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APPLICATION OF LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY
AND TANDEM MASS SPECTROMETRY TO THE IDENTIFICATION OF
ANTHOCYANINS IN THAI BLACK RICE CULTIVARS

KITSADA PITIJA

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EXAMINING COMMITTEE

..........CHAIRPERSON

Dr. Apiwat Baramee

..........MEMBER

Assoc. Prof. Dr. Sugunya Wongpornchai

..........MEMBER

Dr. Prasat Kittakooop

13 February 2009

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Thesis Title Application of Liquid Chromatography-Mass Spectrometry and
Tandem Mass Spectrometry to the Identification of
Anthocyanins in Thai Black Rice Cultivars

Author Mr. Kitsada Pitija

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Thesis Advisor Assoc. Prof. Dr. Sugunya Wongpornchai

ABSTRACT

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The identification of compounds in a group of anthocyanins, which were accumulated in leaves and seed of the black rice cultivar Kumdoisakheth and BGMSN 11, was performed by the use of high performance liquid chromatography (HPLC) having photodiode array as detector together with those techniques employing HPLC combined with an electrospray ionization mass spectrometry (ESI-MS) and a tandem mass spectrometry (MS/MS).

At the first part of the study, bran of the black rice cultivar Khumdoisakheth was used as sample for the selection of a suitable solvent for extraction and for optimization of HPLC conditions for the separation of components in the crude rice bran extract, as well as the conditions of electrospray ionization in LC-MS technique. It was found that 0.5 % formic acid in methanol was the most appropriate solvent among methanol, methanol:dichloromethane (1:4 v/v), and isopropanol used for extraction of anthocyanins from the black rice bran. The optimum HPLC condition

employed Zorbax Eclipse plus C₁₈ with a dimension of 4 × 100 mm and 3 μm particle sizes as a chromatographic column. The mobile phase consisted of methanol and 0.5% acetic acid in water at the ratio of 10:90 (v/v) with a flow rate of 0.4 ml/min. The optimized ESI condition resulted in the following parameters; fragmentor voltage 110 V, capillary voltage 3500 V, drying gas temperature 350 °C, drying gas flow 12 ml/min, and nebulizer pressure 30 psi. The informative product ion mass spectra useful for structural characterization of the black rice anthocyanins were obtained by performing collision induced dissociation (CID) with argon as a collision gas at energies of 15, 20 and 25 V.

Positions of anthocyanins in each HPLC profile of the crude rice sample extracts were determined by the use of data processing in reconstructed ion chromatogram mode monitoring at a specific ion mass corresponding to the characteristic or molecular ion of the anthocyanins of interest. These anthocyanins were confirmed by their UV-Vis spectra obtained by DAD. Structural characterization of the black rice anthocyanins was then performed by analyzing their ESI-MS and ESI-MS/MS spectra, which revealed the presence of ten anthocyanins, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside-5-*O*-rhamnoside, peonidin-3-*O*-glucoside, cyanidin-3-*O*-diglucoside, cyanidin-3-*O*-diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, cyanidin-3-*O*-(feruloyl)glucoside-5-*O*-glucoside, peonidin-3-*O*-diglucoside, malvidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, and peonidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-xyloside, and the two tentatively identified anthocyanins; cyanidin-3-*O*-xyloside glucoside, and cyanidin-3-*O*-xyloside glucoside. Among these identified anthocyanins, cyanidin-3-*O*-

diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, cyanidin-3-*O*-(feruloyl)glucoside-5-*O*-glucoside, peonidin-3-*O*-diglucosid, peonidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-xyloside, and malvidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, were found in leaves or seed of black rices for the first time by this study.

The relative contents of each anthocyanin in the extracts of leaves and seed of the two black rice cultivars at seven growth stages; seeding, tillering, booting, milk grain, dough grain, maturation, and post harvest, were determined by the use of LC-ESI-MS. Results revealed that the accumulation of anthocyanins in each part of the rice plant, leaf or seed, was dependent of their chemical structures with a number of sugar and acylate groups. Monoglycosidic anthocyanins such as cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were only found in seed of the black rice cultivar BGMSN 11 that has green leaves, but found in both leaves and seed of the cultivar Kumdoisakhet, of which its leaves are purple-black. Anthocyanins with two or three sugars or acylate groups tended to stay in the leaf part, generally their contents were highest in leaves at booting stage, except for peonidin-3-*O*-diglucoside and peonidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-xyloside that were found in both leaves and seed. Overall, there was no correlation among the structures of anthocyanins, their relative contents, and growth stages of both black rice cultivars.

