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The distribution of phenolic acids in rice

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Abstract

Phenolic acids were quantified in three cultivars of fresh and aged rice. High levels of ferulic acid (255–362 mg kg^{-1} grain) and *p*-coumaric acid (70–152 mg kg^{-1} grain) were found in brown rice with lower levels (e.g. ferulic acid 61–84 mg kg^{-1} grain) in milled rice. Bound phenolic acids comprised 80–90% of the total phenolic acids for brown rice and 53–74% for milled rice. Storage led to a decrease in total and bound phenolic acid contents in both brown and milled rice and the decline was greater at 37 °C than at 4 °C storage.

Keywords: Rice; Storage; Phenolic acids; Extraction; Distribution; Content

1. Introduction

The phenolic component of plants constitutes a complex mixture, and only a small number of plants have been examined systematically for their phenolic content. Data for rice are limited but suggest that the predominant phenols (Bunzel, Allerdings, Sinwell, Ralph, & Steinhart, 2002; Hudson, Dinh, Kokubun, Simmonds, & Gescher, 2000; Kuroda, Suzuki, Kato, & Imai, 1995) are phenolic acids, primarily ferulic (FA) and *p*-coumaric acids (PCA). Plant phenols have attracted intense interest for a variety of reasons. For instance, the question of the allelopathic potential of rice phenols (Chung et al., 2002; Olofsdotter, Rebulanan, Madrid, Wang, Navarez, & Olk, 2002) remains controversial. In contrast, the level of phenolics in rice has been correlated with UV-B tolerance (Caasilit, Whitecross, Nayudu, & Tanner, 1997).

Phenolic acids can be classified as free phenolic acids and bound phenolic acids (Régnier & Macheix, 1996; Renger & Steinhart, 2000). The level of free phenolic acids provided an index of grain resistance to *Sitophilus oryzae* attack in studies with sorghum (Ramputh, Teshome, Bergvinson, Nozzolillo, & Arnason, 1999). Bound phenolic acids are typically involved in cell wall structure (Bunzel et al., 2002; Sun, Sun, & Zhang, 2001, 2002) where cross-linking of lignin components via phenolic acids appears to have a profound effect on the growth of the cell wall and its mechanical properties and biodegradability. Data reported (Clifford, 1999; Shibuya, 1984) for rice endosperm cell walls are 12 g kg^{-1} of esterified cinnamic acids, comprising 9 g kg^{-1} FA, 2.5 g kg^{-1} PCA and 0.5 g kg^{-1} diferulic esters. Limited data indicate that the distribution of phenolic acids changed following rice storage (Tsugita, Ohta, & Kato, 1983). Changes in antioxidant activity of phenols in grains during processing and storage (Maillard & Berset, 1995) are implicated in lipid oxidation.

These data suggested the importance of characterising the phenolic content of rice. This paper reports a method for the extraction of phenolic acids from high-starch materials and the phenolic acid distribution and content of three fresh and aged rice cultivars.

2. Materials and methods

2.1. Chemicals

Vanillic, gallic, caffeic, syringic, *p*-coumaric and ferulic acids, and α-amylase were obtained from Sigma-Aldrich Co. (Sydney, Australia). HPLC grade methanol and

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acetonitrile were used for all analyses. Solutions of sodium hydroxide and acetic acid were made using analytical grade sodium hydroxide and glacial acetic acid, respectively.

2.2. Rice samples

Three rice cultivars (Koshihikari, medium grain; Kyeema, aromatic long grain; Doongara, long grain) were used in this study. These cultivars, selected because of their commercial importance in the Australian rice industry, were grown in the Murrumbidgee Irrigation Area (MIA) of New South Wales, Australia during the 1999/2000 growing season. Brown rice and milled rice (4 kg bulk samples of each) were placed in air-tight glass bottles and stored in the dark at 4 and 37 °C in thermostatically controlled incubators. Samples were withdrawn at the beginning and after six months of storage.

2.3. Sample preparation

Rice grains (brown or milled, fresh and stored) were ground using a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO) through a 1 mm sieve screen immediately prior to analysis. Moisture was determined by drying at 110 °C to constant mass according to ICC-standard No. 110/1. This and all other analyses were performed using at least duplicate samples and analytical results were expressed on a dry matter basis.

