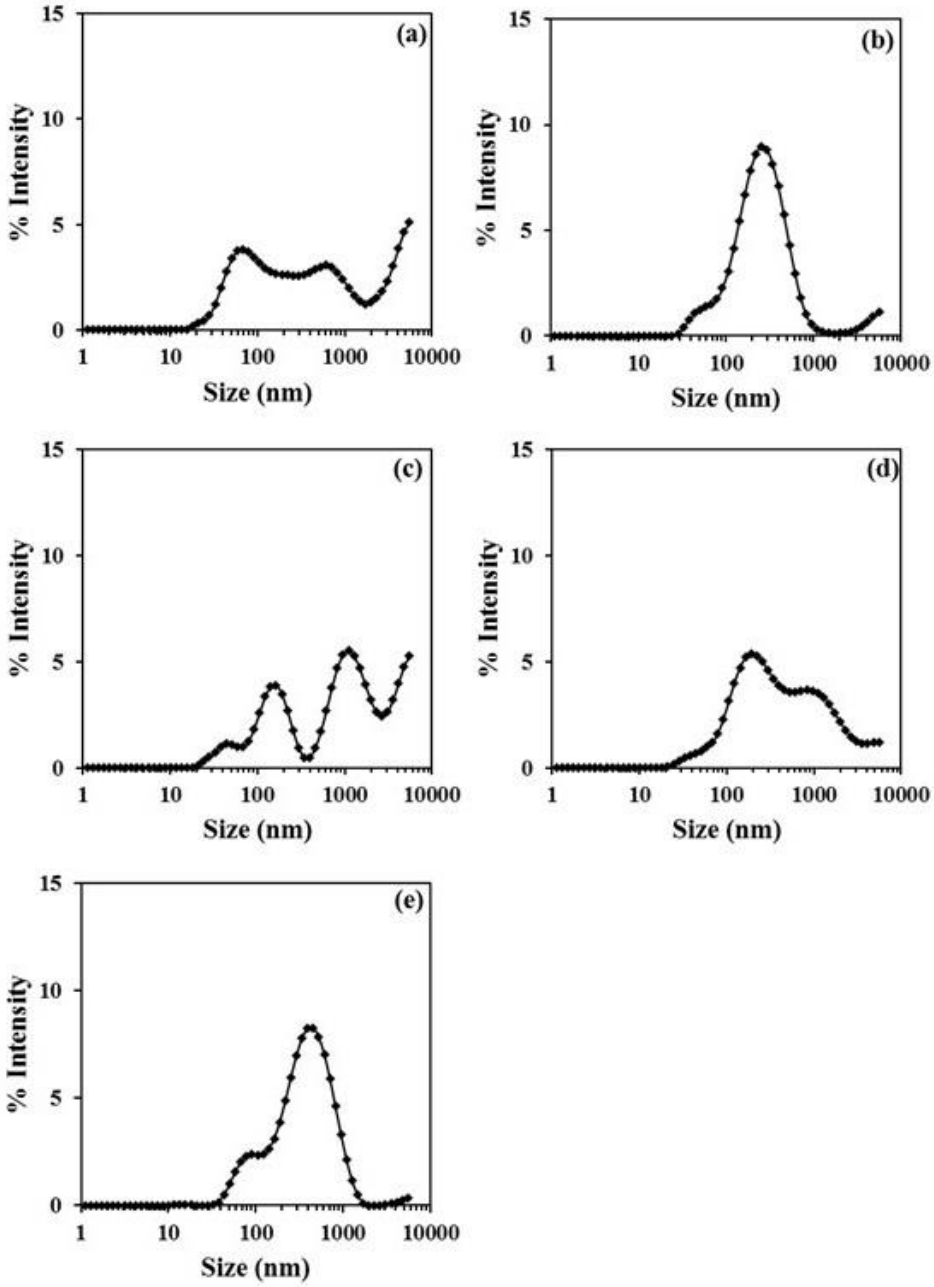

Figure 8 Effect of formulation on the size distribution of vesicles: (a) Tween80/olive oil; (b) Tween80/UMD (RBO-based); (c) Tween80/olive oil/β-sitosterol; (d) Tween80/olive oil/dioleoylglycerol, and (e) Tween80/olive oil/dioleoylglycerol/β-sitosterol.

The *in vitro* digestion model was used to investigate the effect of physiological conditions in the human gut on the stability of Tween80/UMD vesicles. Table 8 showed that the average particle size of Tween80/UMD vesicles significantly increased to 300 nm after the suspension from the stomach condition (very acidic condition) was adjusted to pH 5.8 ($P<0.05$). The size of the Tween80/UMD vesicle remained around 300 nm after the addition of the intestinal enzymes and bile salt, which suggested that increasing of pH close to the neutral pH and intestinal condition induced vesicle flocculation.

Table 8 Effects of physiological conditions during digestion on the average size of Tween80/UMD vesicles

In vitro digestion steps	Average size (nm)
Undigested vesicle in PBS pH 7.4	$237^{bc} \pm 6$
Added salt solution	$228^{c} \pm 5$
Adjusted to pH of 2	$272^{abc} \pm 38$
Digested with pepsin	$270^{abc} \pm 14$
Adjusted to pH of 5.8	$309^{a} \pm 50$
Digested with intestinal enzymes and bile salt	$303^{a} \pm 29$
Adjusted to pH of 6.5	$261^{abc} \pm 21$
Adjusted to pH 7.5	$294^{ab} \pm 48$

Mean ± standard deviation

3.3.3 Cytotoxicity of Tween80/UMD vesicle in Caco-2 cell monolayer and THP-1 macrophage

Figure 9 showed the viability of 21 day–old Caco-2 cell monolayers after 3 h incubation with Tween80/UMD vesicles at different concentrations. The viability of Caco-2 cell was calculated in relation to PBS treated cells (control). The presence of Tween80/UMD vesicle up to 2 mg/mL slightly increased cell viability. However, increasing Tween80/UMD vesicle concentration up to 4 mg/mL significantly lowered the viability of Caco-2 cells to 90% ($P<0.05$). Increasing Tween80/UMD vesicle to high concentration at 5 mg/mL further decreased cell viability down to 80%. In this study, the Tween80 concentration in the tested sample was around 0.125 % (w/v). O'Sullivan *et al.* (2004) reported no cytotoxic effect of Tween80 in the differentiated Caco-2 cell after the cells were treated with Tween80 at 1 mL/L

(0.1 % w/v) concentration for 24 h. Later on, Lu and colleague (2014) showed that Tween80 was not toxic to Caco-2 cells when the concentration was increased to 0.125 %. Nonetheless, the cell viability of Caco-2 decreased when the concentration of Tween80 was increased to 0.25 % (Lu *et al.* 2014).

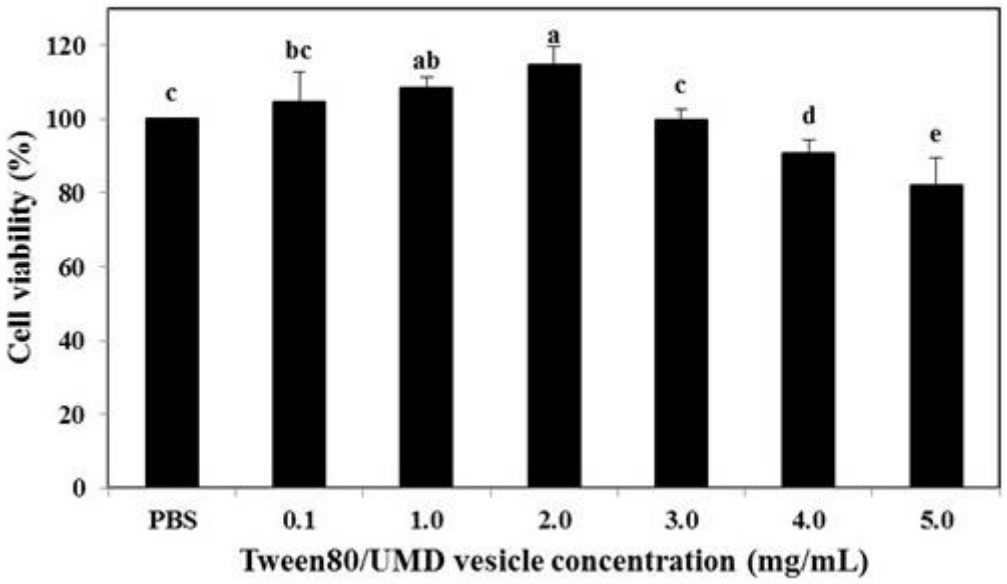

Figure 9 Effect of Tween80/UMD vesicles at the different concentrations on Caco-2 cell viability after 3 h incubation. Bars represent the standard deviation.

