

Quantification of Flavonoids in Black Rice by Liquid Chromatography-Negative Electrospray Ionization Tandem Mass Spectrometry

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ABSTRACT: Systematic identification and structural characterization of flavonoids and their glycosides in bran extracts of seven Thai black rice varieties were performed by sequential uses of reversed-phase HPLC with a photodiode array detector and a combined electrospray ionization tandem mass spectrometer. Eleven flavonoids were detected, and six of these were found for the first time in rice bran. These were taxifolin-7-*O*-glucoside, myricetin-7-*O*-glucoside, isorhamnetin-3-*O*-acetylglucoside, isorhamnetin-7-*O*-rutinoside, 5,6,3',4',5'-pentahydroxyflavone-7-*O*-glucoside, and 5,3',4',5'-tetrahydroxyflavone-7-*O*-glucoside. The quantitative results revealed that different rice varieties possessed flavonoids in different concentrations. The most abundant glycoside derivative of flavonoids widely distributed among the rice varieties was monoglucoside, such as quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, and isorhamnetin-3-*O*-glucoside.

KEYWORDS: flavonoids, black rice, HPLC-ESI-MS, tandem mass spectrometry

INTRODUCTION

Rice is consumed as a staple food by over half of the world's population, and approximately 95% of its production is in Asia.¹ In the Asia-Pacific region, rice is a daily food, and it is believed to provide more health benefits than other carbohydrate-based foods because it contains several nutrients and antioxidative compounds such as vitamin B complex, vitamin E complex (tocopherols and tocotrienols), phytosterols, phytic acid, oryzanol, and phenolic compounds.^{2–6} Rice bran, a byproduct from rice milling, weighs 10% of the total rice grain weight and contains similar phytochemicals and antioxidative compounds as the rice itself.

Although rice is predominantly consumed in milled form as white rice, there are many special varieties of rice that contain pigments, such as black and red, in their bran part. The consumption of pigmented rice is becoming popular in many Asian countries where black rice is often mixed with white rice prior to cooking to enhance the quality and flavor. In addition, red pigment in rice grains is commonly used as a food colorant (e.g., in bread, ice cream, and liquor). In Thailand, black rice has gained increasing popularity in the rice market since the potential anticancer capacity of the extract from the bran of Thai black rice, Riceberry, was recently reported.⁷

Black rice has been regarded as a health-promoting food for its abundant content of phenolic compounds. As in other cereal grains, phenolic compounds in rice exist in soluble and insoluble forms, with the soluble form representing 38% to 60% of the polyphenol content in light brown rice grains and around 81% of the polyphenol content in red and black pericarp grains.^{8,9} Anthocyanins, a group of reddish to purple water-soluble flavonoids that are the primary pigments in red and black cereal grains have been widely identified and characterized.⁵ Cyanidin-3-*O*- β -glucoside and peonidin-3-*O*- β -glucoside have been reported to be the major components of anthocyanins in some colored rice.^{5,10} Many experiments have

been done on biological activities of black rice bran extracts,^{11,12} especially concerning antioxidation,^{13,14} that are related to chemical profile of the pigments. Other than anthocyanins, phytochemicals assumed to be related to human health benefits, such as those in the group of flavonols, flavones, flavanols, and flavanones, have not yet been explored in rice grains¹⁵ as much as other compositions reported in other cereals, fruits, and vegetables. This limitation may be caused by the fact that these groups of flavonoids contain several similar structures that require sophisticated high-resolution analytical techniques for their characterization. In addition, these flavonoids are often found as minor constituents in cereal grains and present in only small amounts.

In this study, an integrated approach consisting of HPLC with a photodiode array detector (DAD), HPLC-ESI-MS, and HPLC-ESI-MS/MS was used for the identification, characterization, and quantification of other flavonoid components apart from anthocyanins in brans of seven Thai black rice varieties. Riceberry was one of the seven. The simultaneous analysis of both free and glycosylated flavonoids in the Thai black rice bran extracts was done preliminarily by reversed-phase HPLC-DAD. To improve the identification and characterization of all flavonoids analyzed and to ensure the absence of the interfering peaks in the samples extracted from black rice bran, a tandem mass spectrometry with an electrospray interface (ESI) was utilized. The quantification of identified rice flavonoids was ultimately accomplished by HPLC-ESI-MS analysis against a standard calibration graph. The outcome of this research in terms of additional information on naturally occurring flavonoids apart from the known anthocyanins should provide

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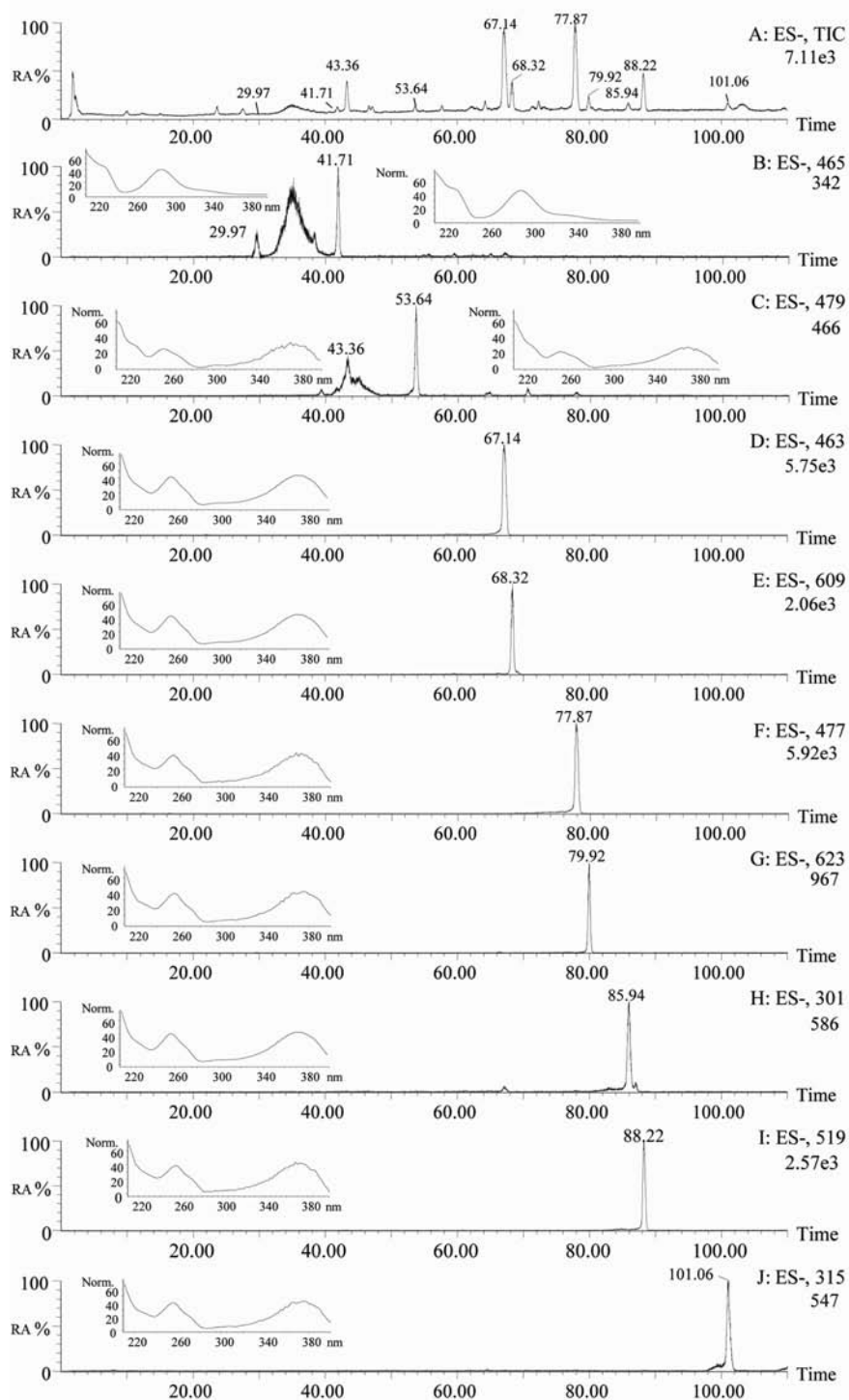


