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Phytochemical Profiles of Black, Red, Brown, and White Rice from the Camargue Region of France

Gema Pereira-Caro,[†] Gerard Cros,[‡] Takao Yokota,[¶] and Alan Crozier*[,†]

[†]Joseph Black Building, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

[‡]Laboratoire de Pharmacologie, CNRS UMR 5247 and Université Montpellier 1 and 2, Institut des Biomolécules Max Mousseron, Faculté de Pharmacie, 15 avenue Charles Flahault, Montpellier 34093 cedex 05, France

[¶]Department of Biosciences, Teikyo University, Utsunomiya 320-8551, Japan

ABSTRACT: Secondary metabolites in black, red, brown, and white rice grown in the Camargue region of France were investigated using HPLC-PDA-MS^2. The main compounds in black rice were anthocyanins (3.5 mg/g), with cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside predominating, followed by flavones and flavonols (0.5 mg/g) and flavan-3-ols (0.3 mg/g), which comprised monomeric and oligomeric constituents. Significant quantities of γ-oryzanols, including 24-methylenecycloartenol, campesterol, cycloartenol, and β-sitosterol ferulates, were also detected along with lower levels of carotenoids (6.5 μg/g). Red rice was characterized by a high amount of oligomeric procyanidins (0.2 mg/g), which accounted >60% of secondary metabolite content with carotenoids and γ-oryzanol comprising 26.7%, whereas flavones, flavonols and anthocyanins were <9%. Brown and white rice contained lower quantities of phytochemicals, in the form of flavones/flavonols (21−24 μg/g) and γ-oryzanol (12.3−8.2 μg/g), together with trace levels of the carotenoids lutein and zeaxanthin. Neither anthocyanins nor procyanidins were detected in brown and white rice. By describing the profile of the heterogeneous mixture of phytochemicals present in different rice varieties, this study provides a basis for defining the potential health effects related to pigmented and nonpigmented rice consumption by humans.

KEYWORDS: *white, red, brown, and black rice, anthocyanins, flavones, flavonols, procyanidins, carotenoids, γ-oryzanol*

INTRODUCTION

Rice (*Oryza sativa* L.) is recognized as the most consumed staple food in the world, especially in Asian countries. The rice grain has a hard husk protecting the inner kernel. After the husk is removed, the remaining product is referred to as brown rice, which comprises the bran, germ, and endosperm. White rice, the most commonly consumed rice around the world, is obtained from brown rice after the commercial rice-milling process removes the bran layer (5−8% of the brown rice weight), which is the major byproduct derived from rice milling and is rich in protein, fiber, oil, vitamins, and other phytochemicals.[1,2] This results in white rice being depleted of most of the nutritional components of the grain. In recent decades, colored varieties including black, brown, and red rice, have increased popularity in Asia. They are rich sources of bioactive compounds, such as tocopherols, tocotrienols, vitamins B and E, γ-oryzanols, and (poly)phenolic compounds, with potential health effects.[3,4]

Several papers have described the beneficial effects of pigmented rice seeds and bran phytochemicals, including the suppression of tumor progression or carcinogenesis in mice and several human cancer cell lines,[5−7] reduction of oxidative stress both in vitro and in animal models,[8,9] reduction of platelet hyperactivity and hyperglyceridemia in dyslypidemic rats,[10] improved serum lipid profiles and enhanced mRNA expression levels of fatty acid metabolism-related genes, and reduction of hyperlipidemia and hyperglycemia in rats.[11]

Among the phytochemicals occurring in pigmented rice, it is important to highlight cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside, which are two of the main anthocyanins in black rice varieties, being localized in the pericarp and aleurone layers of the seeds.[12] However, pigmented rice contains other secondary metabolites with potential health effects including flavonols, flavones, flavan-3-ols, carotenoids, and γ-oryzanols.[13−16] There are several papers that focus on the identification and quantitative analysis of anthocyanins in black rice cultivars, but few studies have examined simultaneously and, in depth, the profile of phytochemicals in black, brown, red, and white rice. The present study analyzed a variety of anthocyanins, chlorogenic acids, flavones, flavonols, flavan-3-ols, carotenoids, and γ-oryzanols in black, brown, red, and white rice, with identifications and quantitative analysis using an integrated approach consisting of HPLC with a photodiode array detector (PDA), a fluorometer (FL), and electrospray ionization tandem mass spectrometry (MS^2).

MATERIALS AND METHODS

Rice. Black (*Riz de Camargue long noir complet Bio*, lot 560 DLUO: 11/2009, cultivar Artemide), long brown (*Riz de Camargue long complet Bio*, cultivar Arelate), and white (*Riz de Camargue long blanc Bio*, cultivar Arelate) rice seeds were supplied by Biosud (Arles, France). Red rice seeds (*Riz de Camargue rouge long complet*, cultivar TamTam) were obtained from Centre Français du Riz (Arles, France).

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All of the rice varieties were cultivated in the Camargue area, south of Arles (France), harvested in 2008, dried, and cleaned. Rice was locally stored in mesh cells individually ventilated with temperature sensors and automatic tracking from 8 to 18 °C. Black, red, and long brown rice samples were dehusked, whereas white rice was dehusked and milled.

Chemicals. Cyanidin 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside, quercetin 3-*O*-glucoside, and quercetin 3-*O*-rutinoside were purchased from Extrasynthese (Lyon, France). Pelargonidin 3-*O*-glucoside and peonidin 3-*O*-glucoside were supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Acetonitrile, acetone, and methanol were purchased from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK). 5-*O*-Caffeoylquinic acid was obtained from AASC Chemicals (Southampton, UK). *β*-Carotene, lutein, hexane, formic acid, acetic acid, and methyl *tert*-butyl ether (MTBE) were supplied by Sigma (Poole, UK). *γ*-Oryzanol was obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Extraction of Flavonoids and Chlorogenic Acids. Two grams of the dry rice seed was imbibed by soaking in 60 mL of water/acetic acid (99:1, v/v) for 12 h. The acidified water was then passed through an SPE cartridge (Strata C18, 55 *μ*m, 70 Å, 10 g) (Phenomenex, Macclesfield, Cheshire, UK) previously preconditioned with 6 mL of methanol, followed by 6 mL of water/formic acid (99:1, v/v), after which it was eluted with 6 mL of methanol/formic acid (99:1, v/v). Imbibed rice seeds were homogenized with 10 mL of 1% formic acid in methanol using a T-25 basic Ultra-Turrax homogenizer (IKA Werke KG, Staufen, Germany) for 2 min at 24000 rpm prior to being centrifuged for 15 min at 4000*g*. The pellet was re-extracted three times with 10 mL of the same solvent. The SPE methanolic eluate and all supernatants were pooled before being reduced to dryness in vacuo at 30 °C. The residues were resuspended in 5 mL of methanol/formic acid (99:1, v/v) and anthocyanins and other flavonoids and chlorogenic acids were analyzed by HPLC-PDA-MS^2 in triplicate.

Extraction of Procyanidins. The method of Robbins et al.[17] was used for the analysis of procyanidins with a degree of polymerization >2. Briefly, 2 g of rice seed was homogenized with 5 mL of an acetone/water/acetic acid mixture (70:29.5:0.5, v/v/v). Samples were centrifuged at 3000*g* during 15 min at 4 °C, and supernatants were collected. The pellet was re-extracted three times with 5 mL of the same solvent as described above. All supernatants were pooled and passed through an SPE cartridge, Strata SCX (55 *μ*m, 70 Å, 500 mg/3 mL) (Phenomenex), following preconditioning of the cartridge with distilled water. Five microliters of the cartridge eluate was analyzed in triplicate by HPLC-FL-MS.