ชื่อเรื่องวิทยานิพนธ์	การประยุกต์ลิกวิด โครมาโทกราฟี-แมสสเปกโตรเมตรีและ แทนเดมแมสสเปกโตรเมตรีในการระบุเอกลักษณ์ของแอนโท ไซยานินในข้าวคั่วพันธุ์ไทย
ผู้เขียน	นาย กฤษดา ปิศาจะ
ปริญญา	วิทยาศาสตรมหาบัณฑิต (เคมี)
อาจารย์ที่ปรึกษาวิทยานิพนธ์	รศ. ดร. สุกัญญา วงศ์พรชัย

บทคัดย่อ

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การพิสูจน์เอกลักษณ์ของสารกลุ่มแอนโทไซยานินที่สะสมในใบและเมล็ดข้าวคั่วพันธุ์ก่ำ
คอบสะเกิดและบีจีเอ็มเอสเอ็ม ๑๑ ทำโดยใช้เทคนิคไฮเพอร์ฟอร์มานซ์ลิกวิด โครมาโทกราฟีที่มีโพ
โตไดโอดแอเรย์เป็นตัวตรวจวัด และเทคนิคคู่ควบระหว่างเทคนิคไฮเพอร์ฟอร์มานซ์ลิกวิด โครมา
โทกราฟี (HPLC) และเทคนิคการแตกตัวเป็นไอออนด้วยไฟฟ้าของแมสสเปกโตรเมตรี (ESI-MS)
และ แมสสเปกโตรเมตรีแบบต่อเรียงกัน (MS/MS)

ในการศึกษาเบื้องต้นร่างของข้าวพันธุ์ก่ำคอบสะเกิดถูกนำมาใช้เป็นตัวอย่างในการหาตัวทำ
ละลายที่เหมาะสมในการสกัดและสภาวะที่เหมาะสมของวิธีการแยกสารสกัดหยาบของข้าวด้วย
HPLC และสภาวะที่เหมาะสมของการแตกตัวเป็นไอออนด้วยไฟฟ้าวิเคราะห์ใน LC-MS พบว่า
สารละลายของกรดฟอร์มิกเข้มข้น 0.5 % ในเมทานอลให้ประสิทธิภาพการสกัดที่ดีกว่า เมทานอล,
เมทานอลผสมไดคลอโรมีเทน (1:4 โดยปริมาตร) และไอโซโพรพานอลในการสกัดแอนโทไซยา
นินจากรำข้าว สภาวะที่เหมาะสมของการแยกด้วยเทคนิค HPLC ใช้คอลัมน์ Zorbax Eclipse plus

C₁₈ ที่มีขนาด 3 × 100 มิลลิเมตร และขนาดอนุภาคเท่ากับ 3 ไมโครเมตร โดยมีเฟสเคลื่อนที่เป็นสารละลายผสมระหว่างเมทานอลและสารละลายกรดอะซิติกเข้มข้น 0.5% ที่สัดส่วน 10:90 โดยปริมาตร และมีอัตราการไหล 0.4 มิลลิลิตรต่อนาที สภาวะที่เหมาะสมของระบบการแตกตัวเป็นไอออนด้วยไฟฟ้าใช้ความต่างศักย์ในการแตกไอออน 110 โวลต์, ความต่างศักย์ที่ในหลอดแคปิลลารี 3500 โวลต์, อุณหภูมิของแก๊สร้อนที่ใช้ในการระเหยแห้ง 350 องศาเซลเซียส, อัตราการไหลของแก๊สร้อนที่ใช้ในการระเหยแห้ง 12 ลิตรต่อนาทีและ ความดันของแก๊สที่ใช้ในผลึกของเหลวให้แตกตัวเป็นละอองแก๊ส 30 ปอนด์ต่อตารางนิ้ว แมสสเปกตรัมของไอออนผลผลิตที่ให้ข้อมูลโครงสร้างทางเคมีหาได้จากการแตกไอออนหลักด้วยเทคนิคการแตกตัวแบบเหนี่ยวนำด้วยการชน (CID) ที่ใช้อาร์กอนเป็นแก๊สตัวชนและใช้พลังงานการชนเท่ากับ 15, 20 และ 25 อิเล็กตรอนโวลต์

ตำแหน่งของสารแอนโทราไซยานินบนโครมาโทแกรมของการแยกสารสกัดหยาบด้วย HPLC หาโดยการสร้างโครมาโทแกรมของไอออน ที่มีมวลที่สนใจซึ่งมีค่าเท่ากับมวลต่อประจุของไอออนที่เป็นลักษณะเฉพาะหรือไอออนโมเลกุลของสารแอนโทราไซยานิน ร่วมกับการยืนยันตำแหน่งดังกล่าวด้วย ยูวี-วิจิเบิล สเปกตรัม ที่ได้จากตัวตรวจวัดแบบไดโอดแอเรย์ การวิเคราะห์แมสสเปกตรัมปกติและแมสสเปกตรัมของไอออนผลผลิต ที่ตำแหน่งเวลาริเทนชันดังกล่าวในขั้นตอนนำไปสู่การวินิจฉัยลักษณะเฉพาะของสารแอนโทราไซยานินในข้าวสาลีทั้งสองพันธุ์ได้จำนวน