Figure 1. LC-MS chromatograms of the extract of mixed Thai black rice brans showing (A) a total ion chromatogram and the selected ion chromatograms overlay of m/z (B) 465, (C) 479, (D) 463, (E) 609, (F) 477, (G) 623, (H) 301, (I) 519, and (J) 315 with their ion abundances and UV-vis spectra.

useful basic information to aid rice breeding programs to produce pigmented rice with enhanced levels of the bioactive compounds that benefit human health.

MATERIALS AND METHODS

Chemicals. Acetonitrile and methanol, HPLC grade, and formic acid, analytical grade, were purchased from Merck (Darmstadt, Germany). Deionized water (Millipore Corporation, Bedford, MA)

was used throughout this experiment. Five flavonoid aglycones; eriodictyol (5,7,3,4'-tetrahydroxyflavanone), taxifolin (3,5,7,3',4'-pentahydroxyflavone), quercetin (3,5,7,3',4'-pentahydroxyflavone), myricetin (3,5,7,3',4',5'-hexahydroxyflavone), and isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavone), purity >99%, were used as standard compounds and purchased from Apin Chemicals (Oxon, U.K.). The stock solutions of these standards (100 $\mu\text{g/mL}$) were individually prepared in 50% methanol in water and stored at $-20\text{ }^{\circ}\text{C}$. A working concentration, 5 $\mu\text{g/mL}$, of each standard compound was obtained by

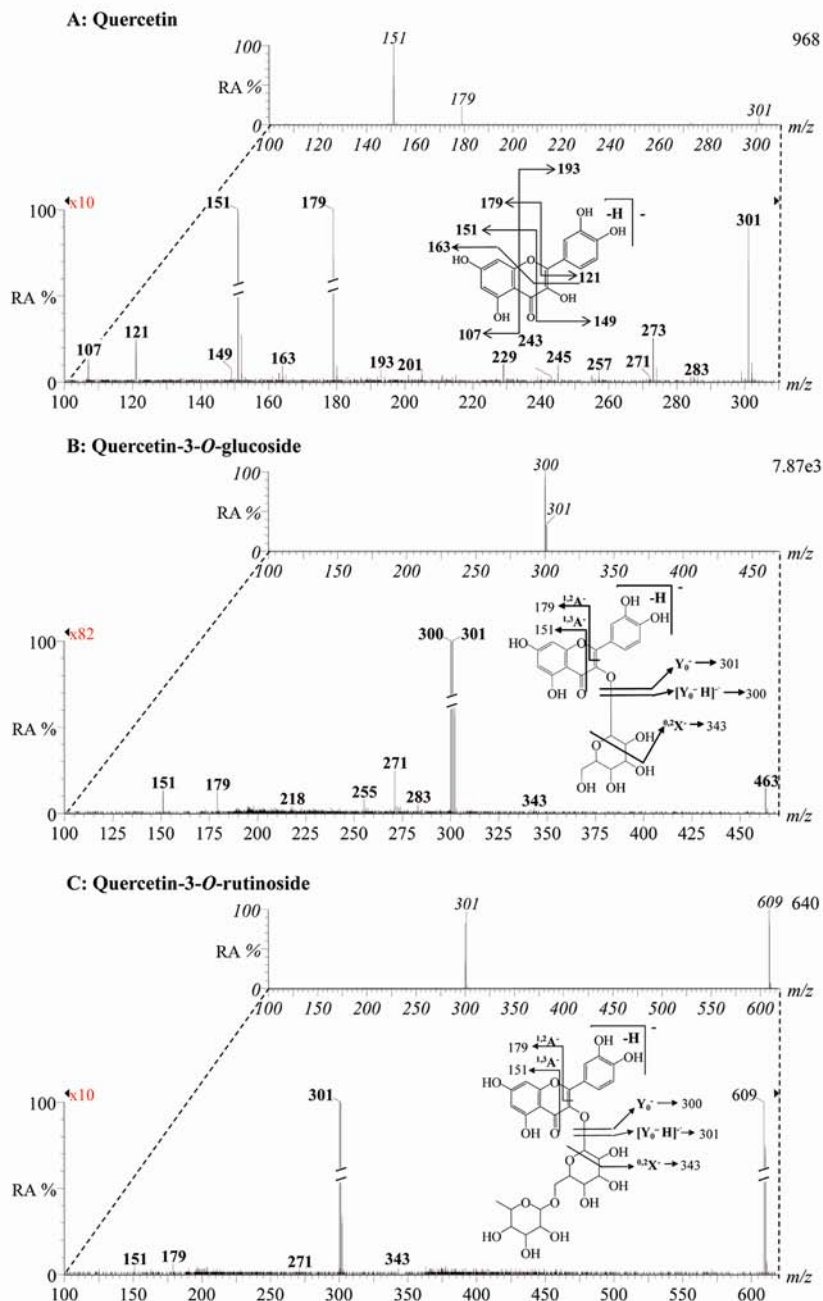


Figure 2. MS/MS spectra of (A) quercetin, $[M - H]^-$, at m/z 301; (B) quercetin-3-*O*-glucoside, $[M - H]^-$, at m/z 463; and (C) quercetin-3-*O*-rutinoside, $[M - H]^-$, at m/z 609.

diluting it with 50% methanol in water. These working solutions were freshly prepared on the day of the experiment. A mixture of all standard flavonoids was also prepared (5 $\mu\text{g}/\text{mL}$) for use in the optimization of the chromatographic separation experiment.