Tween80/UMD vesicle was also investigated for its potential cytotoxicity on THP-1 macrophages after incubation for 24 h. The viability of THP-1 macrophages was calculated as relative to cells grown in the media containing PBS as a control. Figure 10 indicated that the Tween80/UMD vesicle was not toxic to THP-1 macrophages at the concentration of 1.0 mg/mL. At this vesicle concentration, Tween80/UMD contained 32.6 μg/mL phytosterol, 18.1 μg/mL tocols and 8.0 μg/mL γ-oryzanol by calculation. According to Geys *et al.* (2010), Tween80 did not affect the viability of THP-1 macrophages at a concentration of 0.1 %, i. e. 1.0 mg/mL.

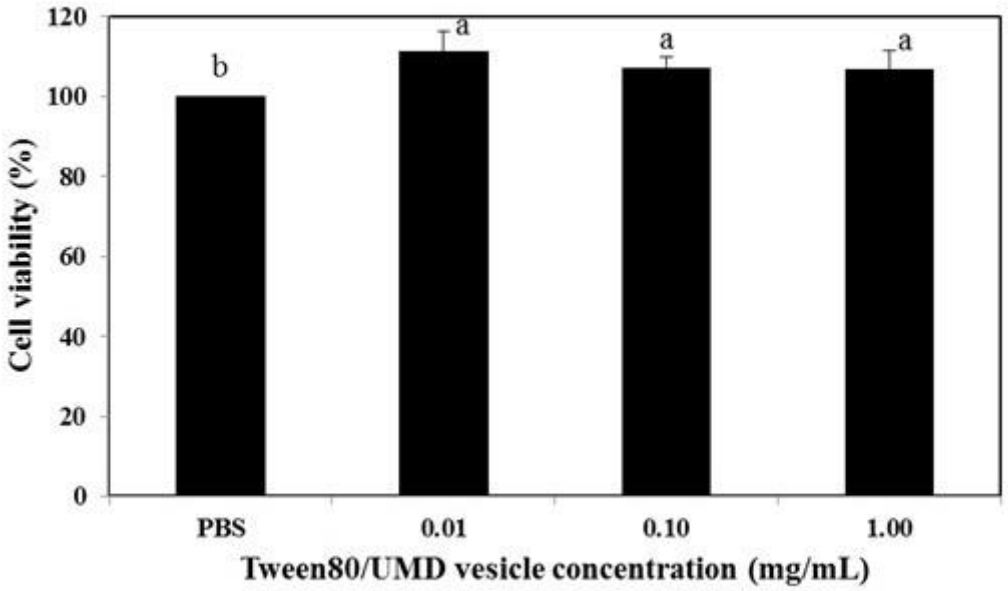

Figure 10 Effect of Tween80/UMD vesicle concentrations on the viability of THP-1 macrophages after 24 h incubation. Bars represent standard deviation.

The 21 day-old Caco-2 monolayer in transwells was exposed to Tween80/UMD vesicles at a final concentration in medium of 0.1, 1, and 5 mg/mL for 3 h. The integrity of the Caco-2 cell monolayer tight junction was monitored using TEER measurement. Transepithelial/transendothelial electrical resistance (TEER) is a quantitative technique to measure the integrity of tight junction dynamics in cell culture models of endothelial and epithelial monolayers. The TEER values are indicators of the integrity of the cellular barriers before they are evaluated for the transport of drugs or chemicals. Figure 11 showed the TEER values of all the treatments, including PBS. It was found that the TEER values gradually decreased over the three-hour incubation period.

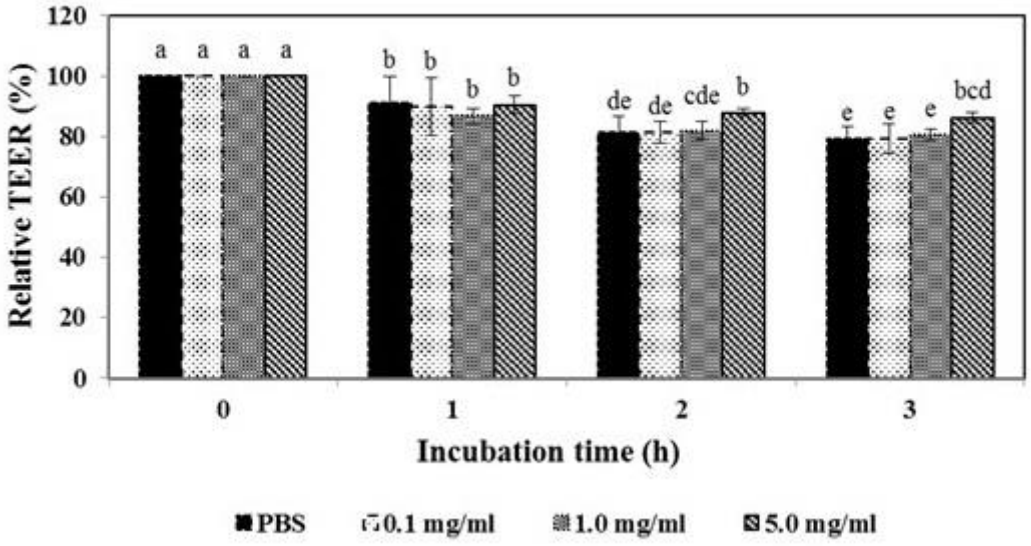

Figure 11 Effect of Tween80/UMD vesicle concentrations on the TEER-values of Caco-2 cell monolayer over incubation time of 3 h.

The Caco-2 cell monolayer could be sensitive to many factors, including the fluctuation of CO_2 and temperature during TEER measurement, which could lead to the reduction of TEER value. Moreover, the incubation time was too short to observe the TEER value change trend. For further investigation, the incubation time should be prolonged in order to investigate the influence of vesicle on the change in TEER value. TEER values are indicators for barrier models such as blood-brain barrier (BBB), gastrointestinal (GI) tract, and pulmonary transports. Variations in TEER value can also arise due to factors such as temperature, medium formulation, and passage number of cells and should be investigated how the Tween80/UMD vesicles are transported into the cells.

Tween80/UMD vesicles loaded with 6-coumarin were used to track the intracellular uptake of vesicles by Caco-2 cell monolayer. Figure 12 illustrates that Tween80/UMD vesicles were present in the cells but mostly on the cell surface. For better results, the nuclei and tight junction should be stained in order to indicate cell structure and vesicle localization.

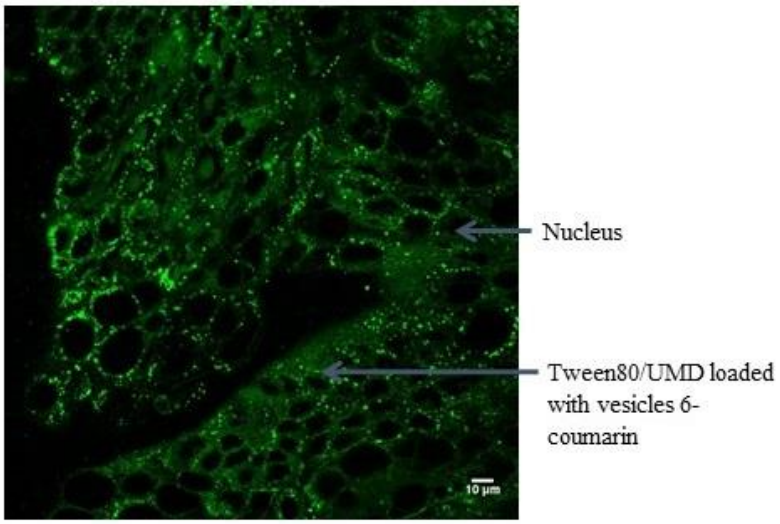

Figure 12 CLSM micrograph of Caco-2 cell monolayer after Tween80/UMD vesicles were uptaken. Tween80/UMD vesicles loaded with 6-coumarin fluoresced in green.

3.3.4 Effect of individual oil-soluble phytochemical and the UMD encapsulated phytochemicals on the immunomodulatory activity of THP-1

The immunomodulatory properties of individual oil-soluble rice phytochemical encapsulated in Tween80/UMD vesicles were investigated, in comparison to the mixed phytochemicals in UMD, by using the THP-1 macrophages model. In a preliminary study, THP-1 macrophages were stimulated with Tween80/UMD vesicles at various concentrations up to 5 mg/mL for 3 and 6 h. Gene expression of inflammatory-related cytokine, i.e., IL-1β, IL-8 and TNF-α remained unchanged compared to cells grown in PBS (data not shown). In this investigation, THP-1 macrophages were exposed with Tween80-based vesicles, i.e., Tween80/UMD (UV), Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol vesicle (EV), Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/α-tocopherol vesicle (TV) and Tween80/dioleoylglycerol/trioleoylglycerol/β-sitosterol/γ-oryzanol vesicle (OV) for 24 h. The gene expression level of a pro-inflammatory cytokine such as IL-1β, IL-8 and TNF-α were investigated compared to cellular response for PBS.