Extraction of Carotenoids and *γ*-Oryzanol. The method of Liu et al.[18] was adapted to extract carotenoids and *γ*-oryzanols from rice seeds. Two grams of rice seed was homogenized with 10 mL of ethanol/hexane (4:3, v/v) using an Ultra-Turrax homogenizer for 2 min at 12000 rpm, prior to being centrifuged for 15 min at 3500*g*. The pellet was re-extracted twice with 10 mL of hexane and centrifuged. The supernatant were pooled and washed first with 10 mL of distilled water and then with 5 mL of a 10% aqueous NaCl solution. The organic phase was retained and reduced to dryness under a gentle stream of nitrogen before being dissolved in 500 *μ*L of MTBE/methanol (90:10, v/v). All procedures were performed quickly, avoiding exposure to light, oxygen, high temperature, and also pro-oxidant metals. Aliquots were analyzed by HPLC-PDA-MS^2.

HPLC-PDA-MS^2 Analysis. Rice extracts were analyzed on a Surveyor HPLC system equipped with a PDA detector scanning from 200 to 600 nm and an autosampler (Thermo Finnigan, San Jose, CA, USA) cooled at 4 °C. Separation of anthocyanins and other secondary metabolites from rice seeds was performed using a 250 mm × 4.6 m i.d., 4 *μ*m, Synergi Max-RP 80 Å reverse phase column (Phenomenex) with a 4 mm × 3 mm i.d. guard column of the same material (Phenomenex) and maintained at 40 °C. The mobile phase, pumped at a flow rate of 1 mL/min, was (i) a 60 min, 10−40% gradient of methanol in 1% aqueous formic acid for the analysis of anthocyanins and (ii) a 75 min, 10−40% gradient for 60 min, followed by a 15 min, 40−50% gradient of methanol in 1% aqueous formic acid for the analysis of flavones and flavonols.

Analysis of carotenoids and *γ*-oryzanols was carried out on a 150 mm × 4.6 mm i.d., 2.6 *μ*m, Kinetex XB-C18 column with a 4 mm × 3 mm i.d. guard column of the same material (Phenomenex) maintained at 35 °C and eluted isocratically with methanol/MTBE/water (85:14:0.5, v/v/v) at a flow rate of 1 mL/min.

After passing through the flow cell of the diode array detector, the column eluate was split, and 0.3 mL/min was directed either to an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan) fitted an electrospray interface (ESI) operating in positive ionization mode for anthocyanins and in negative ionization mode for flavone, flavonols, and *γ*-oryzanols or to an LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan) fitted with ESI operating in positive ionization mode for carotenoids.

Identification of anthocyanins, chlorogenic acids, flavones, and flavonols in all samples was carried out using full scan, data-dependent MS^2 scanning from m/z 100 to 800 and selected reaction monitoring. With ESI in positive ionization mode, the capillary temperature was 300 °C, sheath gas was 50 units, auxiliary gas was 40 units, and source voltage was 3 kV for anthocyanins, whereas for carotenoids, the capillary temperature was set to 325 °C, sheath gas flow was 40 units, auxiliary gas was 20 units, and source voltage was 4.1 kV. For negative ionization, the capillary temperature was set to 300 °C, sheath and auxiliary gas were 70 and 60 units, respectively, and source voltage was 5 kV.

Anthocyanins in rice seed were quantified on the basis of chromatographic peak areas acquired at 520 nm and expressed as available standards or as cyanidin 3-*O*-glucoside equivalents when standards were not available. Chlorogenic acids were quantified on the basis of the absorbance response at 325 nm and expressed as 5-*O*-caffeoylquinic acid equivalents. Quantitative analysis of flavones and flavonols was based on the absorbance response at 365 nm and expressed as available commercial standards or quercetin equivalents. Carotenoids were identified according to the following criteria: retention times and absorbance spectra of commercially available standards or absorbance spectra compared with published data. For identification proposes, full scan data acquisition from m/z 300 to 800 and selective ion monitoring were carried out. The carotenoids were quantified from their chromatographic peak areas acquired at 450 nm. Lutein was quantified by reference to a standard, whereas *β*-carotene, zeaxanthin, and lycopene were quantified in *β*-carotene equivalents. *γ*-Oryzanols were identified on the basis of retention times and absorbance spectra compared with a *γ*-oryzanol standard. These compounds were quantified from their chromatographic peak areas recorded at 325 nm and expressed as *γ*-oryzanol equivalents. The limits of detection ranged from 0.1 to 0.2 ng for anthocyanins, from 0.2 to 0.5 ng for chlorogenic acids, from 0.1 to 0.3 ng for flavones and flavonols, from 0.1 to 0.5 for carotenoids, and from 8 to 10 ng for *γ*-oryzanols. Limits of quantification based on a >3:1 signal to background ratio were 0.6−0.8 ng for anthocyanins, 0.9−2.5 ng for chlorogenic acids, 1−3 ng for flavones and flavonols, 0.9−1.8 ng for carotenoids, and 10−30 ng for *γ*-oryzanols.

HPLC-FL-MS Analysis of Procyanidins. The analysis of procyanidins was carried out using the HPLC system with an FP-920 fluorescence detector (Jasco, UK, Ltd.) and a mass spectrometer. Fluorescence detection was conducted at an excitation wavelength of 230 nm and an emission wavelength of 321 nm. The separation was achieved using a 250 mm × 4.6 mm i.d., 5 *μ*m, Develosil Diol 100 Å column (Phenomenex) with chromatographic conditions that have been previously described.[17]

After passing through the flow cell of the fluorescence detector, the column eluate was split, and 0.3 mL/min was directed to an LCQ Duo IT mass spectrometer (Thermo Finnigan) fitted with an electrospray interface operating in negative ionization mode. With ESI in negative ionization mode, capillary temperature was set to 300 °C, sheath and auxiliary gas were 70 and 60 units, respectively, and source voltage was 5 kV.

Procyanidins were identified on the basis of their MS spectra and quantified in (−)-epicatechin equivalents using fluorescence peak areas, after which estimates were adjusted to account for the reduced fluorescence response for the procyanidins oligomers and polymers.[17]

Table 1. HPLC-PDA-MS^2 Retention Time and Characteristic MS Ions of Anthocyanins, Chlorogenic Acids, Flavones, Flavonols, Carotenoids, and γ-Oryzanols in Different Rice Varieties

peak	t_R (min)	compound	$[M - H]^-$ $(m/z)^a$	MS^2 (m/z)
		Anthocyanins		
1	19.4	cyanidin 3,5-diglucoside	611^+	449, 287
2	21.7	cyanidin 3-*O*-glucoside	449^+	287
3	25.7	cyanidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside	595^+	449, 287
4	26.0	pelargonidin 3-*O*-glucoside	433^+	271
5	29.1	peonidin 3-*O*-glucoside	463^+	301
6	32.6	peonidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside	609^+	463, 301
7	32.7	cyanidin 3-*O*-arabidoside	419^+	287
		Chlorogenic Acids		
8	17.4	3-*O*-feruloylquinic acid	367	193, 134, 173
9	21.1	4-*O*-feruloylquinic acid	367	173, 193, 154, 134
		Flavone and Flavonols		
10	22.1	taxifolin *O*-hexoside	465	303, 285
11	27.2	taxifolin *O*-hexoside	465	303, 285
12	31.4	luteolin 6/8-C-pentoside-6/8-C-hexoside	579	519, 489, 459, 399, 369
13	34.1	luteolin 6/8-C-pentoside-6/8-C-hexoside	579	519, 489, 459, 399, 369
14	36.3	apigenin 6/8-C-pentoside-8/6-C-hexoside	563	503, 473, 443, 383, 353
15	40.1	apigenin 6/8-C-pentoside-8/6-C-hexoside	563	503, 473, 443, 383, 353
16	41.7	apigenin 6/8-C-pentoside-8/6-C-hexoside	563	503, 473, 443, 383, 353
17	47.6	quercetin 3-*O*-glucoside	463	301, 176, 151
18	48.5	diosmetin 8-C-hexoside	461	443, 371, 341
19	50.7	quercetin 3-*O*-rutinoside	609	447, 301
20	57.7	isorhamnetin 3-*O*-glucoside	477	314, 299, 243

peak	t_R (min)	compound	λ_{max}	$[M - H]^-$ (m/z)	MS^2 (m/z)
		Carotenoids			
21	2.6	lutein	420, 445, 470	568^+	
22	4.8	zeaxanthin	320, 470	568^+	
23	15.3	lycopene	410, 475, 505	536^+	
24	21.4	β-carotene	450, 475	536^+	
		γ-Oryzanols			
25	7.8	cycloartenol ferulate	245, 305, 325	615	601, 177
26	8.4	24-methylenecycloarternol ferulate	240, 295, 325	601	587, 542, 177
27	9.3	campesterol ferulate	235, 325	589	574, 542, 177
28	10.2	β-sitosterol ferulate	240, 325	575	560, 545, 177

^{a}+ indicates $[M + H]^+$ rather than $[M - H]^-$.