2.4. Measurement of total phenolic acids

2.4.1. General

Two methods were examined for the determination of total phenolic acids, direct alkaline extraction (McKeehen, Busch, & Fulcher, 1999) and alkaline extraction after enzymatic hydrolysis.

2.4.2. Direct alkaline extraction

Rice flour (2.0 g, dry basis) was combined with aqueous NaOH (4 M; 60 ml) and the mixture was stirred using a magnetic stirrer at room temperature under a nitrogen atmosphere for 4 h. The sample was acidified to pH 1.5–2.5 by gradual addition of ice-cold 6 M HCl and extracted three times with ethyl acetate (3×70 ml). The ethyl acetate fraction was dried by addition of anhydrous sodium sulfate and evaporated to dryness using a rotary vacuum evaporator at 35 °C. The residue was redissolved in aqueous methanol (50% v/v; 4 ml), filtered through a 0.45-μm nylon filter and stored in the dark prior to analysis by HPLC.

2.4.3. Combined enzymatic hydrolysis and alkaline extraction

Rice flour (2.0 g, dry basis) was slurried with distilled water (5 ml). A suspension of α-amylase *Bacillus licheniformis* (5-μl, 26.1 units/μl, Sigma) was added and maintained at room temperature for 15 min. The mixture was hydrolysed in a boiling water bath for 2 min. The hydrolysate was blanketed by N_2 and cooled rapidly under a stream of running water. The hydrolysis degree was monitored by the determination of reducing sugar content (Cui & Oates, 1999). Aqueous NaOH (4 M; 20 ml) was added to the cooled, nitrogen-blanketed mixture which was stirred for 5 min, sealed individually and maintained at 4 °C for either 4 h, 1, 2, 3 or 4 days. The extracts were then neutralised and extracted as described for the direct alkaline extraction method. The enzyme preparation did not contribute to the phenolic acid levels in rice samples.

2.5. Measurement of bound phenolic acids

Bound phenolic acids were determined on rice flour following removal of free phenolic acids, according to the method of Régnier and Macheix (1996). Thus, rice flour (3.0 g, dry basis) was combined with a mixture of methanol, acetone and water (7/7/6 v/v/v; 70 ml), stirred and stored for 1 day at 4 °C under the protection of N_2. The samples were shaken three times during the storage period. The residue was collected by filtration, washed with fresh solvent mixture and dried in a vacuum oven (OVL-570, Gallenkamp, London, England) at 40 °C to remove solvent. The dried residue (moisture content 4%) was used for the determination of bound phenolic acids using the enzymatic procedure described for total phenolic acids. Data are presented as total phenolic acids and bound phenolic acids using a dry mass basis.

2.6. HPLC conditions

An aliquot (250 μl) of the hydrolysed extract was separated using a Varian HPLC System (Model 9012, USA). Peaks were detected with a variable wavelength UV–vis detector (Varian 9050, USA) operated at 280 nm. Although this wavelength is not the optimum for ferulic acid, it allowed the simultaneous detection of hydroxybenzoic and hydroxycinnamic acids. Separations were achieved on a 5-μm Alltima C18 column (150 mm × 4.6 mm; Alltech Associates, Inc, Australia). Gradient elution was performed with a gradient of A (water:acetic acid, 100:1, v/v) and B (methanol:acetonitrile:acetic acid, 95:5:1, v/v/v) as follows: 0–2 min, 5% B; 2–10 min, 5–25% B; 10–20 min, 25–40% B; 20–30 min, 40–50% B; 30–40 min, 50–100% B; 40–45 min, 100% B; 45–55 min, 100–5% B. Solvent flow rate was 1.0 $ml\,min^{-1}$ and the temperature of the column was maintained at 22 °C. Peak identities were confirmed from retention data and by spiking of extracts with authentic standards. Recoveries of ferulic acid and *p*-coumaric acid from spiked samples were 81% and 83%,

respectively. Quantification was achieved by reference to authentic compounds used as external standards.

2.7. Statistical analysis

Experimental data were subjected to analysis of variance using Genstat 5 (release 4.1). Treatment means were tested separately for least significant difference (lsd) at a 5% level of probability.