In all vesicle types, the elevation of the cytokine gene expressions of THP-1 macrophage was found only at a high vesicle concentration of 1 mg/mL (UV. The Tween80/UMD vesicles (UV) contained γ-oryzanol 8.0 μg/mL, tocols 18.1 μg/mL and mixed phytosterols 32.6 μg/mL; EV contained β-sitosterol 32.5 μg/mL; TV contained β-sitosterol 32.5 μg/mL and α-tocopherol 0.2 μg/mL; OV contained β-sitosterol 32.5 μg/mL and γ-oryzanol 8.0 μg/mL (Figure 13). The increase in gene expression of IL-1β after exposed to Tween80-vesicle was the most stand out compared to the gene expression of other cytokines.

The THP-1 macrophages exposed to the Tween80-based vesicle responded to the vesicles differently. The proinflammatory cytokine expression was dependent on the sources of oil-soluble phytochemicals in the vesicles, particularly the types of phytochemicals since the concentrations were quite similar. Expression of the proinflammatory IL-1β gene was the highest when the THP-1 was exposed to the vesicles containing β-sitosterol and γ-oryzanol (OV) and β-sitosterol and α-tocopherol (TV). The TNF-α gene, however, expressed the most when the cells were exposed to vesicles containing β-sitosterol and α-tocopherol (TV).

The expression of the cytokine IL-8 gene was the highest when the THP-1 was exposed to the vesicles containing UMD, which contained a mixture of rice phytosterols, tocols, and γ-oryzanols. The IL-8 cytokine is essential in the activation of neutrophil; a messenger cross-links the inflammation and epithelial-mesenchymal transition (EMT). The influence of the rice bran phytochemicals such as tocols, γ-oryzanol and phytosterol, as well as its mixture on their insight immunomodulation properties, therefore need further investigation.

In summary, this study showed the potential use of UMD as the source of concentrated oil-soluble rice phytochemicals that may find its use in healthy isotonic drinks containing mixed phytosterols, tocols, and γ-oryzanols. Nonetheless, more investigations are needed to increase the productivity of the Tween89/UMD vesicles at a pilot-scale production and rigorous tests for health claims in animal models.

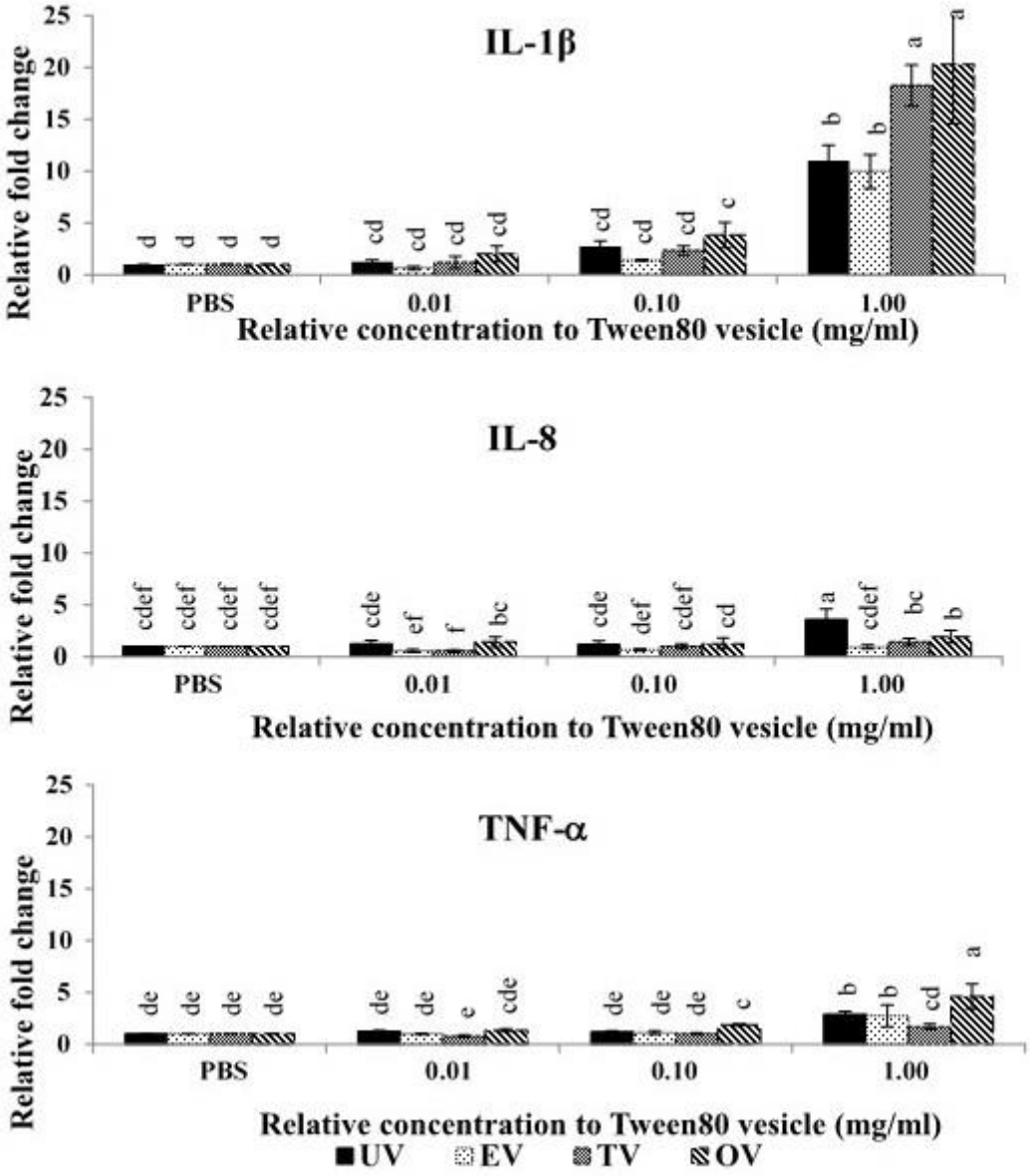

Figure 13 Pro-inflammatory cytokine genes expression of THP-1 macrophages after stimulation with UV, EV, TV, and OV for 24 h. Gene expression was normalized to GAPDH and non-stimulated macrophages at individual concentrations.

บทที่ 4 คุณสมบัติเชิงกลของไขน้ำมันจากกระบวนการกำจัดไข และการใช้โอลิโอเจลน้ำมันรำข้าวในผลิตภัณฑ์เนื้อสัตว์

Abstract

The crude rice bran wax (RBW), a by-product from winterizing step, was explored for its use in the formation of an oil-based three-dimensional network or oleogel capable of holding liquid oil in a solid form at the temperature higher than the melting temperature of RBO. The mechanisms involved in stabilizing the gel network of rice bran oil (RBO) by the RBW were due to the ability of a self-assembled network of high melting point triacylglycerol (TAG), long-chain alcohol and esters of long-chain alcohol in the RBW. The tailored edible oleogel had increased storage modulus (G′), higher viscoelastic transition temperature, and a prolonged time for the oleogel to change from solid to liquid behavior. In a comparative study using crude RBW and ethylcellulose (EC) network as gelator holding liquid oil in the RBO oleogel, it was found that the RBO-EC and RBO-RBW-EC oleogels could prevent the sedimentation of salt and spices in the oil-based marinade sauce at 2 °C for two days and withstand the temperature at 90 °C during grilling ($P<0.05$). Nonetheless, the appearance and the texture of grilled pork marinated with RBO-RBW was more superior than those marinated with the sauce containing EC ($P<0.05$).

4.1 Introduction

Oleogel is a bi-continuous colloidal system consists of a liquid organic phase and a gelator (Co and Marangoni 2012; Vazquez et al., 2007). The liquid organic phase could be organic solvents, mineral oils, or vegetable oils. The liquid phase was trapped in a three-dimensional network of a gelator, which is sometimes called organogelator to be more specific (Dassanayake et al., 2009; Co and Marangoni., 2012). The gelator could be divided into 2 groups. The first group is a low-molecular-weight gelator such as low MW surfactants and a family of lipid molecules such as mono-, di-acylglycerol, and wax (Co and Marangoni, 2012). The oleogels can be prepared by mixing oil and the gelators, followed by heating until the gelator dissolves, and cooling to the temperature lower than the gelation and/or crystallization temperature of the gelators (Gandolfo et al., 2004).