Materials. Three varieties of Thai nonglutinous black rice, 1000-0-01, BT No. 3, and Riceberry and four varieties of Thai glutinous black rice, 1681S, 49-6-6-6-1-0, 132-1-1-3-3-1-4-4, and 132-1-1-4-1-1-1-3, were used in this study. Additionally, a nonglutinous white rice, Khao Dawk Mali 105 (KDML 105), the most popular Thai Jasmine rice was included for comparison. All black rice samples were collected from the experimental field at Kasetsart University, Kamphaengsaen Campus, Nakorn Pathom province in central Thailand in the year 2010. The paddy was dried by modified hot air at a temperature of 40 $^{\circ}\text{C}$ until the moisture content was reduced to approximately 14%

(Wb), measured by a Dole model 400 moisture tester (Seedbuo Equipment Company). Then, the samples were stored in gunny sacks and kept in a controlled room at 15 $^{\circ}\text{C}$. On the day of the experiment, the samples were dehusked and milled by a local milling system (Natawee Technology, Chachoengsao, Thailand) for 30 s in order to obtain a portion of fresh bran weighing approximately 10% (w/w) of the brown rice sample. The bran samples were sieved by passing through an Endecotts test sieve (Endecotts Ltd., London) having an aperture size of 750 μm prior to further experiments.

Sample Preparation. Five grams of each black rice bran sample was placed in a bottle containing 50 mL of 0.1% formic acid in methanol and sonicated for 60 min using an FS30H sonicator (40 kHz, 100W) (Fisher Scientific, Pittsburgh, PA). Following this, the slurry mixture was centrifuged at 2500 rpm for 15 min, and the

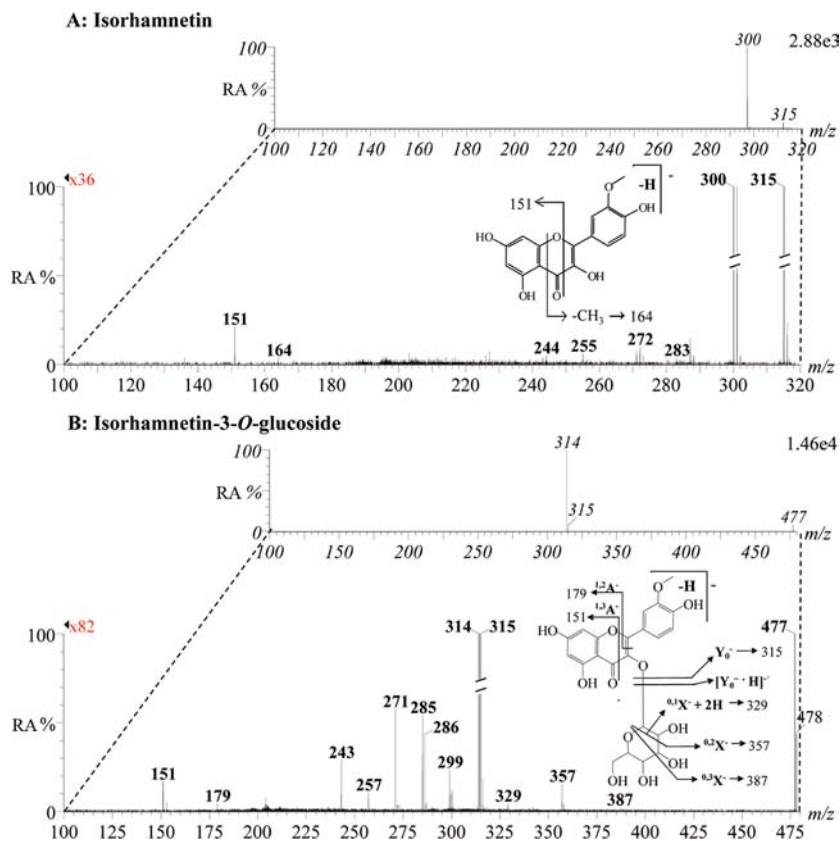


Figure 3. MS/MS spectra of (A) isorhamnetin, $[M - H]^-$, at m/z 315 and (B) isorhamnetin-3-*O*-glucoside, $[M - H]^-$, at m/z 477.

supernatant was filtered through a 0.45 μm pore nylon syringe filter (Whatman Limited, Maidstone, England) and evaporated under reduced pressure at a temperature below 40 °C until its volume was reduced to 2.0 mL. In order to avoid errors arising from unexpected degradation of some flavonoids, the LC-DAD-ESI-MS or MS/MS experiment was completed within 24 h after extraction.

LC-DAD-ESI-MS and MS/MS Analysis. The LC-DAD-ESI-MS/MS system consisted of a Waters Alliance 2695 LC-DAD (Waters Corp., Milford, MA) and a Q-TOF 2 (quadrupole mass filter-time-of-flight) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). All black rice bran extracts were subjected to separation using a 3 \times 100 mm Zorbax Eclipse column (Agilent Technologies) with a particle size of 3.5 μm . The mobile phase consisted of 0.1% formic acid in methanol (solvent A) and 0.1% formic acid in water (solvent B). The gradient varied linearly from 5% to 40% A (v/v) in 120 min with flow rate 0.2 mL/min. The DAD was set at 270, 310, and 350 nm to provide real-time chromatograms and the UV-vis spectra scanned from 200 to 650 nm were recorded for preliminary component identification.

The HPLC effluent was totally delivered into a Z-Spray ES source of a Micromass Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, U.K.), which was operated in a negative ionization mode at a temperature of 80 °C. Nitrogen was used as a nebulizing gas with a flow rate of 12 L/min and as a desolvation gas at a temperature of 150 °C. Meanwhile, capillary and cone voltages were set at 3.00 kV and 30 V, respectively. Ultrahigh purity-grade argon was used as a collision gas at a 10 psi inlet pressure for collision-induced dissociation (CID). Collision energy was mainly set at 35 eV. A scan time of 0.5 s with a m/z range of 50–650 was used to obtain the product ions mass spectra. The software used for data acquisition and processing was MassLynx NT, version 4.0 (Micromass, Manchester, U.K.). The LC system was directly connected with an MS instrument without stream splitting.