3. Results and discussion

3.1. Extraction conditions

Solvent extraction has been established as an efficient method for removal of free phenolic acids from cereal grains (Régnier & Macheix, 1996). On the other hand, the recovery of bound acids is problematic and it has been claimed (Hatfield, Ralph, & Grabber, 1999) that bound (or total) phenolic acids are never fully released by any solvolytic method and are always underestimated. Nevertheless, alkaline hydrolysis represents the most common method for the extraction of total phenolic acids from cereals (Maillard & Berset, 1995) and was compared with a procedure involving a preliminary enzymatic hydrolysis. The standard procedure, involving direct alkaline extraction led to formation of starch pastes with a high viscosity that could inhibit the diffusion of phenolic acids and affect the extraction efficiency. In contrast, preliminary enzymatic hydrolysis minimised formation of starch pastes and allowed a significant reduction in the volume of hydroxide solution. Moreover, in milled rice, optimum yield of ferulic acid, the major phenol, was achieved by enzymatic hydrolysis at about 16% degree of hydrolysis (Table 1). With these conditions, the yield of all phenolic acids in milled rice was significantly greater than with direct alkaline hydrolysis ($P < 0.05$). A much lower content of gallic and vanillic acids (Table 1), determined by direct alkaline treatment, compared with that determined by preliminary enzymatic hydrolysis, is attributed to significant amounts of these phenols being linked by their phenolic groups via ether bonds to arabinoxylans or other constituents.

Using a preliminary enzymatic hydrolysis (16% degree of hydrolysis), maximum recovery of both ferulic and *p*-coumaric acids was achieved after 2 days at 4 °C and levels declined by 15% and 10%, respectively after a total extraction time of 4 days. Extraction time and temperature are important variables in the determination of phenols due to the competing processes of improved solubilisation and potential losses due to ready oxidation of the phenols. The latter must be considered for all phenols but is particularly important in the case of *o*-diphenolic compounds such as caffeic acid. Low temperature extraction was used to minimise the extent of oxidation. All data presented in this paper were collected using a preliminary enzymatic hydrolysis with an extraction time of 2 days at 4 °C.

3.2. Phenolic profiles

The phenolic profiles did not differ significantly between the three cultivars. This is typical of most plants in that the phenolic profile is species-specific (Maillard & Berset, 1995; McKeehen et al., 1999) and is often exploited in chemotaxonomy (Antolovich, Prenzler, Robards, & Ryan, 2000). Furthermore, the phenolic profiles of both brown and milled rice were dominated by ferulic acid and *p*-coumaric acids with lesser amounts of gallic, vanillic, caffeic and syringic acids. Baseline resolution of these compounds was achieved with the chromatographic conditions described. Peak identities were confirmed from retention data (see Table 2) for authentic samples and spiking of samples plus diode array and mass spectral data, as previously described (Ryan, Robards, & Lavee, 1999; Swatsitang, Tucker, Robards, & Jardine, 2000) although detection at 280 nm was routinely used for quantification. The phenolic acids

Table 1
The comparison of extracting efficiencies using direct alkaline extraction and enzymatic hydrolysis for the determination of total phenolic acids in fresh brown and milled rice (cv. Koshihikari)

Sample	Extraction method	Total phenolic acid content (mg kg^{-1} dry grain)						
		Ferulic acid	*p*-Coumaric acid	Gallic acid	Vanillic acid	Caffeic acid	Syringic acid	Sum
Brown rice	Alkaline extraction	259	71	2.1	3.0	Tr[a]	2.9	338
	Enzymatic hydrolysis	255	70	17	12	0.3	2.6	356
Milled rice	Alkaline extraction	52[c]	6.6[c]	2.1	0.3	Tr	Tr	61[c]
	Enzymatic hydrolysis; 9.2[b]	55[c,d]	8.2[d]	5.1	1.5	Tr	Tr	70[c,d]
	Enzymatic hydrolysis; 16.1	66[d]	8.9[d]	3.8	0.4	Tr	Tr	79[d]
	Enzymatic hydrolysis; 20.5	60[d]	8.9[d]	5.7	1.6	Tr	Tr	77[d]

[a] Tr, Trace.
[b] The percentage refers to the degree of hydrolysis (dextrose equivalents). The results obtained by alkaline extraction correspond to "zero" degree of hydrolysis.
[c,d] Data with the same letter indicate no statistical difference ($p > 0.05$).