Statistical Analysis. Data are expressed as the mean value ± standard error ($n = 3$). Multiple comparisons were carried out using one-way analysis of variance (ANOVA) using Statistix 8.0. The level of significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

Identification of Secondary Metabolites in Black, Red, Brown, and White Rice. The secondary metabolite composition of black, red, long brown, and white rice was determined using HPLC-PDA-MS^2. The bases of the proposed identifications are shown in Table 1 and summarized as follows:

Peak 1 (t_R 19.4 min) had a positive charged molecular ion ($[M - H]^+$) at m/z 611, yielding an MS^2 ion at m/z 449, which fragmented with a loss of 162 Da (hexose group) to produce an m/z 287 (cyanidin) daughter ion. This compound is tentatively identified as cyanidin 3,5-*O*-diglucoside, **1** (Figure 1). Peak 2 (t_R 21.7 min) had a $[M - H]^+$ at m/z 449, which fragmented with a loss of 162 Da (hexose group) to produce an m/z 287 (cyanidin) daughter ion. Cochromatography with an authentic standard established that peak 2 is cyanidin 3-*O*-glucoside, **2**. Peak 3 (t_R 25.7 min) produced a $[M - H]^+$ at m/z 595, which on MS^2 yielded product ions at m/z 287 and 449, corresponding to cyanidin and cyanidin *O*-glucoside, respectively. In keeping with published data,[19] this compound is tentatively identified as cyanidin 3-*O*-(6″-*O*-*p*-coumaryl)-glucoside, **3**. Peak 4 (t_R 26.0 min) had a $[M - H]^+$ at m/z 433, which upon MS^2 fragmented with a loss of 162 Da (hexose group) to yield an m/z 271 (pelargonidin) daughter ion. The fragmentation pattern and the cochromatography with authentic standard identified peak 4 as pelagonidin 3-*O*-glucoside, **4**. Peak 5 (t_R 29.1 min) had a $[M - H]^+$ at m/z 463, which upon MS^2 fragmentation yielded a daughter ion at m/z 301 (peonidin). This loss of 162 Da corresponds to cleavage of a hexose moiety. Cochromatography with an authentic standard identified peak 5 as peonidin 3-*O*-glucoside, **5**. Peak 6 (t_R 32.6 min) yielded a $[M - H]^+$ at m/z 609, which gave rise to MS^2 ions at m/z 463 and 301, corresponding to peonidin *O*-glucoside and peonidin, respectively. A comparison with previous findings[20] indicated that peak 6 is probably peonidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside, **6**. Peak 7 (t_R 32.7) produced a $[M - H]^+$ at m/z 419, which yielded a major MS^2 fragment at m/z 287 (cyanidin) that corresponded to a loss of a 132 Da (pentoside ion). On the basis of published fragmentation pattern and data,[21] this compound is tentatively identified as cyanidin 3-*O*-arabidoside, **7**.

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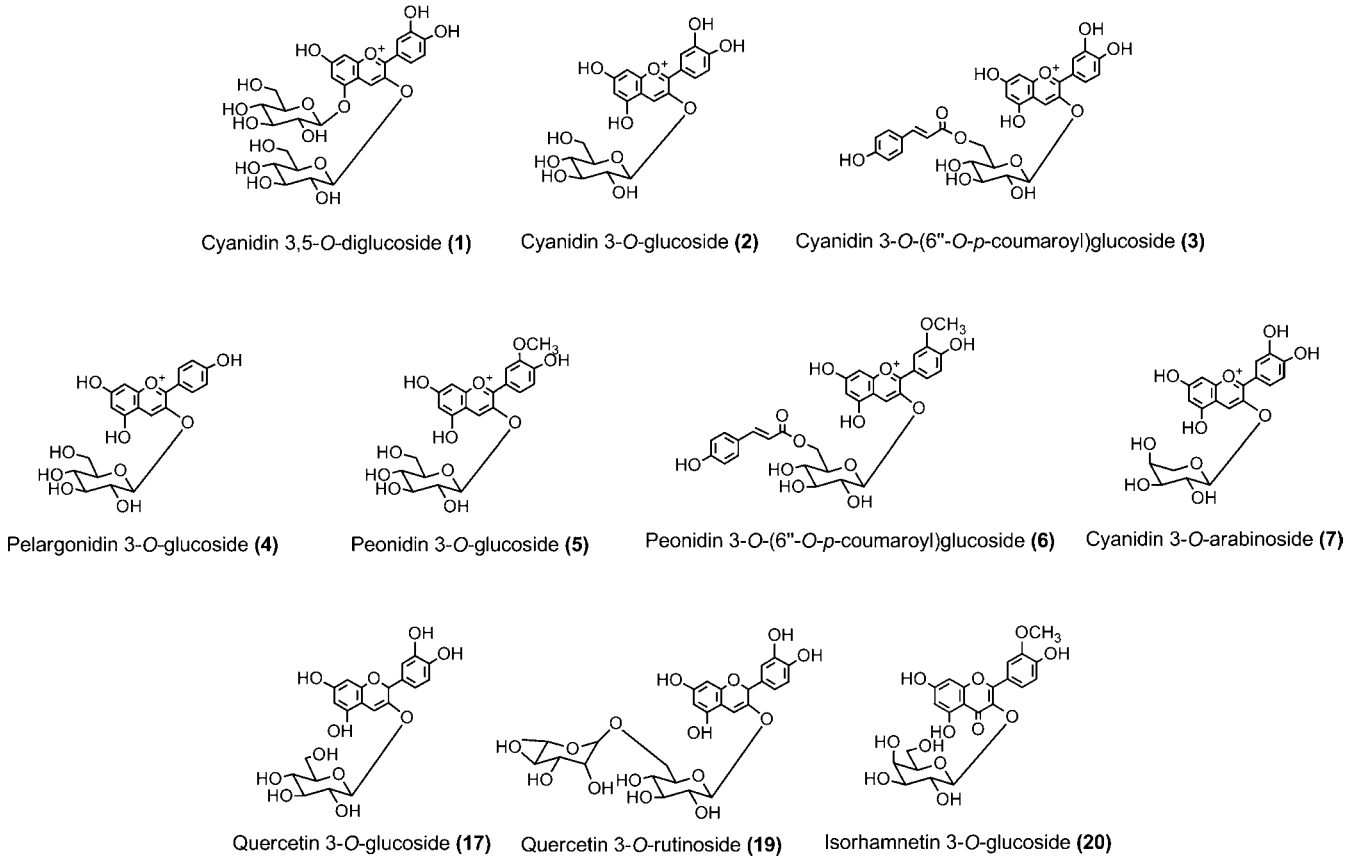

Figure 1. Structures of anthocyanins and flavonols detected in black, red, brown, and white rice.