To determine individual flavonoid components in each black rice bran, the calibration curve of standard quercetin was constructed in the

concentration range of 1.0–1,000 $\mu\text{g}/\text{mL}$. Peak areas of standard quercetin and all detected flavonoids were measured as sums of the areas under peaks in the mass chromatograms of characteristic ions of each flavonoid. Concentrations of each flavonoid detected in the black rice bran samples were calculated based on the percentage recovery of quercetin-3-*O*-glucoside in the bran of BT No. 3 rice used as a representative sample. A consecutive multiple extraction of the flavonoid was performed. Its area under the peak in the first extraction step against the sum of peak areas in the multiple extraction measurements yielded an extraction recovery of 83%. The standard solutions and rice bran samples were prepared and analyzed in triplicate.

RESULTS AND DISCUSSION

Identification of Eleven Flavonoids in Black Rice Bran Extracts. A mixture of seven black rice bran extracts was used as a sample for optimization of the chromatographic system. The components were separated by a Zorbax Eclipse XDB C18 column and eluted by a mobile phase containing (A) 0.1% formic acid in methanol and (B) 0.1% formic acid in water. The LC-MS chromatogram of the mixed black rice bran extract is shown in Figure 1A. According to the UV-vis spectra of the separated components, groups of flavonoids were eluted in the period of retention time 25 to 105 min, after those of anthocyanins.

All black rice flavonoid components were classified based on their UV-vis spectra and deprotonated molecular ions. (A) The total ion chromatogram of the black rice bran extract and the extracted ion chromatograms of flavonoid molecular ions including m/z (B) 465 (29.97 and 41.71 min), (C) 479 (43.36 and 53.64 min), (D) 463 (67.14 min), (E) 609 (68.32 min), (F) 477 (77.87 min), (G) 623 (79.92 min), (H) 301 (85.94 min), (I) 519 (88.22 min), and (J) 315 (101.06 min) are shown

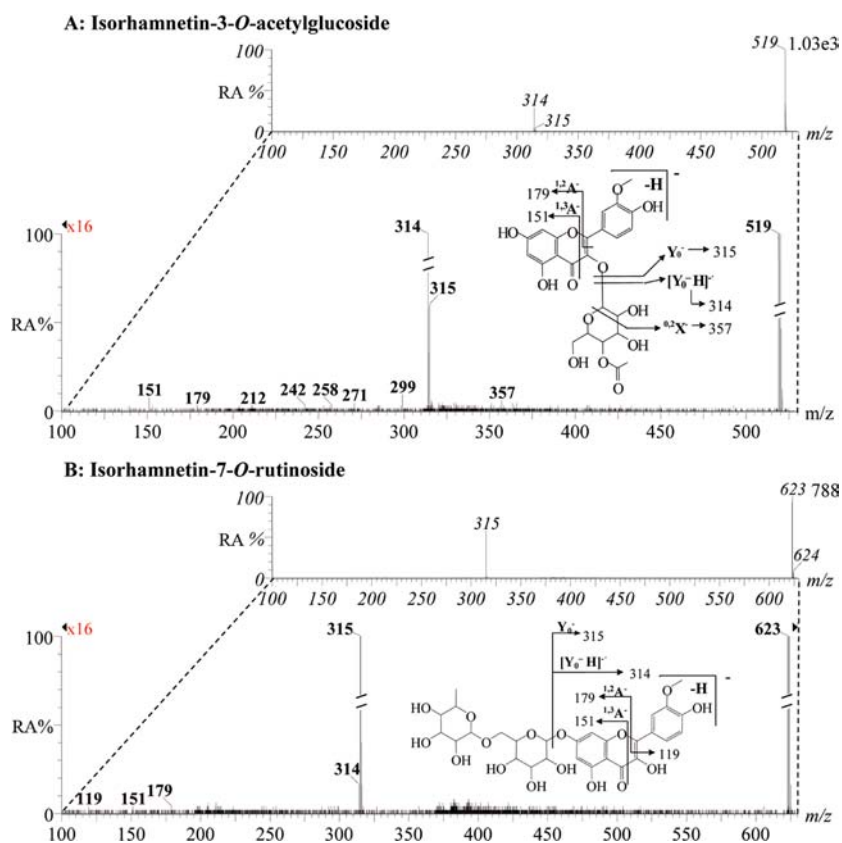


Figure 4. MS/MS spectra of (A) isorhamnetin-3-*O*-acetylglucoside, $[M - H]^-$, at m/z 519 and (B) isorhamnetin-7-*O*-rutinoside, $[M - H]^-$, at m/z 623.

in Figure 1. Among these 11 tentatively identified flavonoids, the components having molecular ions at m/z 465 possessed UV-vis spectra with a maximum wavelength (λ_{\max}) of 288 nm. The other flavonoids, with molecular ions at m/z 479, 463, 609, 477, 623, 301, 519, and 315, had two maximum wavelengths, one at 250 and one at 350 nm. These are in agreement with the characteristic UV-vis spectra of most flavonoids reported in literature.¹⁶

Identification of Flavonoid Aglycones Based On Comparison with Standard Compounds. By comparing the retention times, UV-vis spectra, and deprotonated molecular ions of the peaks of black rice bran extract with those of known standards, it was established that two components at retention times 85.94 (Figure 1I) and 101.06 min (Figure 1J) were quercetin and isorhamnetin, respectively. Further confirmation of this was obtained by the analysis of the quercetin and isorhamnetin characteristic fragmentation patterns obtained from the MS/MS spectra. The structural elucidations of these two flavonoids are shown in Figures 2A and 3A, respectively.

Quercetin possessed a weak deprotonated molecular ion at m/z 301. The dominant product ion at m/z 151 was present as the base peak along with the minor ion of m/z 179, these two ions being specific to retro Diels-Alder reaction (RDA) pathway¹⁷⁻¹⁹ leading to the $^{1,3}A^-$ ion and the $^{1,2}A^-$ ion, respectively. The $^{1,3}A^-$ ion could undergo further CO_2 loss that leads to a product ion at m/z 107. The RDA fragmentation of the C-ring also gave the $^{1,3}B^-$ and $^{1,2}B^-$ fragment ions at m/z 149 and 121, respectively. Another fragmentation pathway concerning neutral losses of H_2O , CO , CH_2O , and CO_2 from the $[M - H]^-$ ion, m/z 301, led to product ions at m/z 283,

273, 271, and 257, respectively. The existence of two ortho OH groups at positions 3' and 4' could be confirmed by this H_2O loss. Additionally, further losses of CO and CO_2 from the $[M - H - CO]^-$ ion, m/z 273, yielded minor product ions at m/z 245 and 229. This observation was consistent with those reported by the previous study.²⁰

Isorhamnetin exhibited the characteristic loss of a CH_3 radical from the pseudomolecular anion $[M - H]^-$, m/z 315, as shown in Figure 3A. This loss gave rise to a base peak ion at m/z 300. Losses of $\cdot OH$, CO , and HCO , which formed minor ions at m/z 283, 272, and 271, respectively, occurred as well as further losses of CO from these three anions. These losses gave rise to low intensity anions at m/z 255, 244, and 243, respectively. An ion at m/z 164 was attributed to the further loss of CO from a $[^{0,4}B - CH_3]^-$ ion. A fragment ion of m/z 151 was produced by the cleavage of the C ring and was assigned to be the RDA fragments $^{1,3}A^-$.