Table 2
Retention data for phenolic acids

Phenolic acid	Retention time (min)
Gallic acid	7.1
Vanillic acid	16.9
Caffeic acid	17.7
Syringic acid	18.0
p-Coumaric acid	22.0
Ferulic acid	23.0

listed in all tables were the only phenols detected in the samples at significant concentrations (>0.1 mg kg^{-1} dry grain) using the specified detection conditions which represent a reasonable compromise for biophenols (Antolovich et al., 2000). The levels of phenolic acids (Tables 3–5) were much higher than those that have been reported in barley bran, barley endosperm and whole wheat (Clifford, 1999). The concentration of individual phenolic acids was, not surprisingly, higher in all instances in brown rice than in milled rice.

The distribution of total and bound phenolic acids in brown and milled rice is presented in Tables 3–5 which also show the changes in phenolic acid content associated with storage. Ferulic acid was the dominant phenolic acid, both in total and bound states, in brown and milled rice, followed by *p*-coumaric acid. With the exception of gallic acid, the concentrations of other phenolic acids rarely exceeded the trace level in either state in milled rice. The distribution of phenolic acids differed between brown rice and milled rice. Although ferulic acid remained the dominant phenolic acid in both brown and milled rice, in the bound state (see Tables 3–5) the proportion of *p*-coumaric acid was higher in brown rice (ratio of ≈0.3–0.5 in Koshihikari, Kyeema, and Doongara) than in corresponding milled rice samples (≈0.1 in all cultivars).

Bound phenolic acids comprised the bulk of the phenolic acids in both brown and milled rice. Thus, the bound phenolic acids, as a proportion of total phenolic acids, were 87%, 85% and 89% in brown Koshihikari, Kyeema and Doongara, respectively, with corresponding values of 56%, 74% and 53% in milled Koshihikari, Kyeema and Doongara. The proportion of bound ferulic acid relative to total ferulic acid was very similar to these values for the three cultivars in both milled and brown rice. In the case of *p*-coumaric acid, the percentage of bound to total acid in milled rice was again similar at 66%, 73% and 55%, respectively, for the three cultivars. However, in brown rice, the bound state comprised approximately 100% of the total *p*-coumaric acid.

The total phenolic acids in the "bran" (calculated as the difference in contents of brown and milled rice) typically contributed 70–90% of the total phenolic acids in the grain, depending on cultivar and the particular phenolic acid. In the case of ferulic acid, the bran contained 74% for Koshihikari, 79% for Kyeema and 78% for Doongara. The corresponding values for *p*-coumaric acid were 87% for Koshihikari, 93% for Kyeema and 93% for Doongara. These results suggest that the concentrations of phenolic acids increased from endosperm to the aleurone. This is consistent with *p*-coumaric acid being primarily associated with the lignin, as the cell walls of the hulls are considerably more lignified than those of the rest of the kernel (Salomonsson, Theander, & Aman, 1980). On the other hand, ferulic acid is linked to cell wall constituents (McKeehen et al., 1999). As the walls of aleurone cells are rich in arabinoxylans, this can be used to explain why the cell walls of the aleurone layer have the highest concentration of ferulic acid. In contrast to the distribution of *p*-coumaric acid in the rice kernel, that of ferulic acid is more uniform throughout

Table 3
The contents of bound and total phenolic acids in brown and milled rice cv. Koshihikari

Phenolic acid	Phenolic acid content (mg kg^{-1} dry grain)[a]											
	Brown rice						Milled rice					
	Fresh		Stored (six months, 4 °C)		Stored (six months, 37 °C)		Fresh		Stored (six months, 4 °C)		Stored (six months, 37 °C)	
	Bound	Total	Bound	Total	Bound	Total	Bound	Total	Bound	Total	Bound	Total
Ferulic acid	259	289	224	255	202	214	58	71	38	66	36	55
p-Coumaric acid	82	85	70	70	59	62	8.9	9.5	5.9	8.9	5.5	8.0
Gallic acid	16	19	11	16	11	14	3.2	4.0	0.11	3.8	0.2	3.8
Vanillic acid	11	15	2.1	12	3.0	13	0.3	0.6	Tr[c]	0.2	Tr	0.2
Caffeic acid	2.7	3.5	2.1	3.9	2.0	2.2	0.2	0.2	Tr	Tr	Tr	Tr
Syringic acid	2.0	2.8	1.4	2.6	1.5	2.4	Tr	Tr	Tr	Tr	Tr	Tr
Sum	373	415	311	360	279	308	71	86	44	79	42	67
Ratio[b]	0.32		0.31		0.29		0.15		0.15		0.15	

[a] Standard error for measurement of individual acids 5%.
[b] Ratio = *p*-coumaric acid content/ferulic acid content.
[c] Tr, Trace.