10 โครงสร้าง ได้แก่ cyanidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside-5-*O*-rhamnoside, peonidin-3-*O*-glucoside, cyanidin-3-*O*-diglucoside, cyanidin-3-*O*-diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, cyanidin-3-*O*-(feruloyl)glucoside-5-*O*-glucoside, peonidin-3-*O*-diglucoside, malvidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, และ

peonidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-xyloside และสารประกอบแอนโทไซยานิน 2 โครงสร้างที่ยังไม่สามารถระบุเอกลักษณ์ได้แน่นอนคือ cyanidin-3-*O*-xyloside glucoside และ cyanidin-3-*O*-xyloside-glucoside โดย cyanidin-3-*O*-diglucoside-5-*O*-glucoside, cyanidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucoside, cyanidin-3-*O*-(feruloyl)glucoside-5-*O*-glucoside, peonidin-3-*O*-diglucosid, peonidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-xyloside, และ malvidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-glucosiden ถูกพบในใบและเมล็ดของข้าวสาลีเป็นครั้งแรกในการศึกษา

นี้

การวิเคราะห์ปริมาณสัมพัทธ์ของสารแอนโทไซยานินแต่ละตัวในสารสกัดของใบและเมล็ดข้าวสาลีทั้งสองพันธุ์ ที่เจริญระยะการเจริญเติบโต ได้แก่ ระยะที่เป็นต้นกล้า ระยะแตกกอ ระยะตั้งท้อง ระยะนํ้านม ระยะแป้งอ่อน ระยะแก่ของเมล็ด และระยะหลังการเก็บเกี่ยวด้วย LC-ESI-MS พบการสะสมของสารแอนโทไซยานินในแต่ละส่วนของใบและเมล็ดของข้าวขึ้นอยู่กัลักษณะโครงสร้างทางเคมีกับจำนวนโมเลกุลของน้ำตาลและหมู่อัลคิลเลต โดยแอนโทไซยานินที่มีน้ำตาลโมเลกุลเดียว ได้แก่ cyanidin-3-*O*-glucoside และ peonidin-3-*O*-glucoside พบเฉพาะในส่วนของเมล็ดของข้าวพันธุ์บีจีเอ็มเอสเอ็ม ๑๑ ซึ่งมีใบสีเขียว แต่พบทั้งในใบและเมล็ดของข้าวพันธุ์ก่ำดอยสะเก็ดซึ่งมีใบสีม่วงดำ ส่วนแอนโทไซยานินที่มีน้ำตาลสองหรือสามโมเลกุลหรือหมู่อัลคิลเลตส่วนใหญ่พบเฉพาะในใบข้าว โดยเฉพาะแล้วมีปริมาณสูงที่ระยะตั้งท้อง ยกเว้น peonidin-3-*O*-diglucoside และ peonidin-3-*O*-(*p*-coumaroyl)glucoside-5-*O*-xyloside ที่พบทั้งในส่วนของใบและเมล็ดข้าวโดยรวมแล้วไม่พบความสัมพันธ์ระหว่างโครงสร้างของแอนโทไซยานินปริมาณสัมพัทธ์ และระยะการเจริญเติบโตของข้าวสาลีทั้งสองพันธุ์

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ABBREVIATIONS AND SYMBOLS

MeCN	acetonitrile
C ₁₈	octadecyl
°C	degree Celsius
CID	collision-induced dissociation
DCM	dichloromethane
ESI	electrospray ionization
eV	electron volt
FI	flow injection
G	gram
HPLC	high performance liquid chromatography
Kg	kilogram
kV	kilovolt
l	liter
LC	liquid chromatography
LC-ESI-MS	liquid chromatography- electrospray ionization mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
MS	mass spectrometry
MS/MS	mass spectrometry-mass spectrometry
min	minute

ml	milliliter
m	meter
MW	molecular weight
m/z	mass-to-charge ratio
MeOH	methanol
mg	milligram
MCP	micro channel plate
nm	nanometer
PDA	photodiode array
ppm	part per million
psi	pound per square inch
Q-TOF	hybrid quadrupole time-of-flight
TICs	total ion chromatograms
TOF	time-of-flight
μ l	micro liter
μ m	micrometer
UV	ultraviolet
V	volt