Continuous gel network having numerous structures can be categorized depending on the structure forming process of oleogels (Roger, 2009; Marangoni and Garti, 2011), i.e., self-assembled crystalline surfactants that can form a spontaneous reverse bilayer, the rod-shaped tubules of inverse bilayer or hexagonal II structure in pure oil system (Roger, 2009) due to its amphiphilic structure. Therefore, after cooling and aging, the surfactant can form β crystal network.

The self-assembled of isoprenoids such as β-sitosterol and γ-oryzanol could promote the formation of oleogel by stacking phytosteryl moieties into nanotubuls (Bot and Agterof, 2006). The sterol part plays an essential role in the formation of the helical ribbon structure of the self-assembled tubules while the OH group in the phytosterol conformation limits the solubility of phytosterol in the oil phase.

The second group of gelator for oleogel formation is the carbohydrate polymers. The polymers absorb the solvent and swell to form a polymer network. The amount of swelling depends on the temperature and molecular interactions between the polymers and solvents (Li et al., 2012), similar to the aqueous thermo-reversible gels, in which the nature of the junctions is physical entanglement (Laredo et al., 2011). One polymer which can be used as an organogelator is ethyl cellulose (Co and Marangoni, 2012). The gel network was stabilized by H-bonds between ethoxy and OH groups on the ethylcellulose strands (Laredo et al., 2011). Ethylcellulose can be used in the food, pharmaceutical, and cosmetic industry. Zetzl et al. (2012) described that the strength of oleogel depends on polymer molecular weight, the concentration of polymer, and the fatty acid composition of the liquid oil.

Rice bran oil (RBO) is composed of 71.10 % unsaturated fatty acid which is mostly oleic acid (Nukit et al., 2014) and is considered nutritious since it contains many phytochemicals such as γ-oryzanol, tocotrienols, tocopherols, and phytosterols (Prasad, 2006; Sawadikiat et al. 2015). Rice bran wax (RBW), a by-product of the rice bran oil refining process, which consists of long-chain alcohol, the esters of saturated fatty acid and a saturated fatty alcohol, hydrocarbon chain, and triacylglycerol (Yoon and Rhee, 1982), can be used as a gelator for RBO oleogel (Dassanayake et al., 2009). The RBW can form a needle or platelet shape crystal in the edible oil of various crystal size and strength, depending on the concentration of the RBW.

Meat marinating is a method to extend the shelf-life and proof organoleptic properties and sensory quality such as tenderness, juiciness, and flavor of meat steak, grilled meat, and meat products. Oil-based marinade suited for a meat product because the oil can protect the meat as a meat moisturizer and delivers flavors into meat fibers. Seasoning such as salt, spice, and herb enhances the flavor and taste of marinated meat. However, salt and seasonings often sediment at the bottom of the container during storage and transportation. The objective was to evaluate the ability of ethyl cellulose (EC), in comparison with the crude RBW from winterizing step, to from oleogels capable of suspending salt and spices during marination and grilling of pork steak.

4.2 Materials and methods

4.2.1 Influence of ethyl cellulose (EC) and rice bran wax (RBW) concentration on rheological properties of rice bran oil oleogel

Preparation of RBO oleogel containing different gelator

The RBO-EC, RBO-RBW, and RBO-EC-RBW samples were prepared based on the weight of RBO as 100 %. The RBO blends containing added ethyl cellulose (EC) at 2.50 %, 3.75 %, and 5.00 % were prepared by gradually mixed EC in RBO and heated the blends to 180 °C within 30 min. RBO-EC blends were stirred continually with a magnetic stirrer to ensure that the RBO-EC was a clear liquid. RBO mixed with RBW and EC were prepared to obtain a final concentration of RBW of 0.65 %, 1.30 %, and 1.95 % RBW. The RBO-RBW blends were heated up to 90 °C until the RBW melted, then the EC was gradually dispersed in the hot oil blends to obtain a final concentration of 2.50 %, 3.75 % and 5.00 %EC in the RBO-RBW-EC mixtures. The mixtures were further heated to 180 °C within 30 min and stirred continuously with a magnetic bar to obtain a clear liquid. After that, the samples were cooled at room temperature (25 °C) for 24 h.

Effect of rice bran wax (RBW) and ethyl cellulose (EC) concentration on rheological properties of rice bran oil oleogels containing different types of gelator

A controlled stress rheometer (Anton Parr, Germany) equipped with glass plate-and-plate geometry (43 mm diameter) with a gap of 1.2 mm was used to determine the rheological properties of RBO-RBW, RBO-EC, and RBO-RBW-EC oleogels prepared as described above. Frequency sweep tests at 0.5% strain, which was within the linear viscoelastic range of all samples, were performed from 0.1-100 Hz at 25 °C: the oscillatory rheological parameters storage modulus (G′) and loss modulus (G″) were recorded.

4.2.2 Use of rice bran oil oleogel containing rice bran wax and ethyl cellulose (RBO-RBW-EC) in preventing salt sedimentation

Influence of EC and RBW concentrations on (resistance to) salt sedimentation

RBO containing different concentrations of RBW and EC were prepared as described above. After the clear liquid oil blends were obtained, 5 g of oil blends were poured into a glass centrifuge tube and cooled at room temperature (25 °C). Salt (NaCl) was dispersed in the oil blends to obtain the final concentration of 15 % salt manually using a spatula. The samples were stored at 90 °C overnight.

For the salt sedimentation test, samples were centrifuged at 500 rpm at 25 °C for 10 min to separate salt to the bottom of the tubes. After that, the supernatant RBO was removed from the tubes to get salt sediment. The sedimented salt was washed using petroleum ether to remove residual oil, dried in a hot air oven at 90°C (1h), and weighed. The % salt sedimentation was calculated, as shown in Eq. 8:

$$W_s = \frac{[W_{tube+salt} - W_{tube}]}{W_{salt}} \times 100 \qquad (8)$$

where W_s is the weight of sedimented salt, $W_{tube+salt}$ is the weight of the centrifuge tube and sedimented salt in the tube after washing the oil out with petroleum ether and drying at 90 °C for 1 h. W_{tube} is the weight of the initial centrifuge tube, and W_{salt} is the weight of the initial salt added at the beginning.

Effect of liquid RBO, RBO-RBW oleogel, and RBO-RBW-EC oleogel on the qualities of marinating pork steak before and after grilling

The marinade sauce containing different forms of RBO, i.e., liquid RBO, RBO-RBW oleogel, and RBO-EC oleogel, was prepared. The RBO-RBW oleogel contained 1.3 % RBW, while the RBO-EC oleogel contained 3.75 % EC. The RBO-RBW-EC oleogel was prepared using 1.3 % RBW and 3.75 % EC. The liquid RBO and RBO oleogels of various types were mixed with 19 % salt, 10 % dried spice, and 2 % paprika extract and kept overnight at room temperature (25 °C) before marinating a pork steak manually. The dried spice treatment was used as a control sample. The pork steaks before marinade were kept at 2-4 °C. The steak marinated with 2.1 % salt, and 1.1 % dried spice was used as a control sample designated as "dried spice" treatment.

After marinating and aging at 2 °C for 48 h, the samples were grilled at 260 °C for 5 min on each side until the internal temperature of steak reached 75 °C. The grilled samples were cooled down to room temperature and analyzed for cooking loss and cooking yield.

Determination of marinade retention in raw marinated pork steak

The retention of marinade sauce on the raw pork steak was characterized by putting the marinated pork on a sieve tray during aging for 24 h at 2 – 4 °C. The marinated pork was weighed before and after aging. The difference in the weight before and after aging was calculated and reported as % marinade retention shown in Eq. 9:

$$\%\, marinade\ retention\ = \frac{w_1 - w_2}{w_1} \times 100 \qquad (9)$$

where w_1 is the weight of marinade used, and w_2 is the weight of marinade dripped through the sieve collected on a tray.

Determination of weight loss in grilled marinated pork steak

The weight loss after cooking the pork steak marinated different types of RBO and RBO oleogels was determined after grilling the steak at 260°C until core temperature reached 75 °C, and calculated using Eq. 10:

$$\%\, weight\ loss\ = \frac{w_3 - w_4}{w_3} \times 100 \qquad (10)$$

where w_3 is the weight of pork steak before grilling, and w_4 is the weight of grilled steak.