Table 4
The contents of bound and total phenolic acids in brown and milled rice cv. Kyeema

Phenolic acid	Phenolic acid content (mg kg^{-1} dry grain)[a]											
	Brown rice						Milled rice					
	Fresh		Stored (six months, 4 °C)		Stored (six months, 37 °C)		Fresh		Stored (six months, 4 °C)		Stored (six months, 37 °C)	
	Bound	Total	Bound	Total	Bound	Total	Bound	Total	Bound	Total	Bound	Total
Ferulic acid	330	362	287	350	270	333	76	84	61	74	60	68
p-Coumaric acid	117	121	107	113	101	103	9.1	10	6.0	8.2	5.7	7.5
Gallic acid	22	30	5.7	27	6.0	23	6.5	7.1	Tr[c]	5.6	Tr	5.0
Vanillic acid	4.3	6.1	3.3	4.9	3.1	5.0	3.0	3.2	Tr	2.5	Tr	1.5
Caffeic acid	5.1	5.8	3.9	4.6	3.1	3.5	0.10	0.10	Tr	0.03	Tr	0.10
Syringic acid	3.7	4.0	1.5	3.1	1.7	2.9	0.10	0.10	Tr	0.10	Tr	0.10
Sum	482	528	408	502	385	470	95	105	67	90	66	82
Ratio[b]	0.35		0.37		0.37		0.12		0.10		0.10	

[a] Standard error for measurement of individual acids 5%.
[b] Ratio = *p*-coumaric acid content/ferulic acid content.
[c] Tr, Trace.

Table 5
The contents of bound and total phenolic acids in brown and milled rice cv. Doongara

Phenolic acid	Phenolic acid content (mg kg^{-1} dry grain)[a]											
	Brown rice						Milled rice					
	Fresh		Stored (six months, 4 °C)		Stored (six months, 37 °C)		Fresh		Stored (six months, 4 °C)		Stored (six months, 37 °C)	
	Bound	Total	Bound	Total	Bound	Total	Bound	Total	Bound	Total	Bound	Total
Ferulic acid	240	278	238	272	221	258	66	70	35	61	33	55
p-Coumaric acid	139	152	138	139	119	124	9.5	11	5.0	9.1	4.6	9.0
Gallic acid	17	18	6.6	16	6.2	15	5.7	6.6	0.1	5.6	0.1	5.0
Vanillic acid	2.8	3.1	2.0	2.1	1.9	2.0	1.2	2.0	0.5	1.6	0.5	1.5
Caffeic acid	6.3	6.9	2.2	6.1	2.0	5.5	0.4	0.6	Tr[c]	0.5	Tr	0.5
Syringic acid	3.2	3.3	2.6	2.6	2.5	2.5	Tr	Tr	Tr	Tr	Tr	Tr
Sum	409	463	389	437	353	408	83	91	41	78	38	71
Ratio[b]	0.58		0.58		0.54		0.14		0.14		0.14	

[a] Standard error for measurement of individual acids 5%.
[b] Ratio = *p*-coumaric acid content/ferulic acid content.
[c] Tr, Trace.

the whole grain, although the content in the outer layer is still dominant.

3.3. Storage effects

There was a consistent decrease in bound phenolic acid content in both brown and milled rice following storage of the three cultivars. Furthermore, the decline in bound phenolic acids was greater at 37 than at 4 °C. These changes were significant ($P < 0.05$) and are consistent with previous observations (Mod, Conkerton, Chapital, & Yatsu, 1983) regarding oxidation of ferulate esters of hemicellulose, causing a reduction in bound phenolic acids. Given the contribution of bound phenolic acids to the total acid content, it is not surprising that similar trends were observed for total phenolic acids during storage. In contrast, the free phenolic acids (calculated as the difference between total and bound phenolic acids) increased significantly ($P < 0.01$) during storage of milled rice, presumably as a result of enzymatic and non-enzymatic release of bound phenolic acids. It is notable that the linoleic acid content of the free-lipid fraction of the three rice cultivars decreased during storage (Zhou, Blanchard, Helliwell, & Robards, 2003). However, the magnitude of the changes in lipid content was relatively small and it was not possible to establish a relationship between the change in free-lipids and free phenolic acids.

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