Peaks 8 and 9 (t_R 17.4 and 21.1 min, respectively) had a $[M - H]^-$ ion at m/z 367 that fragmented to yield a MS^2 spectrum with ions at m/z 193, 173, and 134. These peaks were identified as isomers of feruloylquinic acid, which have previously been detected in red rice.[13] On the basis of the MS^3 fragmentation patterns of chlorogenic acids previously reported,[22] peaks 8 and 9 are tentatively identified as 3-*O*-feruloylquinic acid and 4-*O*-feruloylquinic acid, respectively.

Peaks 10 and 11 (t_R 22.1 and 27.2 min, respectively) produced a $[M - H]^-$ at m/z 465, yielding MS^2 ions at m/z 285 and 303, dihydroquercetin $- H_2O$ and dihydroquercetin $- H$ structures, respectively, which in accordance with published data[23] is in keeping with these peaks being taxifolin *O*-hexoside isomers.

Peaks 12 and 13 (t_R 31.4 and 34.1 min, respectively) had a $[M - H]^-$ at m/z 579, which yielded MS^2 ions at m/z 489, 369, 399, 459, and 519. The fragments at m/z 489 (loss of 90 Da) and 459 (loss of 120 Da) indicated the presence of a *C*-linked hexosyl unit, whereas the fragment at m/z 519 (loss of 60 Da) is produced by partial cleavage of a pentosyl unit. The ions at m/z 369 (m/z 286 aglycone + m/z 83) and 399 (m/z 286 aglycone + m/z 113) suggest the aglycone is luteolin. In keeping with Figueirinha et al.,[24] peaks 12 and 13 were tentatively identified as luteolin 6/8-*C*-hexoside-6/8-*C*-pentosides. Both of these flavones have been reported to occur in pigmented wild rice varieties.[25]

Peaks 14−16 (t_R 36.3, 40.1, and 41.7 min, respectively) had a $[M - H]^-$ at m/z 563, which produced major fragments ions at m/z 443, 473, 503, 383, and 353. The fragments at m/z 503, 473, and 443 correspond to the loss of 60, 90, and 120 Da, obtained by cross-ring cleavages in hexose and pentose residues. These typical mass losses indicate that peaks 14−16 were *C*-glycosyl flavonoids. Unlike *O*-glycosides, the intact aglycone of *C*-glycosides is not efficiently cleaved from the parent ion. Also, the fragments at m/z 353 and 383 are, respectively, indicative that the molecular weight of the aglycone is 270 Da, in keeping with apigenin. On the basis of previously published data[18] and because flavone *C*-glycosyl bonds have been found only at the 6- and 8-positions,[26] peaks 14−16 were tentatively identified as apigenin 6/8-C-pentoside-8/6-C-hexoside isomers. These flavone conjugates are known to occur in red rice[27] and in pigmented wild rice.[24]

Peak 17 (t_R 47.6 min) produced a $[M - H]^-$ at m/z 463, which on MS^2 yielded products ions at 301 (quercetin aglycone), 176, and 151. On the basis of the fragmentation pattern and by comparison with an authentic standard, this compound was identified as quercetin 3-*O*-glucoside, **17**. Peak 18 (t_R 48.5 min) had a $[M - H]^-$ at m/z 461, which gave a MS^2 fragmentation at m/z 341, 371, and 443, typical of chrysoeriol *C*-hexoside and diosmetin *C*-hexoside. The more prominent m/z 371 ion[28] suggests that this peak is a diosmetin *C*-glucoside. Full MS and MS-MS spectra of peak 18 did not provide any further evidence regarding the nature of the aglycone. However, the presence of $[(M - H) - 90]^-$ (m/z 371) and $[(M - H) - 120]^-$ (m/z 341) confirms that this compound is mono-C-hexosylated. The position of the sugar residue can be assigned by observation of the peak for the $[(M - H) - 18]^-$ fragment (m/z 443), which is indicative of the hexose substituent being located at the C6-position rather than at the C3- or 8-position.[28] Peak 18 is, therefore, tentatively identified as a diosmetin 8-*C*-hexoside.

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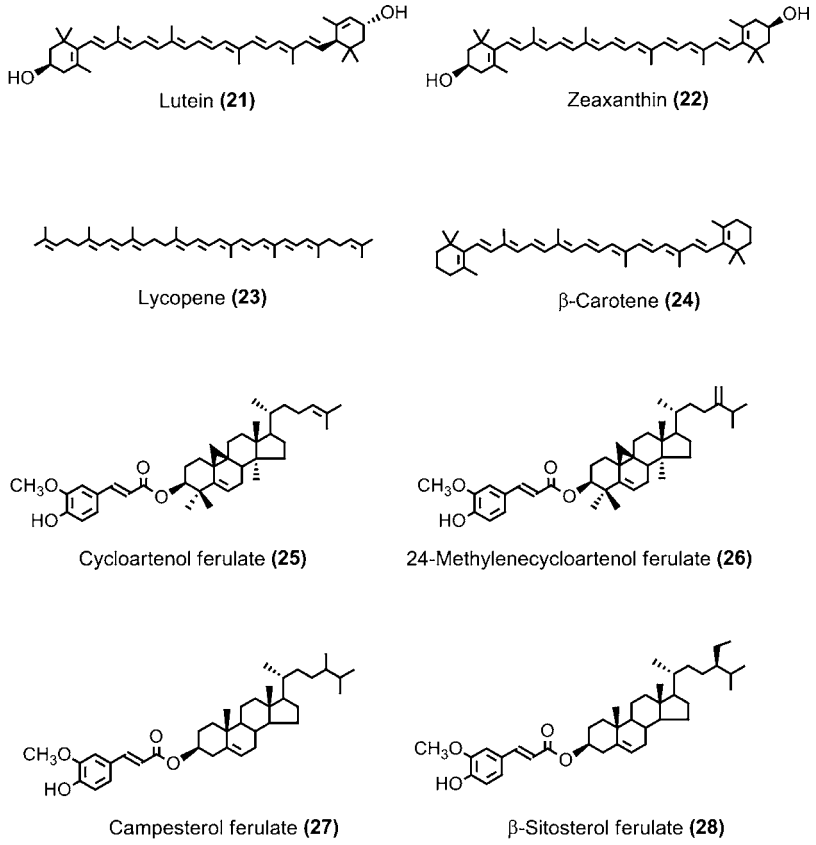

Figure 2. Structures of carotenoids and γ-oryzanols detected in black, red, brown, and white rice.

Peak 19 (t_R 50.7 min) produced a $[M - H]^-$ at m/z 609, which gave on MS^2 two peak ions at m/z 301 (quercetin ion) and m/z 447. This fragmentation pattern and cochromatography with a reference compound identified this peak as quercetin-3-*O*-rutinoside, **19**.

Peak 20 (t_R 57.7 min) had a $[M - H]^-$ at m/z 477, which gave a MS^2 ion at m/z 314 with a loss of 162 Da, corresponding to a methoxyflavonol-3-*O*-glucoside structure. On the basis of the product ion mass spectrum and in keeping with previous findings,[16] this peak was tentatively identified as isorhamnetin 3-*O*-glucoside (**20**), and this was subsequently confirmed by cochromatography with a reference compound.

Peak 21 (t_R 2.6 min; λ_{max} at 420, 445, and 470 nm) had a $[M - H]^+$ at m/z 568. This peak was identified as lutein, **21** (Figure 2), on the basis of the absorbance and MS characteristics. This was confirmed by coelution with a lutein standard.

Peak 22 (t_R 4.8 min; λ_{max} at 320 and 470 nm) presented a $[M - H]^+$ at m/z 568. On the basis of the retention time, absorbance and MS characteristics, and previously published data,[29] peak 22 was identified as zeaxanthin, **22**.