Determination of sensorial attributes of raw marinated steak and grilled marinated steak

Twenty untrained panelists at the University of Hohenheim, evaluated raw marinated pork steak, and grilled marinated pork steak resided in Stuttgart, Germany. Samples were labeled with a random three-digit number and using a 9-point hedonic scale for evaluation. For raw marinated pork steak, the questions on appearance, odor, overall liking, and % likelihood of buying were asked. For grilled marinated pork steak, the panelists were asked to evaluate their acceptance for appearance, odor, taste, juiciness, and overall liking.

4.2.3 Statistical analysis

The data were analyzed by analysis of variance (ANOVA) at a significance level of $P<0.05$. Significant differences between the treatments were analyzed by Duncan's multiple range test (DMRT). All statistical analyses were performed using SPSS software version 12 (SPSS, Chicago, Illinois, USA).

4.3 Results and discussion

4.3.1 Effect of ethyl cellulose (EC) and rice bran wax (RBW) concentrations on rheological properties of RBO oleogel

In the absence of RBW, the RBO remained liquid flow behavior shown as a higher value of loss modulus (G'') than the storage modulus (G') over the frequency of 0.1 – 100 s^{-1} (Figure 14a). The increase of RBW at the level of 0.65 – 3.25% increased both moduli of the mixtures (Figures 14b-f) and resulted in an increase in crossover point at a higher angular frequency, indicated that raising RBW concentration helped to form a stronger or more elastic structure of RBO oleogel.

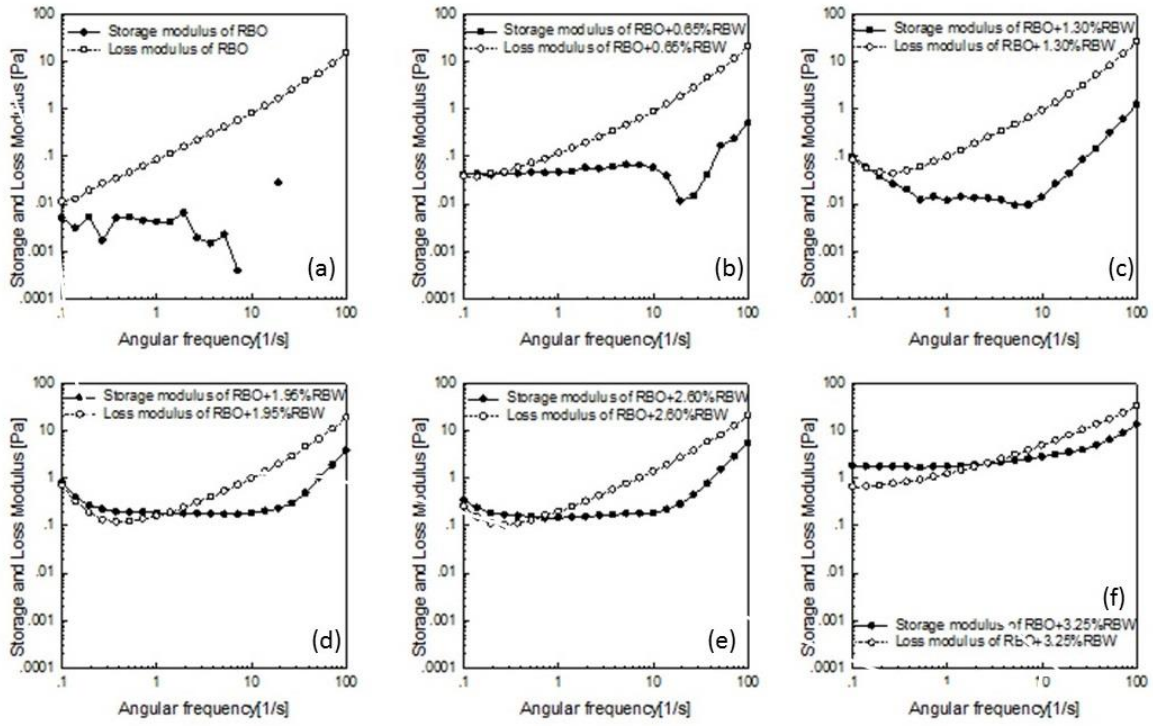

Figure 14 Effect of RBW concentration on storage modulus (G′) and loss modulus (G″) of RBO (a) 0 %RBW; (b) 0.65 %RBW; (c) 1.30 %RBW; (d) 1.95 %RBW; (e) 2.60 %RBW; (f) 3.25%RBW.

Data at low frequencies describes the behavior of the materials at a slow change of stress, while the behavior at fast load is expressed at high frequencies. The frequency sweep test is important for studying polymer melts and the processibility of the materials, i.e., oleogels containing different gelators. When RBW was added at the level above 1.95 %, the G′ was higher than the G″, suggesting that the particles added into the oleogel structure can disperse within the gel structure and do not sediment. However, the three-dimensional network of RBO-RBW oleogel was quite weak, observed as the crossover point at G′ within the range of 0.1-2.2 Pa.

However, when the EC was used as a gelator at the range of 2.5 – 5%, both G′ and G″ of RBO-EC oleogels were much higher than RBO-RBW (Figure 15). The structure of RBO-EC oleogel was quite strong. The RBO-EC containing 2.5% EC had the crossover point at the G′ at 33 Pa and a frequency of 50 s^{-1} (Figure 15a). The G′ of RBO-EC oleogel could be as high as 100 – 400 Pa with no crossover point when the EC concentration was increased to 3.75 – 5 % (Figures 15c-e). Increasing the EC concentration up to 3.75% resulted in a solid structure that gelled instantaneously when the mixed RBO-EC was cooled down to 25 °C after solubilizing at 180 °C during the preparation of oil blends.

The frequency sweep suggested that the three-dimensional network of RBO having different solid behavior could be fabricated by using RBW or EC gelator. However, the appropriate types and concentrations of gelators depend entirely on the targeted food and their industrial processing parameters, such as a series of temperature changes in the whole process.

The differences in the mechanisms involved in the oleogel formation induced by RBW and EC gelators suggested that the mixed gelators could be beneficial in terms of practical applications. Figure 16 illustrates that the solid behavior of RBO-EC oleogel and the magnitude of storage modulus (G′) could be reduced in the presence of RBW, which offers the direct use of RBO-based gels induced by mixed gelators to the wide range of foods.

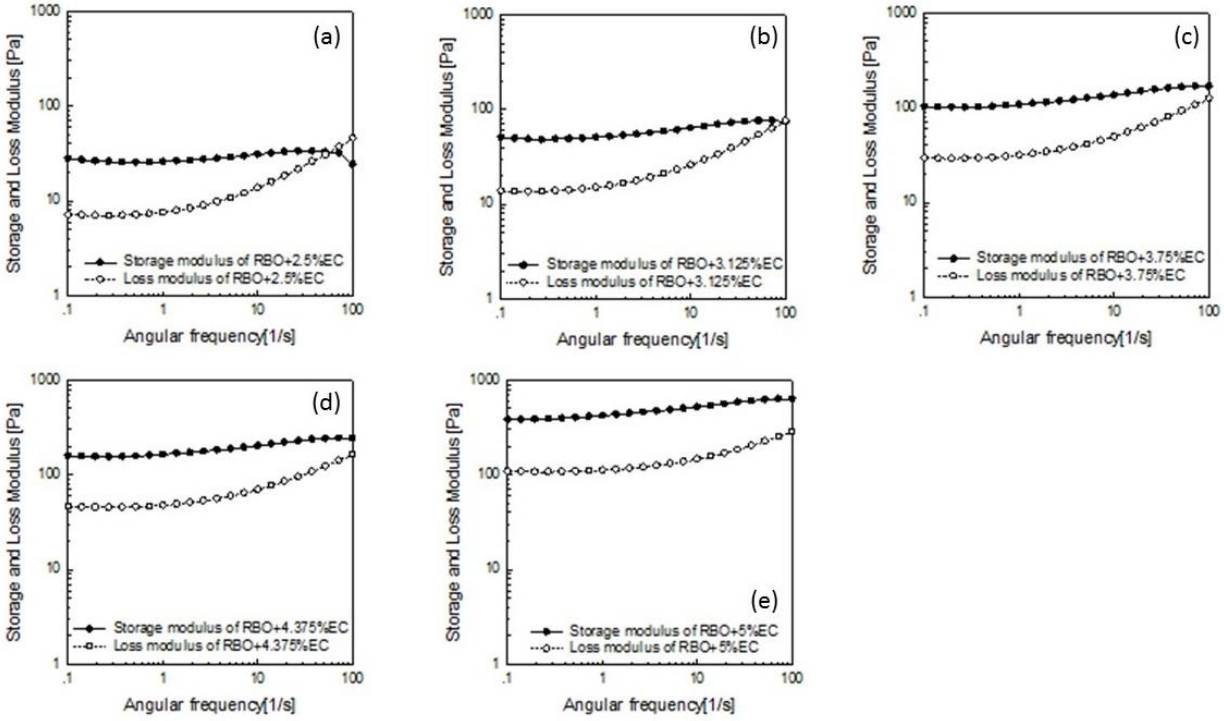

Figure 15 Effect of ethyl cellulose (EC) concentration on storage modulus (G′) and loss modulus(G″) of RBO-EC blends: (a) 2.5 %EC; (b) 3.125 %EC; (c) 3.75 %EC; (d) 4.375 %EC; (e) 5 %EC.