Structural Characterization of the Black Rice Flavonoid Glycosides. Quercetin-3-*O*-glucoside eluted at a retention time of 67.14 min. Its deprotonated molecular ion appeared weak at m/z 463, as shown in the enlarged scale of Figure 2B, which included the fragment ions of the aglycone part at m/z 151 and m/z 179 that are similar to those of quercetin. For the glycan part, the $[Y_0 - H]^-$ ion at m/z 300 showed significantly higher abundance than the Y_0^- ion, m/z 301. This revealed that the precursor ion at m/z 463 was the deprotonated molecular ion of flavonoid-3-*O*-glucoside.^{21,22} The product ions of m/z 283 and 255 were consistent with the further fragmentation of the Y_0^- ion by the successive losses of H_2O and CO molecules. The $[Y_0 - H]^-$ ion could undergo further fragmentation by loss of the CHO radical and C_2H_2O

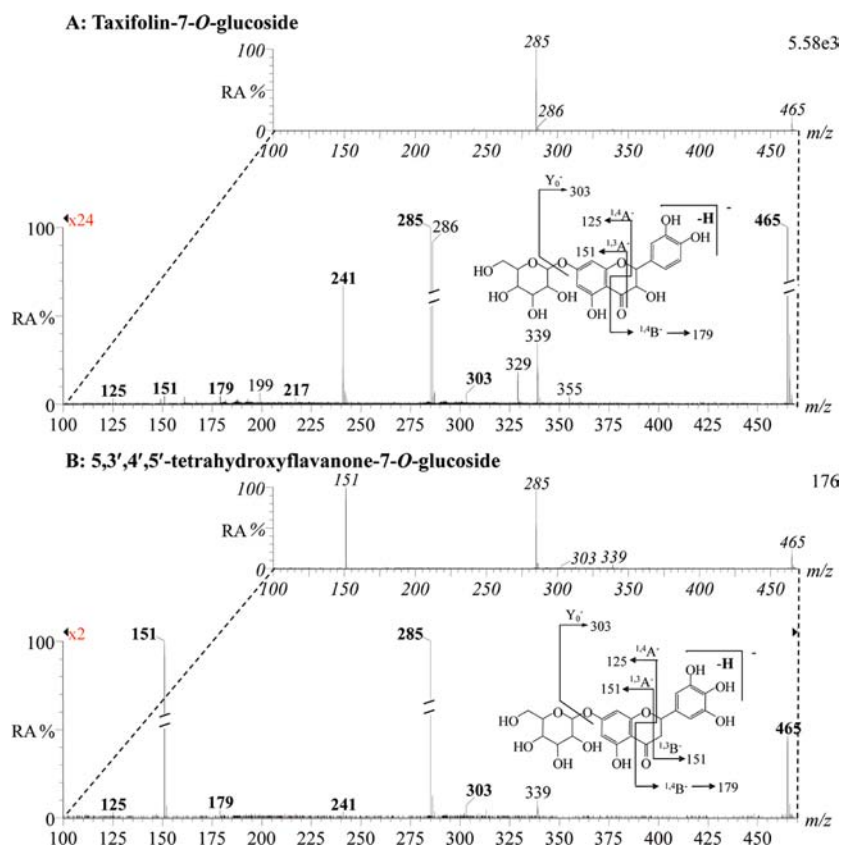


Figure 5. MS/MS spectra of (A) taxifolin-7-*O*-glucoside, $[M - H]^-$, at m/z 465 and (B) 5,3',4',5'-tetrahydroxyflavanone-7-*O*-glucoside, $[M - H]^-$, at m/z 465.

molecule, thus giving rise to the product ions at m/z 271 and 218, respectively. A similar structural determination based on mass fragmentation of this flavonoid was also shown in a previous study.²³

Quercetin-3-*O*-rutinoside, another derivative of quercetin, was detected at a retention time 68.32 min. Its MS/MS spectrum, shown in Figure 2C, possesses the highly abundant molecular ion, $[M - H]^-$, at m/z 609 together with the base peak ion at m/z 301. The fragmentation pattern of its aglycone was similar to that of quercetin, except the slight abundance difference of the Y_0^- and $[Y_0 - H]^-$ ions at m/z 301 and 300, respectively, showed the structure of flavonoid-monodiglycoside. Therefore, the structure of this quercetin derivative was assigned as quercetin-3-*O*-rutinoside.

Isorhamnetin-3-*O*-glucoside was detected at a retention time of 77.87 min. Its product-ion mass spectrum in Figure 3B contained a weak molecular ion at m/z 477. A mass difference of 162 and the significantly higher abundance of the $[Y_0 - H]^-$ ion at m/z 314 than that of the Y_0^- ion at m/z 315 clearly revealed the structure of methoxylated flavonoid-3-*O*-glucoside. The loss of the CH_3 radical from the Y_0^- ion, followed by the further loss of CHO and the combined loss of 2CHO and CO molecules, yielded the product ions m/z 271 and 243. These two ions are shown in the expanded scale spectrum. The ions at m/z 299, 285, and 257 resulted from successive losses of CH_3 , CHO, and CO molecules of the $[Y_0 - H]^-$ ion, respectively. Meanwhile, the existence of product ions of m/z 387, 357, and 329 were consistent with the ions $^{0,3}X^-$, $^{0,2}X^-$, and $^{0,1}X^-$, respectively, which were formed by scission of the glucoside moiety. Again, the characteristic C-ring cleavage fragmentation,

including $^{1,2}A^-$ and $^{1,3}A^-$ ions, was also detected at m/z 179 and 151, respectively.

Isorhamnetin-3-*O*-acetylglucoside with its molecular ion at m/z 519 as the base peak was identified at a retention time of 88.22 min. Figure 4A shows the product ion mass spectra of this flavonoid which was obtained using a collision energy of 25 eV. Both Y_0^- , m/z 315, and $[Y_0 - H]^-$, m/z 314, ions and the other product ions obtained from the cleavage of the C-ring were observed; most of these ions were similar to those appearing in the mass spectrum of isorhamnetin-3-*O*-glucoside. The difference of molecular ion masses between these two flavonoids of 42 amu could correspond to the acetyl group. Since flavonoid acetylglucoside generally occurs in natural sources,²⁴ the structure of isorhamnetin-3-*O*-acetylglucoside was suggested for this black rice flavonoid.