Peak 23 (t_R 15.3 min; λ_{max} at 410, 475, and 505 nm) presented a $[M - H]^+$ at m/z 536. On the basis of the retention time and UV−visible and MS characteristics of lycopene extracted from ketchup as well as comparison with previously published data,[30] this peak was identified as lycopene, **23**.

Peak 24 (t_R 21.4 min; λ_{max} at 450 and 475 nm) had a $[M - H]^+$ at m/z 536. This peak was identified as β-carotene, **24**, considering the absorbance and MS characteristics, which was confirmed by coelution with a standard.

The identification of peaks 25−28 (t_R 7.8, 8.4, 9.3, and 10.2 min) was based on a comparison of cochromatography and absorbance spectra of the unknown peaks with those of an γ-oryzanol standard. The peaks were identified as cycloartenol ferulate, **25**, 24-methyenecycloartenol, **26**, campesterol ferulate, **27**, and β-sitosterol, **28**.

Peak 29 (t_R 5.2 min) was identified as catechin by comparison of its absorbance spectrum and retention time with those of an authentic standard. This was confirmed by MS-MS, which yielded a $[M - H]^-$ at m/z 289, a prominent MS^2 ion at m/z 245, and a minor fragment at m/z 205.

Procyanidins with a degree of polymerization of >3 do not separate satisfactorily on reversed phase HPLC columns. They were, therefore, analyzed by HPLC using a diol support, identified according to their molecular mass, and quantified by fluorometry in (−)-epicatechin equivalents, due to a lack of reference compounds. Fluorescence peaks 30, 32, 34, 36, and 38 (t_R 7.4, 14.9, 21.6, 27.9, and 33.2 min) with a $[M - H]^-$ at m/z 577, 865, 1153, 1441, and 1729 were identified as type B dimeric, trimeric, tretrameric, pentameric, and hexameric procyanidins of (epi)catechin, whereas peaks 31, 33, 35, and 37 (t_R 14.7, 21.3, 27.2, and 33.0 min) with a $[M - H]^-$ at m/z 863, 1151, 1439, and 1727 were identified as type A dimeric, trimeric, tretrameric, pentameric, and hexameric procyanidins of (epi)catechin as have been previously described.[17] Fluorescence peaks 39−42 were outside the upper mass range of the mass spectrometer and were tentatively identified as heptameric to decameric procyanidins of (epi)catechin, as established previously.[17]

Profile of Secondary Metabolites in Black, Red, Brown, and White Rice. Typical chromatographic profiles of each group of compounds are illustrated in Figures 3−5. Tables 2 and 3 provide information about the quantities of a total of 42 identified/tentatively identified compounds

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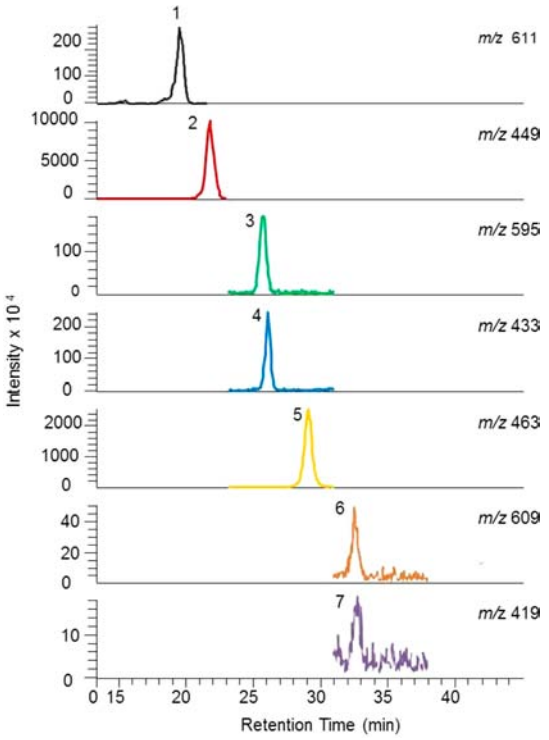

Figure 3. HPLC-MS^2 profile of extract of black rice with detection of anthocyanins by SRM at *m/z* 611 (cyanidin 3,5-*O*-diglucoside), *m/z* 449 (cyanidin 3-*O*-glucoside), *m/z* 595 (cyanidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside), *m/z* 433 (perlargonidin 3-*O*-glucoside), *m/z* 463 (peonidin 3-*O*-glucoside), *m/z* 609 (peonidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside), and *m/z* 419 (cyanidin 3-*O*-arabidoside).

including anthocyanins, chlorogenic acids, flavone, flavonols, carotenoids, γ-oryzanols, and procyanidins in black, red, brown, and white rice.

Anthocyanins. Cyanidin 3-*O*-glucoside (2.8 mg/g) and peonidin 3-*O*-glucoside (0.5 mg/g) were the main anthocyanins detected in black rice, representing 96.6% of the total anthocyanins, with the remaining 3.4% consisting of five minor components such as cyanidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside, cyanidin 3-*O*-arabidoside, peonidin 3-*O*-(6″-*O*-*p*-coumaryl)-glucoside, cyanidin 3,5-*O*-diglucoside, and pelargonidin *O*-glucoside. In red rice cyanidin 3-*O*-glucoside (3 μg/g) and cyanidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside (1.3 μg/g) were the only anthocyanins detected in significant amounts, whereas no anthocyanins were detected in brown and white rice (Table 2).

Significant differences in the concentration of total anthocyanins in black and red rice cultivars have been reported previously.[12,31] The data obtained in the present study is in keeping with these studies as the anthocyanin content of black rice was 3.5 mg/g, whereas that of red rice was only 4.3 μg/g (Table 2). Cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside have been also detected in Korean and Chinese black rice grains.[31−33] The concentrations of cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside of black rice previously reported[12] are similar to those obtained in the current study. In contrast, much higher concentrations and different profiles of anthocyanins were detected in Chinese black-purple rice, which contained cyanidin 3-*O*-glucoside (6.3 mg/g), peonidin 3-*O*-glucoside (3.6 mg/g), delphindin 3-glucoside (0.7 mg/g), and petunidin 3-glucoside (0.9 mg/g).[31]

It is interesting to note that some studies have reported that red rice[15,27,34] and white rice[15] do not contain any anthocyanins, whereas one study detected malvidin as the anthocyanin red rice.[35] Our data differ slightly from these findings in detecting two cyanidin derivatives in red rice and an absence of anthocyanins in white rice. The variations of the total anthocyanin content and the individual anthocyanins in rice might be due to the use of different cultivars of black and red rice and variable local growing conditions.[4]

Flavone and Flavonol Glycosides. The rice varieties analyzed in this study contained five flavones, namely, luteolin-6/8-*C*-pentoside-8/6-hexoside (two isomers) and apigenin-6/8-*C*-pentoside-8/6-hexoside (three isomers), with their concentrations in red (13.8 μg/g), brown (21.6 μg/g), and white (24 μg/g) rice being similar and significantly lower than that in black rice (77 μg/g) (Table 2). Other compounds such as taxifolin *O*-hexoside, quercetin 3-*O*-glucoside, diosmetin 8-*C*-hexoside, quercetin 3-*O*-rutinoside, and isorhamnetin 3-*O*-glucoside were detected only in black rice (0.47 mg/g total flavonols), although small quantities of diosmetin 8-*C*-hexoside were also identified in red rice. Likewise, the content of these compounds increased in pigmented rice, with black rice containing the highest levels of flavones and flavonols. In the black rice extract, taxifolin *O*-hexoside and quercetin 3-*O*-rutinoside were the predominant compounds, followed by diosmetin 8-*C*-hexoside, apigenin isomers, quercetin *O*-glucoside, and small quantities of luteolin and isorhamnetin 3-*O*-glucoside. Our results are consistent in terms of identification with those obtained in earlier studies,[4,16] which reported the detection of quercetin 3-*O*-glucoside and isorhamnetin 3-*O*-glucoside in seven Thai bran black rice extracts and which identified quercetin 3-*O*-glucoside in black rice grains, respectively.