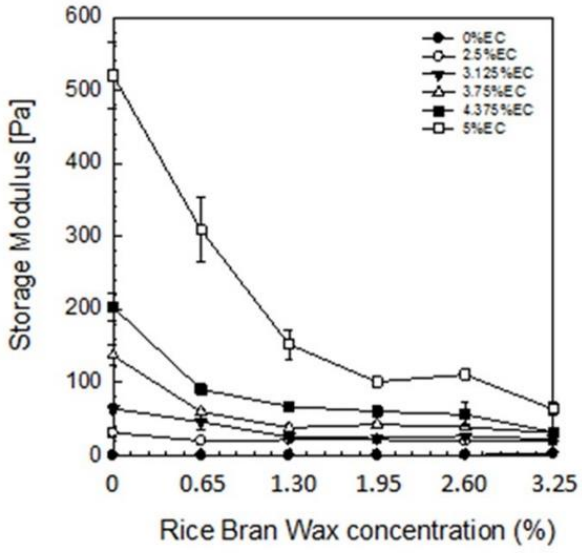

Figure 16 Storage modulus of RBO added with different concentrations of rice bran wax (RBW), and ethyl cellulose (EC) measured at 10 s^{-1} angular frequency. Bars represent standard deviation.

Figure 17 illustrates that when the RBO-RBW oil blends became turbid when the concentration of RBW was above 2.60 % and the solid structure at 25 °C could not be achieved. All RBO-RBW mixture remained liquid after the tubes were inverted (Figure 17a). The use of EC, however, enhanced the formation of RBO-EC oleogels when the concentration of EC was up to 4.375% (Figure 17b). The RBO-EC oleogel formed at 25°C and stay solid for more than 24 h, which is in good agreement on the frequency sweep results (Figures 15 and 16) that the RBO-EC oleogel was quite strong as the G′ was 200 Pa when the EC was used at 4.375 % as a gelator.

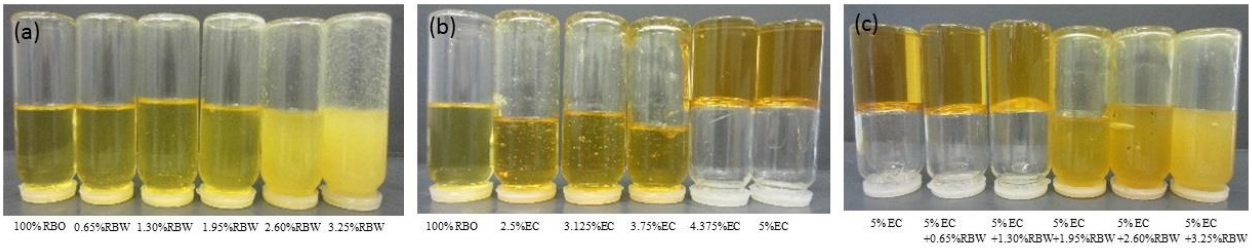

Figure 17 Effect of RBW and EC concentration on the appearance of oil blends at 25 °C: (a) RBO-RBW; (b) RBO-EC; and (c) RBO-EC-RBW.

At a higher concentration of EC (i.e., 5%) in the oleogel, the addition of RBW between 0.65 – 1.30 % helped reduce the storage modulus but retained gel network structure that was relatively stable at 25°C for more than 24 h. The appearance of RBO-RBW-EC oleogel was in agreement with the results shown in Figure 16 that mixed gelators creating networks having G′ of 150-300 Pa and could offer their use as thermostable oleogel network.

4.3.2 Use of rice bran oil oleogel containing rice bran wax and ethyl cellulose (RBO-RBW-EC) in preventing salt sedimentation in the oil marinade formula for pork steak

Industrial marinades in the meat industry are essential in improving meat yields by increasing water retention, as well as to add flavor and taste compounds. Although general marinades are composed of water, salt, and phosphate for both vacuum-tumbling and injecting application systems, sometimes the oil marinades with particulates of spice and herbs are used in marinated steak industries.

Oil-based marinades require the dispersion of dried ingredients such as salts and spices in the oil phase. Sedimentation of salt is considered a defect of the marinades. The result in Table 9 shows that using RBO-EC oleogel at high EC concentration above 3.75 % resulted in salt suspending in the oleogel structure. Such an RBO-EC oleogel structure was quite stable and did not melt at 90 °C. Higher EC concentration could result in the oleogel having high storage modulus (G′) of solid behavior of RBO oleogel, and may not be suitable for tumbling raw meat with the marinades before grilling.

Table 9 Effect of gelators on salt sedimentation in rice bran oil oleogel after storage at 90°C for 12 h.

Types of oleogel	% Salt sedimentation of salt after storage at 90 °C
RBO + 2.5%EC	96.2 ± 2.1
RBO + 2.5%EC + 0.65%RBW	96.3 ± 1.1
RBO + 2.5%EC + 1.30%RBW	98.2 ± 1.2
RBO + 2.5%EC + 1.95%RBW	99.10± 0.4
RBO + 3.75%EC	No sedimentation
RBO + 3.75%EC + 0.65%RBW	No sedimentation
RBO + 3.75%%EC + 1.30%RBW	No sedimentation
RBO + 3.75%EC + 1.95%RBW	78.1 ± 1.6
RBO + 5%EC	No sedimentation
RBO + 5%EC + 0.65%RBW	No sedimentation
RBO + 5%EC + 1.30%RBW	No sedimentation
RBO + 5%EC + 1.95%RBW	No sedimentation

Using a lower concentration of EC at 2.5 % resulted in almost 100 % of salt sedimentation, although RBW was added up to 1.95% (Figure 18). Nevertheless, using 3.75 % EC could disperse salt within the RBO-RBW-EC network structure at 90 °C when the RBW was present at 0.65 – 1.30 %. Such a weak network could help retain coated marinade on the surface of steak during heating up to 90 °C before the meat is fully cooked.