Isorhamnetin-7-*O*-rutinoside's product-ion mass spectrum was obtained at a retention time of 79.92 min. This mass spectrum had a molecular ion at m/z 623 as its base peak. Another product ion that appeared at m/z 315 revealed the existence of isorhamnetin. The residue loss of m/z 308 that occurred was consistent with the molecular weight of rutinoside. Scale enlargement showed some weak product ions. Among these, the ions m/z 179, 151, and 119 were identified as $^{1,2}A^-$, $^{1,3}A^-$, and $^{1,2}B^-$, respectively. The loss of the glycan part from the molecular ion generated two intense ions, Y_0^- and $[Y_0 - H]^-$, at m/z 315 and 314, respectively. The relatively high abundance of the Y_0^- ion indicated the position of this glycan as 7-*O*-glucoside. Therefore, these characteristic ions could be regarded as the fragment ions of isorhamnetin-7-*O*-rutinoside. Its fragmentation pattern is shown in Figure 4B.

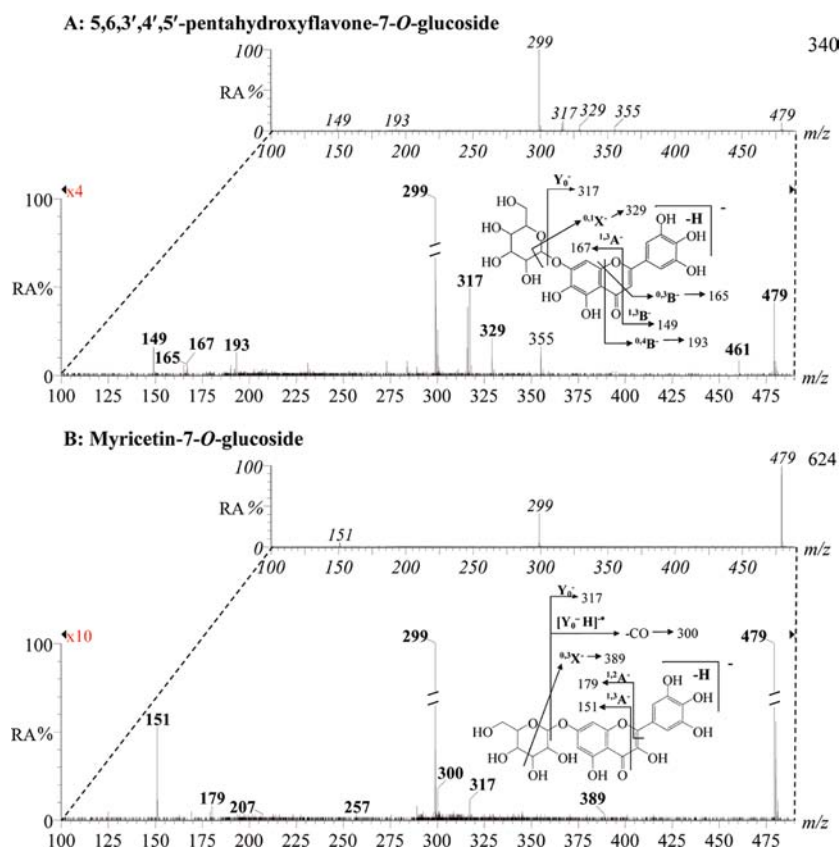


Figure 6. MS/MS spectra of (A) 5,6,3',4',5'-pentahydroxyflavone-7-O-glucoside, $[M - H]^-$, at m/z 479 and (B) myricetin-7-O-glucoside, $[M - H]^-$, at m/z 479.

Table 1. Molecular Weight, Retention Time, Characteristic Fragment Ions, Type of Glycoside, and Position of Glycosidic Bond of 11 Flavonoids Found in Black Rice Bran Extracts

flavonoid component	molecular weight	retention time (min)	class	characteristic ions (C-ring cleavage)	ratio of $Y_0/[Y_0 - H]^-$	position of glycosidic bond
quercetin	302	88.22	flavonol	$^{1,2}A^-$, $^{1,2}B^-$, $^{1,3}A^-$, $^{1,3}B^-$, $^{0,2}A^-$, $^{1,4}A^-$, $^{1,4}B^-$		
quercetin-3-O-glucoside	464	67.14	flavonol	$^{1,2}A^-$, $^{1,3}A^-$, $^{0,4}B^-$	<1	3-O-glucoside
quercetin-3-O-rutinoside	610	68.32	flavonol	$^{1,2}A^-$, $^{1,3}A^-$	<1	3-O-rutinoside
isorhamnetin	316	101.06	flavonol	$^{1,3}A^-$, $^{0,2}A^-$		
isorhamnetin-3-O-glucoside	478	77.87	flavonol	$^{1,2}A^-$, $^{1,3}A^-$, $^{0,4}B^-$	<1	3-O-glucoside
isorhamnetin-3-O-acetylglucoside	520	85.94	flavonol	$^{1,2}A^-$, $^{1,3}A^-$	<1	3-O-acetylglucoside
isorhamnetin-7-O-rutinoside	624	79.92	flavonol	$^{1,2}A^-$, $^{1,3}A^-$, $^{1,2}B^-$	>1	7-O-rutinoside
taxifolin-7-O-glucoside	466	29.97	flavanone	$^{1,4}A^-$, $^{1,4}B^-$, $^{1,3}A^-$	>1	7-O-glucoside
5,3',4',5'-tetrahydroxyflavanone-7-O-glucoside	466	41.71	flavanone	$^{1,4}A^-$, $^{1,4}B^-$, $^{1,3}A^-$, $^{1,3}B^-$	>1	7-O-glucoside
5,6,3',4',5'-pentahydroxyflavone-7-O-glucoside	480	43.36	flavone	$^{1,3}A^-$, $^{1,3}B^-$, $^{0,3}B^-$, $^{0,4}B^-$	>1	7-O-glucoside
myricetin-7-O-glucoside	480	53.64	flavonol	$^{1,2}A^-$, $^{1,3}A^-$	>1	7-O-glucoside

Taxifolin-7-O-glucoside, with a molecular ion of m/z 465, was the first flavonoid eluted among all flavonoid components and was obtained at a retention time of 29.97 min. Its production mass spectrum in Figure 5A shows the loss of a glycan part, 162 Da, which gives rise to the product ion of m/z 303. The occurrence of this Y_0^- ion, m/z 303, and the nonoccurrence of the $[Y_0 - H]^-$ ion, m/z 302, indicates that this compound has a 7-O-glucoside moiety. Additionally, UV-vis spectrum of this compound implies the structure of a flavanone with a 5 hydroxyl substituent group. In Figure 5A, the characteristic ion of flavonoid fragmentation, m/z 151, was observed with the

other characteristic ions of taxifolin fragmentation, m/z 179 and 125, which are attributed to $^{1,4}B^-$ and $^{1,4}A^-$ ions, respectively. Moreover, the product ions obtained from neutral and radical losses were also observed at the m/z 285, 241, and 217 that were identified as $[Y_0 - H_2O]^-$, $[Y_0 - (H_2O + CO_2)]^-$, and $[Y_0 - (C_2H_2O + CO_2)]^-$ ions, respectively.