It is noteworthy that three isomers of apigenin 6/8-*C*-pentoside-8/6-*C*-hexoside and two isomers of luteolin 6/8-*C*-arabinosyl-6/8-*C*-glucoside represent a major portion of the total flavone/flavonol content of red, brown, and white rice. These compounds have been previously found in non-pigmented, black, and red rice grains,[4,27] although the levels were lower than those obtained in the current study.

Chlorogenic Acids and Related Compounds. Chlorogenic acids in the form of two feruoylquinic acid isomers (3-*O*-feruloylquinic and 4-*O*-feruloylquinic acids) were detected exclusively in black rice at a concentration of 0.8 ± 0.2 μg/g (Table 2). No caffeoylquinic acids were detected, although the presence of 5-*O*-caffeoylquinic acid and *p*-coumaric acid in one red and two white varieties of grain rice has been reported.[36] A range of phenolic acids including gallic, protocatechuic, 4-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, and cinnamic acids in brown, red, and black rice have been detected.[4] In addition, caffeic, *p*-coumaric, ferulic, and sinapinic acids have been detected in eight red-grained and three brown-grained rice varieties,[37] with ferulic acid together with gallic, hydroxybenzoic acid, and protocatechuic acid being the dominant phenolic acids in black and red rice bran.[38] None of these phenolic compounds were detected in any of the four rice varieties analyzed in the present study.

Carotenoids. The major carotenoids in black rice were the xanthophylls lutein and zeaxanthin, which comprised >94% of the total carotenoids with the carotenoids, lycopene, and β-carotene occurring as minor components. In red, brown, and white rice xanthophylls lutein and zeaxanthin were the only carotenoids detected, with lutein being the most abundant. The

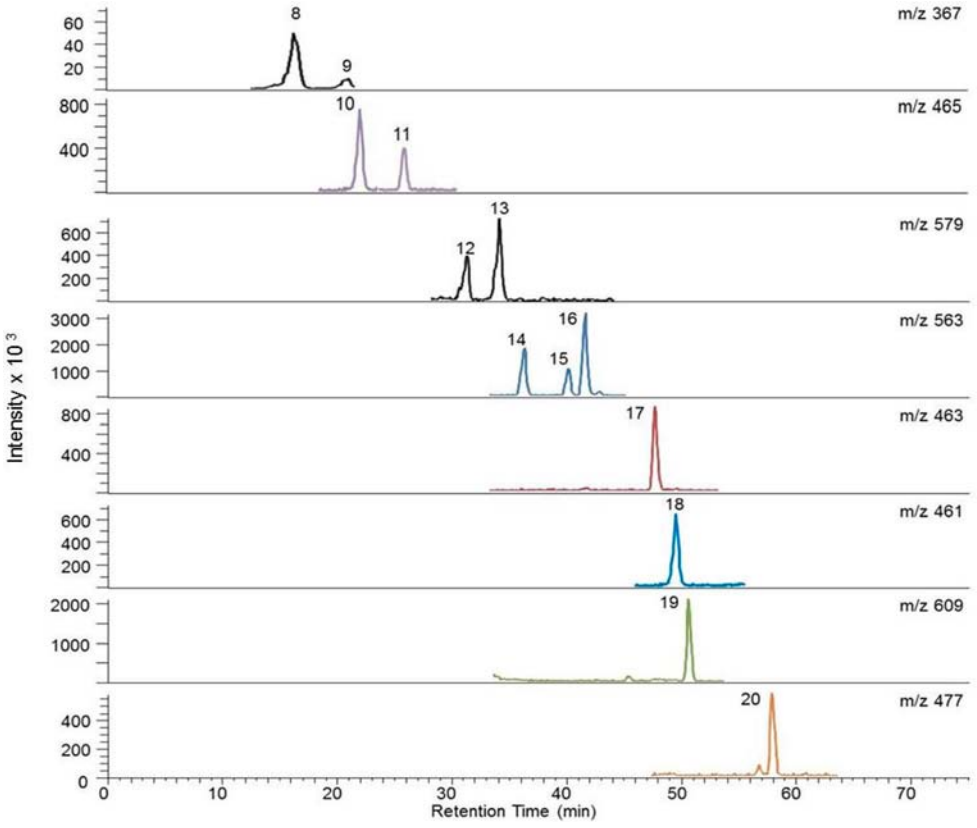

Figure 4. HPLC-MS^2 analysis of extract of black rice with detection of chlorogenic acids, flavones, and flavonols by SRM at m/z 367 (3/4-*O*-feruloylquinic acid), m/z 579 (luteolin 6/8-*C*-arabinosyl-6/8-*C*-glucoside), m/z 563 (apigenin 6/8-*C*-pentosyl-6/8-*C*-glucoside), m/z 463 (quercetin 3-*O*-glucoside), m/z 461 (diosmetin 8-*C*-hexoside), m/z 609 (quercetin 3-*O*-rutinoside), and m/z 477 (isorhamnetin 3-*O*-glucoside).

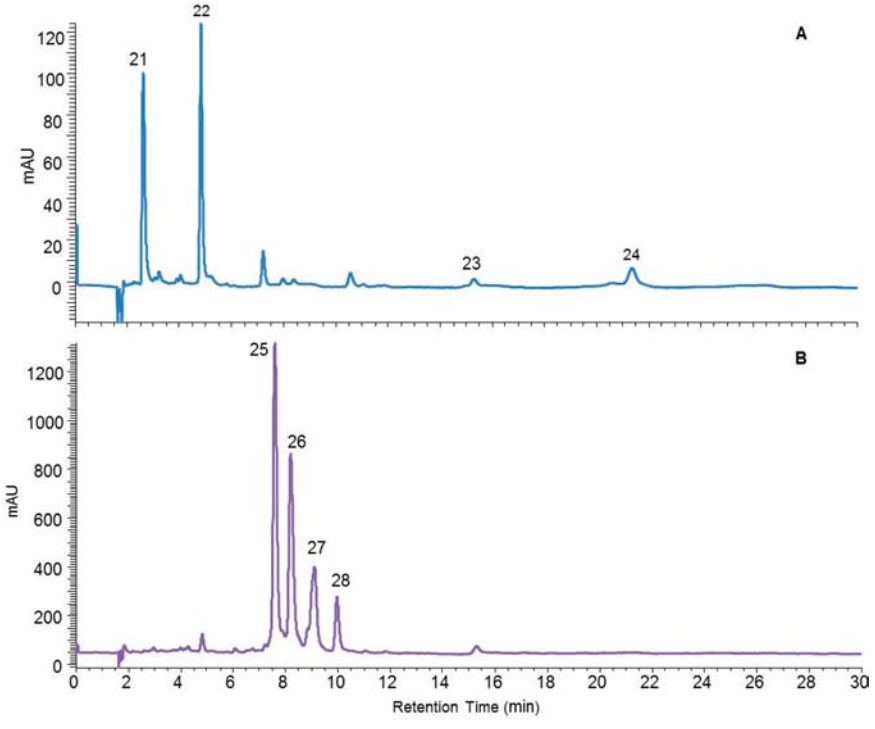

Figure 5. HPLC profile of a black rice extract at (A) 450 nm of carotenoids (peaks: 21, lutein; 22, zeaxanthin; 23, lycopene; 24, β-carotene) and at (B) 325 nm of γ-oryzanols (peaks: 25, cycloartenol ferulate; 26, 24-methylenecycloarternol ferulate; 27, campesterol ferulate; 28, β-sitosterol ferulate).