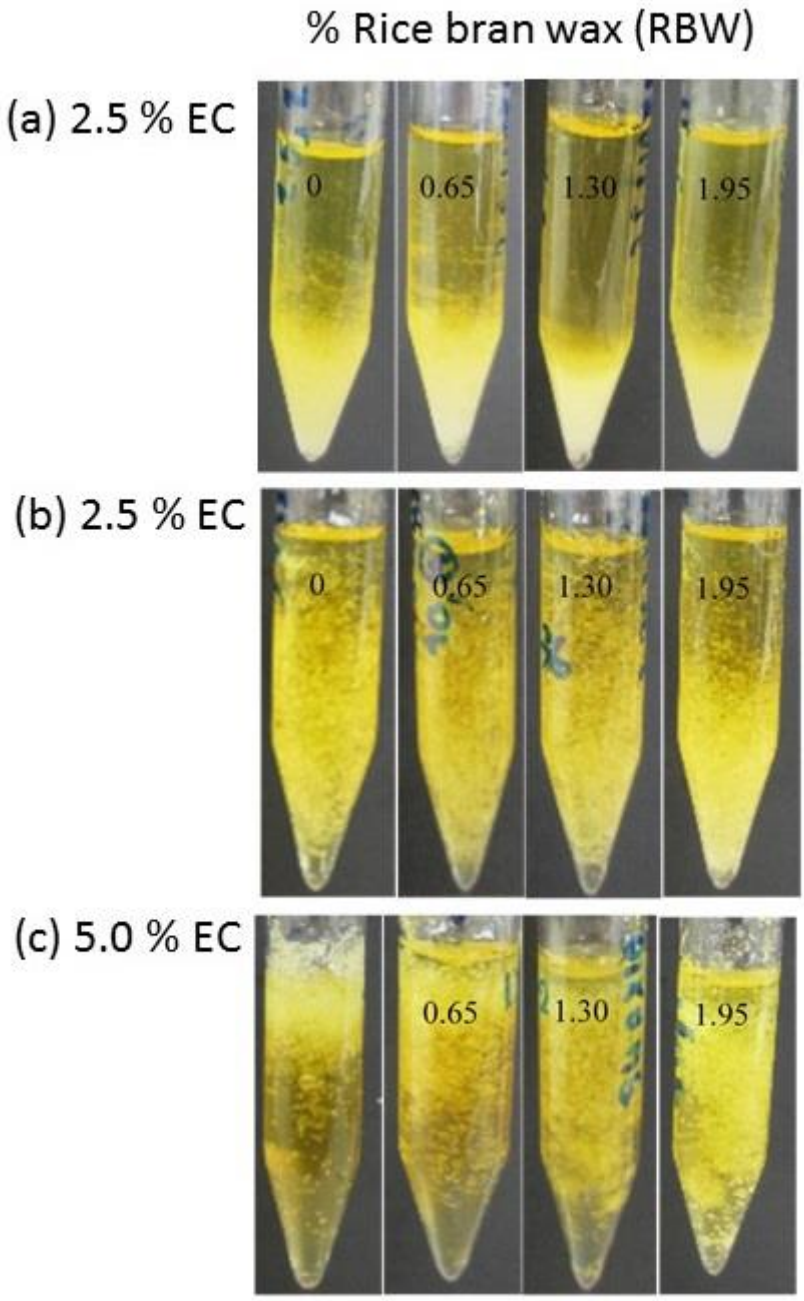

Figure 18 Effect of gelators on salt sedimentation in rice bran oil oleogel after storage at 90 °C for 12 h.

Results on the marinade retention in raw marinated pork steak and the weight loss of grilled marinated pork steak (Table 10) indicated that using 1.30 % RBW and 3.75 % EC in RBO-RBW-EC oleogel marinade resulted in a lower weight loss of grilled steak compared with the use of liquid RBO in marinating ($P<0.05$).

Table 10 Marinade retention of raw marinated pork steak and weight loss of grilled pork steak.

Treatments	% Marinade retention in raw steak	% Weight loss in grilled steak
Liquid oil		
RBO+spice+salt	$46.25^{b} \pm 3.74$	$32.58^{a} \pm 0.73$
Oleogel		
RBO+1.3%RBW+spice+salt	$63.46^{a} \pm 0.56$	$28.44^{b} \pm 1.96$
RBO+3.75% EC+spice+salt	$64.12^{a} \pm 1.47$	$28.23^{b} \pm 1.47$
RBO+3.75%EC + 1.3%RBW+spice+salt	$63.89^{a} \pm 1.03$	$27.44^{b} \pm 0.33$

Mean within a column followed by different letters are significantly different ($P<0.05$).

Sensorial attributes of raw marinated pork and grilled marinated pork steak were evaluated by 20 panelists using a 9-point hedonic scaling (Tables 11, 12, respectively). The liking score of raw pork steak marinated with RBO-EC and RBO-RBW-EC oleogel in appearance was higher than those marinated with RBO-RBW oleogel and marinade containing liquid oil and dried spices ($P<0.05$). The oleogel marinade was able to spread and cling onto the surface of the pork steak after marination, while the liquid oil marinade flew and spread on the containers (Figure 19).

On the other hand, steak marinated with liquid oil had a lower score due to the flow of RBO. Moreover, RBO-RBW oleogel was more opaque due to crystallization of RBW at low temperature, dried spice in the marinade exhibited greenish color of herb, resulting in the lowest liking score in appearance characteristics. For odor characteristic, pork steak marinated with RBO, and dried spice had the lowest liking score ($P<0.05$) due to the strong smell of herbs. The overall liking and likelihood of buying scores indicated that steak marinated with RBO-EC oleogel had the highest liking score and likelihood of buying.

Table 11 Result of 9-point hedonic scale score and %likelihood of buying of marinade raw pork steak. All of the treatments mixed with 19 % salt, 10 % dried spice and 2 % paprika extract.

Treatments	Appearance	Odor	Overall Liking	% Likelihood of buying
Dried spice	3.50^{c} ± 1.64	6.00^{b} ± 1.56	4.40^{d} ± 1.63	15%
RBO+spice+salt	5.55^{b} ± 1.54	6.85^{a} ± 1.35	5.95^{c} ± 1.39	50%
RBO+1.3% RBW +spice+salt	5.80^{b} ± 1.77	6.95^{a} ± 1.10	6.30^{bc} ± 1.26	70%
RBO+3.75% EC +spice+salt	7.60^{a} ± 0.99	7.15^{a} ± 1.04	7.45^{a} ± 1.00	95%
RBO+3.75%EC+ 1.3%RBW+spice+salt	6.95^{a} ± 1.43	6.85^{a} ± 1.14	7.00^{ab} ± 1.12	85%

Mean within a column followed by different letters are significantly different ($P<0.05$), n=20 panelist.

Table 12 Effects of different oil marinades on sensory attributes of grilled pork steak

Treatments	Appearance	Odor	Taste	Juiciness	Overall Liking
Dried spice	4.90^{b}± 1.77	5.60^{a}±1.60	5.65^{c}± 1.69	6.30^{a}±1.22	5.45^{d}±1.47
RBO+spice +salt	7.10^{a}± 1.36	6.50^{a}±1.50	6.95^{ab}±1.54	6.80^{a}±0.99	6.98^{ab}±1.38
RBO+1.3% RBW +spice+salt	6.85^{a}± 1.53	6.65^{a}±0.81	7. 30^{a}±0.86	6.60^{a}±1.39	7.18^{a}±0.94
RBO+3.75% EC +spice+salt	5.15^{b}± 1.63	6.40^{a}±1.27	6.40^{bc}±0.88	6.70^{a}±1.26	6.00^{cd}±1.26
RBO+3.75%EC+ 1.3% RBW+spice+salt	5.60^{b}± 1.47	6.65^{a}±1.18	6.80^{ab}±0.95	5.90^{a}±1.55	6.35^{bc}±1.09

Mean within a column followed by different letters are significantly different ($P<0.05$), n=20 panelist.