5,3',4',5'-Tetrahydroxyflavanone-7-O-glucoside, shown at retention time 41.83 min, has the product ion mass spectrum (Figure 5B) similar to that of taxifolin-7-O-glucoside except for the existence of the characteristic ion of C-ring cleavage at m/z 151 present as the base peak. This high stability product ion

could be attributed to the C-ring cleavage that led to the formation of the $^{1,3}B^-$ ion rather than the $^{1,3}A^-$ ion, both of which possessed a m/z of 151. Therefore, the hydroxyl substitution pattern of this compound was more likely to be matched with flavones rather than flavonols.

5,6,3',4',5'-Pentahydroxyflavone-7-O-glucoside was obtained at a retention time of 43.36 min. This gave a product-ion mass spectrum that possessed a weak molecular ion at m/z 479. In this mass spectrum (Figure 6A), the occurrences of ions at m/z 317 and 316 represent the formation of the Y_0^- and $[Y_0 - H]^-$ ions, respectively. The greater abundance of the Y_0^- ion indicates the position of the glycosidic bond that a flavonoid-7-O-glycoside was assigned. The product ion of m/z 299 present as the base peak resulted from the loss of H_2O from the Y_0^- ion at m/z 317. The loss of H_2O from the precursor ion at m/z 479 was observed at m/z 461. The formation of product ion at m/z 329 is related to the cleavage of the glycan part and is identified as ion, $^{0,1}X^-$. Other minor product ions were obtained from C-ring cleavage. These included m/z 193, 165, 167, and 149, which were identified as $^{0,4}B^-$, $^{0,4}A^-$, $^{1,3}B^-$, and $^{1,3}A^-$, respectively.

Myricetin-7-O-glucoside, at a retention time of 53.64 min, produced a MS/MS spectrum with the deprotonated molecular ion of m/z 479 as the base peak. The relatively intense product ion of m/z 299 was obtained from further loss of H_2O from the Y_0^- ion, m/z 317. Characteristic ions, including $^{0,3}X^-$, $^{1,2}A^-$, and $^{1,3}A^-$, were also observed at m/z 389, 179, and 151, respectively. These ions are consistent with the structural assignment of this black rice flavonoid.

Table 1 summarizes structural information obtained by HPLC-ESI-MS/MS analysis of the 11 black rice flavonoids.

Quantitative Analysis of the Flavonoids in Black Rice Bran Extracts. Fresh bran extracts of each variety of Thai black rice, 1000-0-01, BT No. 3, Riceberry, 16815, 49-6-6-6-1-0, 132-1-1-3-3-1-4-4, and 132-1-1-4-1-1-1-3, together with a white rice variety, Khao Dawk Mali 105 (KDML 105), were subjected to quantitative analysis by an LC-ESI-MS system. Concentrations of each flavonoid in the extracts of black rice brans were calculated against the calibration curve of standard quercetin which was linear in the concentration range of 1.0–1,000 $\mu\text{g}/\text{mL}$ and with a regression equation of $y = 1.1439x + 4.6143$ ($r^2 = 0.9983$). Results obtained from this analysis are shown in Figures 7–9, which are expressed as μg of quercetin equivalent/ g of black rice bran ($\mu\text{g QE g}^{-1}$). The analysis used three aliquots of each extract that were measured in triplicate, with the average value calculated in each case.

Figure 7 shows that quercetin-3-O-glucoside was the most abundant quercetin derivative found in all black rice samples. Its contents in bran of most rice varieties were more than 200 $\mu\text{g QE g}^{-1}$ of black rice bran except for those of 132-1-1-4-1-1-1-3 (126 $\mu\text{g QE g}^{-1}$) and 132-1-1-3-3-1-4-4 (26 $\mu\text{g QE g}^{-1}$). The highest amount of this compound occurred at 305.20 $\mu\text{g QE g}^{-1}$ of black rice bran variety BT No. 3. Lower amounts, in the range of 10 to 90 $\mu\text{g QE g}^{-1}$ of black rice bran, were obtained for quercetin-3-O-rutinoside. However, this derivative was not detected in some rice varieties as well as its aglycone, quercetin, which also appeared in low amounts.

A series of isorhamnetin and its glycoside derivatives showed the same trend as quercetin in that the isorhamnetin-3-O-glucoside was more widely distributed among the black rice varieties and was found in greater amounts than the other derivatives (Figure 8). Isorhamnetin contents in the black rice bran extracts were in the range of 160–270 $\mu\text{g QE g}^{-1}$ of black

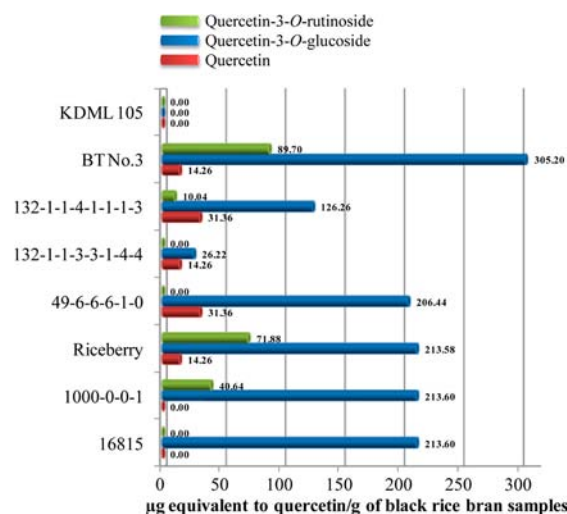


Figure 7. Contents of (A) quercetin, (B) quercetin-3-O-glucoside, and (C) quercetin-3-O-rutinoside in the bran of seven Thai black rice varieties and a white rice, KDML 105.