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Table 2. Quantities of Anthocyanins, Flavones, Flavonols, Carotenoids, and γ-Oryzanols in Black, Red, Brown, and White Rice[a]

compound	black (μg/g)	red (μg/g)	brown (μg/g)	white (μg/g)
cyanidin 3,5-diglucoside	20 ± 2	nd	nd	nd
cyanidin 3-*O*-glucoside	2857 ± 73	3.0 ± 1.0	nd	nd
cyanidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside	57 ± 2	1.3 ± 0.4	nd	nd
pelargonidin 3-*O*-glucoside	8 ± 1	nd	nd	nd
peonidin 3-*O*-glucoside	500 ± 11	nd	nd	nd
[eonidin 3-*O*-(6″-*O*-*p*-coumaryl)glucoside	23 ± 2	nd	nd	nd
cyanidin 3-*O*-arabidoside	9 ± 1	nd	nd	nd
total anthocyanins	**3474 ± 92a**	**4.3 ± 1.4b**	**nd**	**nd**
feruloylquinic acid (2 isomers)	0.8 ± 0.2	nd	nd	nd
total chlorogenic acids	**0.8 ± 0.2a**	**nd**	**nd**	**nd**
taxifolin *O*-hexoside (2 isomers)	162 ± 23	nd	nd	nd
luteolin 6/8-*C*-pentoside-8/6-*C*-hexoside (2 isomers)	14 ± 3	6.7 ± 1.8	3.3 ± 1.8	8 ± 3
apigenin 6/8-*C*-pentoside-8/6-*C*-hexoside (3 isomers)	63 ± 11	7.1 ± 5.7	18 ± 4	16 ± 5
quercetin 3-*O*-glucoside	41 ± 4	nd	nd	nd
diosmedin 8-*C*-hexoside	87 ± 16	8 ± 2	nd	nd
quercetin 3-*O*-rutinoside	107 ± 19	nd	nd	nd
isorhamnetin 3-*O*-glucoside	8.2 ± 0.2	nd	nd	nd
total flavone and flavonols	**482 ± 76a**	**22 ± 9b**	**21 ± 6b**	**24 ± 8b**
lutein	4.3 ± 3.4	0.4 ± 0.1	0.07 ± 0.02	0.006 ± 0.001
zeaxanthin	1.9 ± 0.2	0.1 ± 0.1	0.01 ± 0.01	0.002 ± 0.001
lycopene	0.16 ± 0.04	nd	nd	nd
β-carotene	0.20 ± 0.01	nd	nd	nd
total carotenoids	**6.6 ± 3.6a**	**0.5 ± 0.2b**	**0.08 ± 0.03c**	**0.008 ± 0.001d**
cycloartenol ferulate	26 ± 1	21 ± 0	2.8 ± 0.3	2.1 ± 0.2
24-methylenecycloarternol ferulate	20 ± 1	28 ± 2	3.3 ± 0.3	3.1 ± 0.1
campesterol ferulate	12 ± 1	23 ± 3	3.8 ± 0.7	2.1 ± 0.3
β-sitosterol ferulate	4.8 ± 0.6	6.6 ± 0.1	2.4 ± 0.6	0.9 ± 0.2
total γ-oryzanols	**63 ± 3b**	**79 ± 5a**	**12 ± 2c**	**8.2 ± 0.8d**

[a] $n = 3$; values are expressed as μg/g ± standard error. nd, not detected. All values within a row with different letters are significantly different, $p < 0.05$

Table 3. Diol HPLC-FL-MS Characteristics and Quantities of Flavan-3-ols in Black and Red Rice[a]

peak	t_R (min)	flavan-3-ol	$[M - H]^-$ (m/z)	black (μg/g)	red (μg/g)
29	5.2	catechin[b]	289	20 ± 5	92 ± 17
30	7.4	dimer, type B	577	25 ± 8	42 ± 15
31	14.7	trimer, type A	863	11 ± 3	nd
32	14.9	trimer, type B	865	41 ± 8	12 ± 1
33	21.3	tetramer, type A	1151	19 ± 3	2 ± 1
34	21.6	tetramer, type B	1153	22 ± 7	4 ± 2
35	27.2	pentamer, type A	1439	31 ± 10	7 ± 3
36	27.9	pentamer, type B	1441	29 ± 9	5 ± 1
37	33.0	hexamer, type A	1727	36 ± 12	9 ± 2
38	33.2	hexamer, type B	1729	22 ± 8	4 ± 2
39	37.9	heptamer		29 ± 11	8 ± 2
40	41.9	octamer		23 ± 9	4 ± 2
41	45.5	nonamer		17 ± 5	2 ± 1
42	48.7	decamer		0.2 ± 0.2	nd
		total flavan-3-ols		**325 ± 98a**	**191 ± 49b**

[a] $n = 3$; nd, not detected; for HPLC peak numbers, see Figure 4. Different letters indicate significant difference, $p < 0.05$. [b] Analysis of catechin is based on C12 reverse phase HPLC, which, unlike diol HPLC, is able to separate catechin and epicatechin. .

carotenoid content varied substantially among black, red, brown, and white rice grains, indicating distinct differences depending upon grain color. The levels of carotenoids were 6.6, 0.5, 0.08, and 0.008 μg/g in black, red, brown, and white rice, respectively (Table 2). These concentrations are in keeping with previously findings.[15]

The carotenoid content in black rice, 6.6 μg/g, differed markedly from the 33−41 μg/g found in the bran extracts of four varieties of Thai black rice.[14] This indicates that carotenoids are located mainly in rice bran, as suggested by Kong and Lee,[39] who elucidated the distribution and quantitation of the major antioxidant compounds in the milling fractions (whole grain, bran, and endosperm) of two black rice varieties. In addition, and in contrast to our results, there is a report that the main brown rice carotenoids are *β*-carotene and lutein (both ∼100 ng/g), with zeaxanthin levels lower at ∼30

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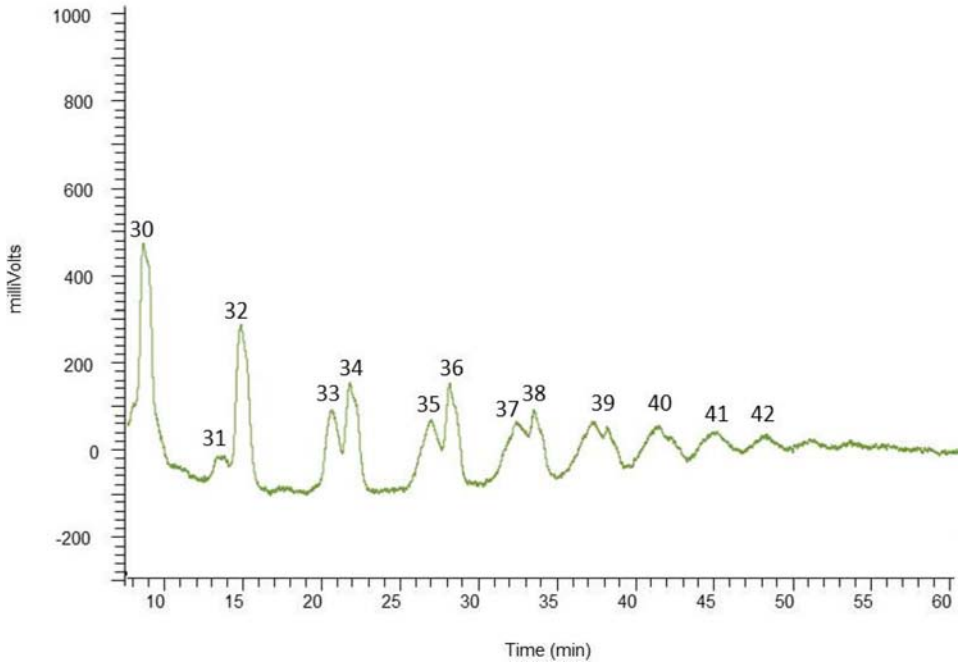

Figure 6. Diol HPLC profile with fluorescence detection of procyanidins in black rice. For MS data and peak identification, see Table 3.