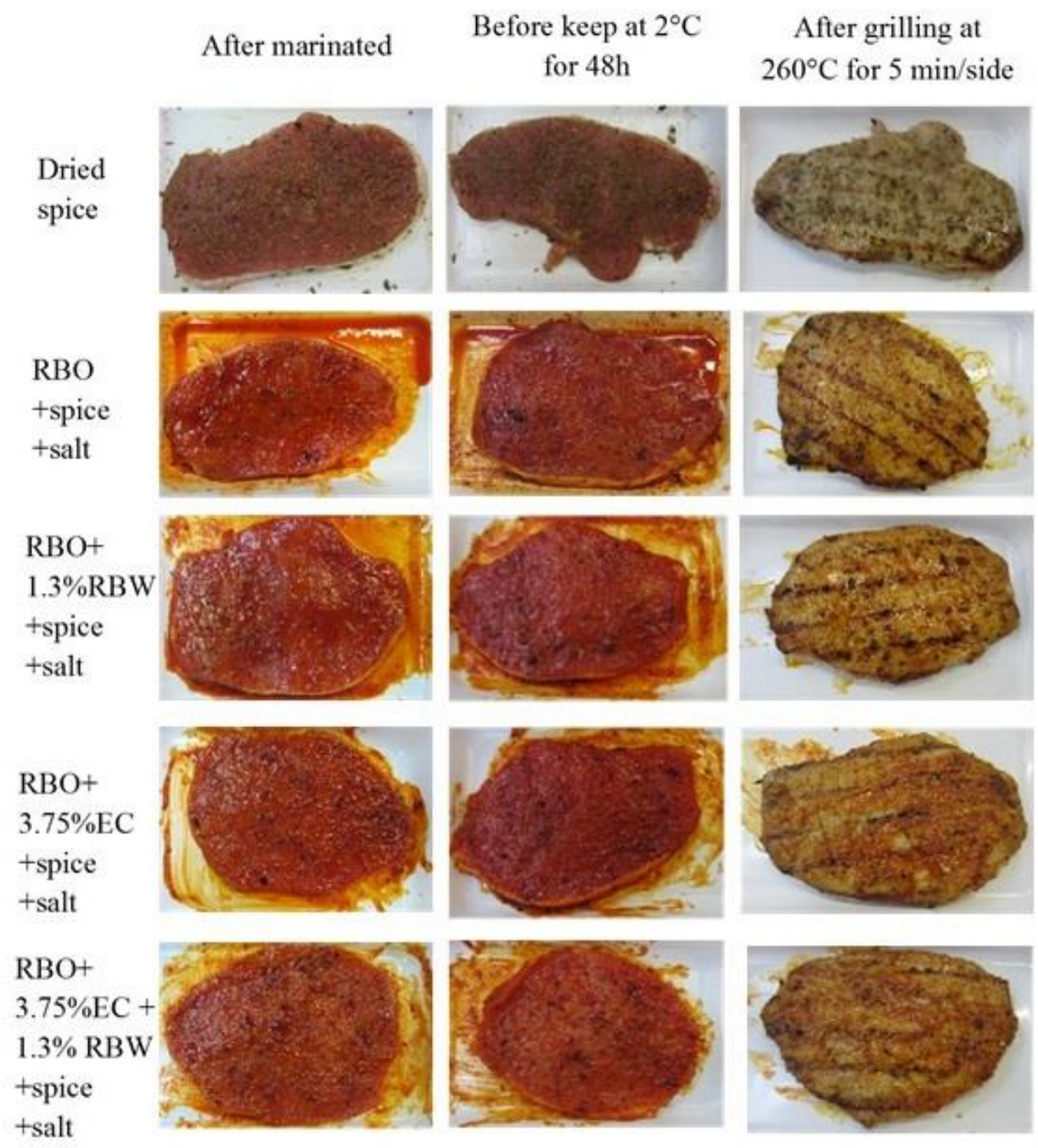

Figure 19 Appearance of raw and grilled pork steak.

Sensorial evaluations of grilled steak, however, revealed that the odor and juiciness characteristics of all samples were not significantly different ($P \geq 0.05$). In contrast with raw marinated steak, the grilled steak marinated with RBO-RBW oleogel had the highest liking score.

The differences in the appearance of pork steak marinated with different formulas are shown in Figure 19. Although the panelists indicated that they likely bought raw steak marinated with RBO-EC oleogel the most, they liked the grilled steak marinated with RBO-RBW oleogel the most and that marinated with dried spice the least. This was because the presence of 3.75 %EC resulted in the appearance of thin-film coated on the surface of grilled pork steak since the carbohydrate polymers did not melt upon grilling.

It is apparent that the EC could help to form the thermostable RBO-EC oleogels at a temperature higher than the melting temperature of RBO but below 90°C. Further heating of pork steak to 260°C during grilling, however, could result in the flow of the bulk oil initially entrapped within the EC network, leaving the thin film of EC coated on the surface of grilled steak, which resulted in an unappealing appearance of grilled pork steak.

Using different gelators or their mixture in RBO blends resulted in the interplay among three structure-forming mechanisms, namely self-assembled of surfactant, fat crystal network of TAGs, and a polymeric strand of carbohydrates. Each mechanism resulted in different thermo-physical properties of solidified RBO blends. The success of oleogel fabrication by self-assembled surfactant depended on the difference in the conformation of fatty acid of liquid oil and hydrophobic part of surfactants. When molecular conformations of TAGs in liquid oil and hydrophobic parts of surfactant were different, such structure could induce phase separation of surfactants, followed by surfactant network entrapping liquid oil within the solidified structure. Nonetheless, this structure could be modified to hold liquid RBO at a temperature higher than the melting temperature of RBO by adding RBW.

The carbohydrate EC could be used to increase the strength and oil further holding capacity of RBO oleogel at high temperatures (90 °C). However, the strong structure of RBO-EC oleogel may not be suitable in all food products, although it can help disperse salts and spices homogenously without sedimentation. The rheological properties of RBO-EC could be further modified by adding RBW to decrease the strength of the EC network. It was most likely that the RBW could interact with EC by H-bondings, resulting in fewer interactions between EC and bulk liquid RBO.

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บทที่ 5 ข้อเสนอแนะ

จากแนวทางการใช้ประโยชน์ของผลพลอยได้จากน้ำมันรำข้าวที่ได้ศึกษานี้ ผู้ประกอบการจากบริษัทน้ำมันรำข้าวสุรินทร์ จำกัด ซึ่งเป็นบริษัทขนาดกลางที่ร่วมศึกษาวิจัยในโครงการนี้ ได้รับองค์ความรู้จากการที่คณะผู้วิจัยและนักศึกษาปริญญาเอกทำการศึกษาสภาวะการกลั่นแยกกรดไขมันด้วยเครื่อง MD ขนาดโรงงานต้นแบบของบริษัทในการทำให้สารพฤกษเคมีใน DD มีความเข้มข้นเพิ่มขึ้น นอกจากนั้น การตีพิมพ์วารสารทางวิชาการในเรื่องผลการตรวจวิเคราะห์องค์ประกอบที่มีอยู่ในผลพลอยได้ทั้งในส่วนของของเหลวผลกลั่นจากกระบวนการกำจัดกลิ่น (DD) และในไขน้ำมันจากกระบวนการกำจัดไข (RBW) ทำให้ผู้ประกอบการสามารถนำข้อมูลที่ได้ไปใช้ในการเจรจาธุรกิจต่อไปได้

ในการผลิตสารพฤกษเคมีเข้มข้นโดยการใช้เครื่อง MD ระดับโรงงานต้นแบบ ณ บริษัทน้ำมันรำข้าวสุรินทร์ จำกัด นั้น หัวหน้าโครงการวิจัยและนักศึกษาปริญญาเอกได้ร่วมมือกับบุคลากรของบริษัทในการปรับสภาวะการกลั่นแยกด้วยเครื่อง MD ได้แก่ อุณหภูมิ ความดัน และอัตราการไหล เพื่อทดสอบการผลิตในระดับโรงงานต้นแบบ (Technology Readiness Level (TRL) ระดับ 4) และประมวลความเป็นไปได้ในการขยายกำลังการผลิตไฟโตสเตอรอล โทคอล และแกมมาออไรซานอลผสมเข้มข้นเชิงพาณิชย์ ซึ่งจะใช้เป็นวัตถุดิบในอุตสาหกรรมอาหารเพื่อสุขภาพและเครื่องสำอางต่อไปเมื่อผู้ประกอบการหาลูกค้าที่ประสงค์จะใช้สารพฤกษเคมีจากรำข้าวเข้มข้นในธุรกิจอุตสาหกรรมต่อเนื่องได้ การทดสอบในเซลล์โมเดลจากการศึกษานี้ แม้ยังไม่ได้ตีพิมพ์ในวารสารทางวิชาการ แต่เป็นการศึกษาที่จะยืนยันการใช้ประโยชน์ของ UMD เมื่อทำให้อยู่ในรูปละลายน้ำ อย่างไรก็ตาม วิธีการห่อหุ้มสารออกฤทธิ์ทางชีวภาพใน UMD ยังจำเป็นต้องได้รับการศึกษาเพิ่มเติมในการขยายกำลังการผลิตเวซิเคิลในระดับโรงงานต้นแบบ (TRL 4) เพื่อให้มีกำลังการผลิตที่มากเพียงพอสำหรับการศึกษาใน animal model สำหรับการผลิตเพื่อทดสอบและกล่าวอ้างด้านสุขภาพ (health claim) ในระดับ TRL 5

อย่างไรก็ตาม ผู้ประกอบการได้ขอขึ้นทะเบียนและได้รับเลขที่ อย. ในการผลิตและจำหน่ายไขน้ำมันในปี 2561 ซึ่งยังคงมีคุณภาพในเรื่องสัดส่วนของไตรกลีเซอไรด์และสารที่ไม่ซาปอนนิไฟด์รวมทั้งค่ากรด ที่เป็นไปตามข้อกำหนดในประกาศกระทรวงสาธารณสุขว่าด้วยเรื่องไขมันและน้ำมัน เพื่อขยายผลการนำไปใช้ในระดับอุตสาหกรรมสำหรับอุตสาหกรรมต่อเนื่องเนื่องจากมีความปลอดภัยเพียงพอสำหรับผู้บริโภค

กล่าวโดยสรุป ข้อมูลทางวิชาการที่ได้รับการตีพิมพ์จากโครงการนี้ ทำให้บริษัทมีแนวทางในการใช้ประโยชน์ในเรื่องการผลิต Deodorizer distillate ที่มีวิตามินอีและไฟโตสเตอรอลเข้มข้น และการวางแผนการผลิตไขมันชนิดแข็ง ทดแทนไขมันจากขบวนการ partially hydrogenation ได้ เนื่องจาก อย.ห้ามนำเข้าผลิตภัณฑ์ไขมันแข็งมาในราชอาณาจักรตั้งแต่ปี 2562 เป็นต้นมา

ภาคผนวก

การตีพิมพ์ผลงานในวารสารวิชาการจากโครงการวิจัยนี้

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