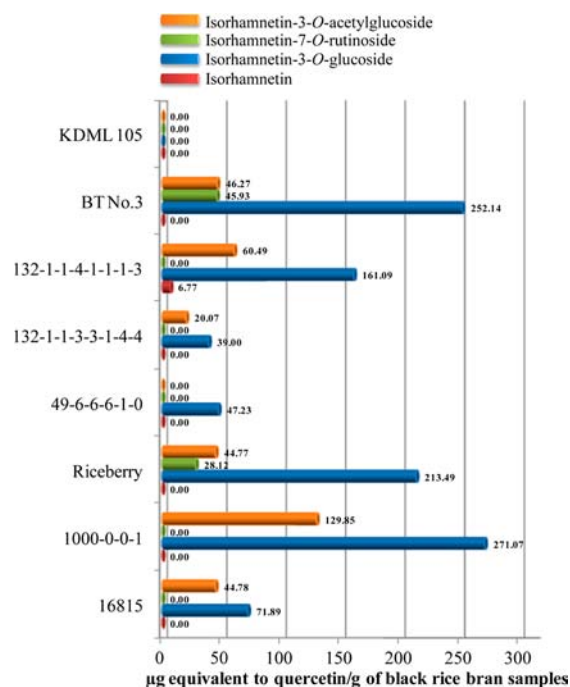


Figure 8. Contents of (A) isorhamnetin, (B) isorhamnetin-3-O-glucoside, (C) isorhamnetin-7-O-rutinoside, and (D) isorhamnetin-3-O-acetylglucoside in the bran of seven Thai black rice varieties and a white rice, KDML 105.

rice bran for 1000-0-0-1, BT No. 3, Riceberry, and 132-1-1-4-1-1-1-3 and in the range of 40–70 $\mu\text{g QE g}^{-1}$ of black rice bran for 16815, 49-6-6-6-1-0, and 132-1-1-3-3-1-4-4 rice varieties. This was followed by the contents of isorhamnetin-3-O-acetylglucoside ranging between 0 and 130 $\mu\text{g QE g}^{-1}$ of black rice bran. Isorhamnetin-7-O-rutinoside was only detected in bran extracts of the nonglutinous BT No. 3 and Riceberry samples at concentrations of 28 and 46 $\mu\text{g QE g}^{-1}$, respectively. Its aglycone, isorhamnetin, occurs only in the 132-1-1-4-1-1-1-3 variety at a very low concentration of 7 $\mu\text{g QE g}^{-1}$.

In Figure 9, the wide distribution of myricetin-7-O-glucoside and 5,6,3',4',5'-pentahydroxyflavone-7-O-glucoside among the black rice varieties occurred with higher concentrations of

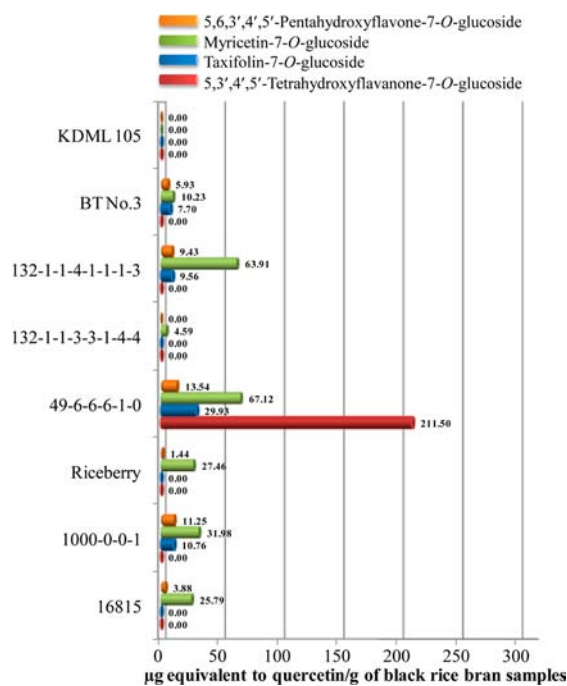


Figure 9. Contents of (A) taxifolin-7-*O*-glucoside, (B) 5,3',4',5'-tetrahydroxyflavanone-7-*O*-glucoside, (C) 5,6,3',4',5'-pentahydroxyflavone-7-*O*-glucoside, and (D) myricetin-7-*O*-glucoside in the bran of seven Thai black rice varieties and a white rice, KDML 105.

myricetin-7-*O*-glucoside in all extracts of the black rice bran. For the flavanone glucosides, taxifolin-7-*O*-glucoside was not detected in some black rice bran extracts, while 5,3',4',5'-tetrahydroxyflavanone-7-*O*-glucoside was found only in the extract of 49-6-6-6-1-0 at a relatively high amount of 212 $\mu\text{g QE g}^{-1}$. Concentrations of myricetin-7-*O*-glucoside ranged from 5 to 67 $\mu\text{g QE g}^{-1}$, while that of taxifolin-7-*O*-glucoside ranged from 0 to 30 $\mu\text{g QE g}^{-1}$ and concentrations of 5,6,3',4',5'-pentahydroxyflavone-7-*O*-glucoside ranged from 0 to 14 $\mu\text{g QE g}^{-1}$.

These results demonstrated that the amount of each flavonoid component was varied with the variety of rice. In the same rice variety, some flavonoids present in significantly higher concentrations than the others and the flavonoid-3-*O*-glucosides, if found, are usually a majority. It is remarkable that none of these eleven flavonoids were detected in the bran extract of the white rice KDML105.

In terms of method validation, the method developed in this study gave limits of detection (LOD) at 0.75, 0.50, and 1.00 $\mu\text{g/mL}$ for quercetin, myricetin, and isorhamnetin, respectively. For accuracy of analysis higher than 95%, the LODs of quercetin, myricetin, and isorhamnetin were 2.5, 2.0, and 3.5 $\mu\text{g/mL}$, respectively. The precision of the method was evaluated after inter- and intraday analysis in triplicates. Its relative deviation ranged from 2.43 to 4.76%.

In conclusion, black rice flavonoids, as well as their glycosides, that accumulated in the bran part of some Thai black rice varieties were identified by systematic instrumental analysis. The data processing in the mass chromatogram mode was used to monitor the specific ions at the *m/z* that corresponded to the characteristic or molecular ions of the flavonoids of interest. The presence of these flavonoids was also confirmed by their UV-vis spectra obtained by DAD. Structural analysis of these black rice flavonoids was then performed by analyzing their

ESI-MS and ESI-MS/MS spectra. This revealed the presence of 11 flavonoids. Among these, taxifolin-7-*O*-glucoside, myricetin-7-*O*-glucoside, isorhamnetin-3-*O*-acetylglucoside, isorhamnetin-7-*O*-rutinoside, and 5,6,3',4',5'-pentahydroxyflavone-7-*O*-glucoside were found for the first time in black rice bran. However, none of the black rice varieties possessed all of these 11 flavonoids. Also, none of these 11 flavonoids was found in the bran of white rice KDML105. The quantitative analyses also suggested that different rice varieties possessed flavonoids in different concentrations. The most abundant glycoside derivative of flavonoids that was widely distributed among the rice varieties was monoglucoside, such as quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, taxifolin-7-*O*-glucoside, and myricetin-7-*O*-glucoside.

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