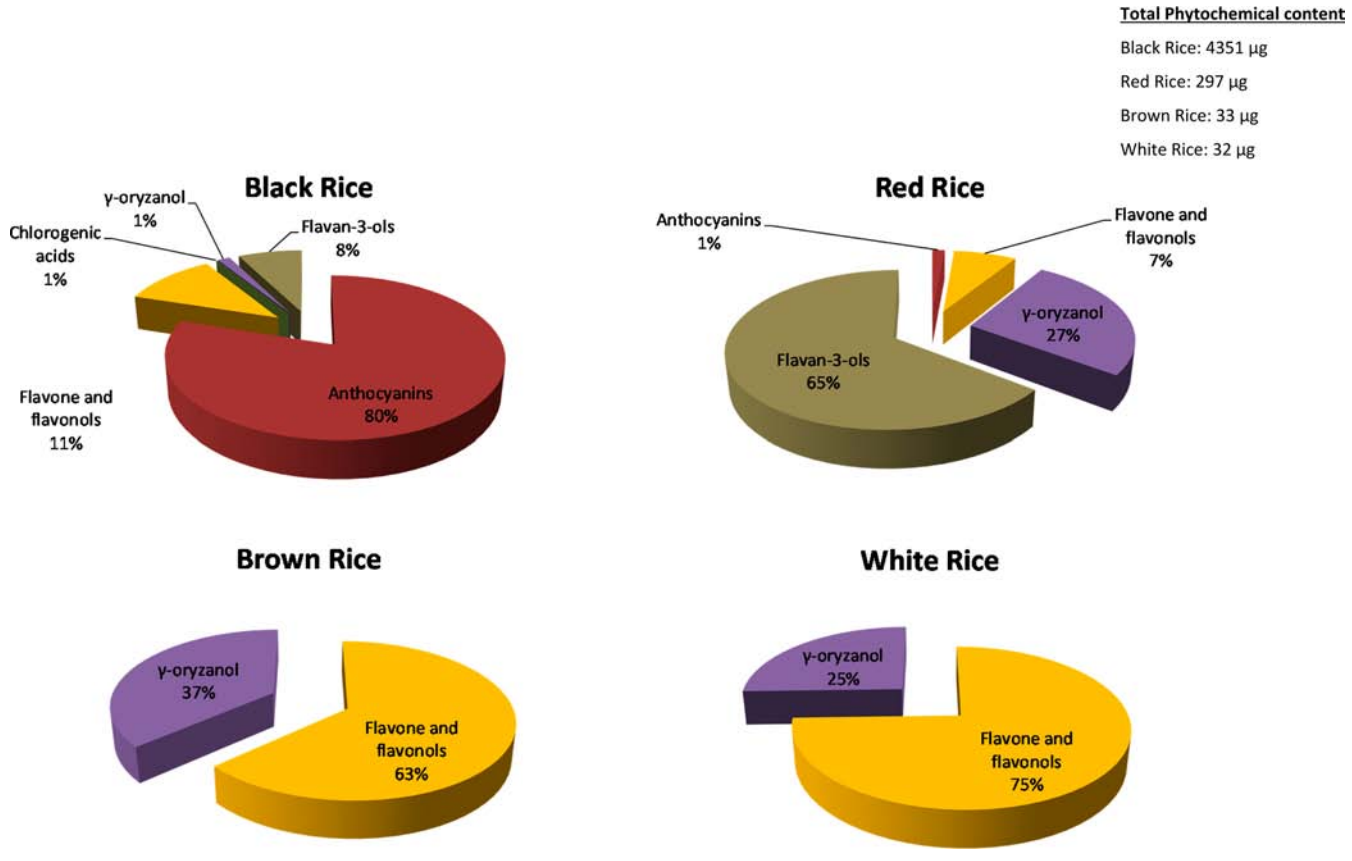

Figure 7. Global percentage of anthocyanins, chlorogenic acids, flavones and flavonols, carotenoids, γ-oryzanols, and flavan-3-ols in black, red, brown, and white rice.

ng/g.[29] Many factors can contribute to this difference; for example, maturity, cultivar, geographic, and environmental conditions may all have an impact on the carotenoid profile in rice.

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γ-Oryzanol. The analysis of the black, red, brown, and white rice revealed total γ-oryzanol contents of 63, 79, 12, and 8.2 μg/g, respectively (Table 2), including the presence of four different components: ferulate derivatives of 24-methylenecycloartenol (27−38%), cycloartenol (23−41%), campesterol (19−31%), and β-sitosterol (7−20%). The proportion of the individual sterol ferulates exhibited variability as a function of the variety. In black rice cycloartenol ferulate was the major component of γ-oryzanol, followed by 24-methylenecycloarternol, campesterol, and β-sitosterol ferulate; whereas in red and white rice 24-methylenecycloarternol was the predominant oryzanol, followed by cycloartenol and campesterol ferulates, with a lower concentration of β-sitosterol ferulate (Table 2).

The profiles and content of oryzanols in black, red, and brown rice are similar to those reported in 30 European brown rice varieties.[40] However, other studies have found higher total oryzanol levels, notably reports of 246−330 μg/g in brown rice of different Taiwan cultivars,[41] 269−1727 μg/g in pigmented and nonpigmented rice,[42] and 3894−5911 μg/g[43] and 2483−4057 μg/g[6] in bran extracts of different black rice varieties. These results are in keeping with reports that bran fractions have the greatest phytochemical content, with decreasing amounts occurring in the bran rice and even less in the seeds,[7,39] and the expected low levels in white rice as a consequence of the milling process.

Procyanidins. A total of 14 flavan-3-ols were identified and quantified in black and red rice, but were not detected in either brown or white rice. A typical HPLC-FL chromatogram is shown in Figure 6. The total procyanidin content in black rice, 325 μg/g, was more abundant than the 191 μg/g detected in red rice (Table 3). Interestingly, black and red rice contained only one flavan-3-ol monomer, catechin, which is partially in line with the findings of Qiu et al.,[24] who found that the amount of catechin was 4 times higher than that of epicatechin in a Canadian black rice variety, Manomin. Furthermore, the concentration of catechin was much higher in red rice (92 μg/g) than in black rice (20 μg/g), whereas, conversely, procyanidins with a 2−10 degree of polymerization were more abundant in black rice than in red rice (Table 3).

The total levels of procyanidins in red rice are in line with those reported by Finocchiaro et al.,[44] who found that five red dehulled rice grains contained between 94 and 118 μg/g of procyanidins. In addition, the same investigators observed that three Italian white rice had no procyanidins, as previously reported[13,24] and in keeping with our results. It is noteworthy that procyanidins have been typically observed in red but not black rice varieties.[13,34,37,43,45] However, a black Italian dehulled rice (cv. Arteminde)[44] and some colored commercial wild rice[24] have been reported to contain large amounts of oligomeric procyanidins (72−181 μg/g), results in agreement with those observed in the current study.

In summary, 42 secondary metabolites, comprising anthocyanins, flavones, flavonols, chlorogenic acids, carotenoids, and γ-oryzanols, have been qualitatively and quantitatively analyzed in the seeds of black, red, brown, and white rice. Black rice was the richest source of phytochemicals, with anthocyanins, most notably cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside, being the major compounds (Figure 7). In contrast, the predominant components in red rice are procyanidins (Figure 7). Brown and white rice contained neither anthocyanins nor procyanidins but are characterized by the occurrence of flavones and γ-oryzanols. All these observations indicate that pigmented rice and, in particular, black rice are rich sourceS of diverse phytochemicals that may have positive potential effects on human health.

AUTHOR INFORMATION

Corresponding Author

*(A.C.) Phone: +44-141 330 4613. Fax: +44-141 330 4613. E-mail: alan.crozier@glasgow.ac.uk.

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Notes

The authors declare no competing financial interest.

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