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High-performance liquid chromatographic determination of phenolic compounds in rice

Su Tian[a], Kozo Nakamura[b,*], Tong Cui[c], Hiroshi Kayahara[b]

[a] Department of Science of Biological Resources, United Graduate School of Agricultural Sciences, Gifu University, Gifu 501-1193, Japan
[b] Division of Food Functional Analysis, Science of Functional Foods, Graduate School of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan
[c] College of Food Science and Technology of Agricultural University of Hebei, Hebei 071001, China

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Abstract

A method has been developed for the determination of 6′-*O*-feruloylsucrose, 6′-*O*-sinapoylsucrose, ferulic acid, sinapinic acid, *p*-coumaric acid, chlorogenic (3-caffeoylquinic) acid, caffeic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid, and syringic acid in rice. The rice samples were extracted with 70% ethanol, filtered, and defatted. The defatted aqueous solution was subjected to solid-phase extraction using a C_{18} silica gel cartridge; no analyte was lost in this procedure. The 70% acidic methanol elution was analyzed directly by HPLC and HPLC-ESI-MS. Phenolic compounds were separated with a C_{18} reversed-phase column by gradient elution using 0.025% trifluoroacetic acid in purified water (A)—acetonitrile (B) (0 min, 5% B; 5 min, 9% B; 15 min, 9% B; 22 min, 11% B; and 38 min, 18% B) as the mobile phase at a flow rate of 0.8 ml/min. Detection limits ranged from 0.10 to 0.35 ng per injection (5 μl). Relative standard deviations of 0.22–3.95% and recoveries of 99–108% were obtained for simultaneous determination of these phenolic compounds. This method was applied to analysis of phenolic compounds in brown rice and germinated brown rice soaked in 32 °C water for varying durations.
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1. Introduction

Rice (*Oryza sativa* L.) is a cereal food that serves as a staple in many countries of Asia. Research indicates that cereal grains contain special phenolic compounds, such as ferulic acid and diferulates, which are not present in significant quantities in fruit and vegetables [1]. Similarly, these phenolic compounds have antioxidant [2,3], antimutagenic [4], anticancer [5], and other positive effects and play an important role in maintaining health. Ferulic acid and *p*-coumaric acid are the major phenolic compounds in rice and exist in the form of free, soluble conjugated, and insoluble bound [6]. Most of these compounds are bound to polysaccharides containing glucose, arabinose, xylose, galactose, rhamnose, and mannose residues in the cell wall [7–9]. Two main hydroxycinnamic acid derivatives, β-D-fructofuranosyl-α-D-(6-*O*-(*E*)-feruloylglucopyraniside) (6′-*O*-feruloylsucrose) and β-D-fructofuranosyl-α-D-(6-*O*-(*E*)-sinapoylglucopyraniside) (6′-*O*-sinapoylsucrose), containing ferulic acid and sinapinic acid covalently linked to sucrose through ester linkages, were separated and identified from a methanol extract of rice [10]. Hydroxycinnamate sucrose esters, also called sucrose phenylpropanoid esters, are common secondary metabolites in plants [11], and may possess antioxidant [12], anticancer, and antiviral biological activities [13] as a radical scavenger and protein kinase C inhibitor [14].

* Corresponding author. Tel.: +81 265 771638; fax: +81 265 771638.
E-mail address: knakamu@gipmc.shinshu-u.ac.jp (K. Nakamura).

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Early studies on hydroxycinnamic acid analysis employed TLC for separation, after which the spots were scraped off the plates and quantified using spectrophotometric techniques [15]. Later, GC procedures were developed, but they required derivatization of the acids to provide volatility [16]. However, RP-HPLC is presently the most useful tool for the qualitative and quantitative analyses of phenolic acids, including their esterified (conjugated) forms [17]. Bäumker et al. [18] reported the HPLC analysis of 6′-*O*-feruloylsucrose, ferulic acid, and *p*-coumaric acid as the degradation products of triferuloyl sucrose and diferuloyl sucrose catalyzed by the protein extracts from *Tulipa* Apeldoorn. More recently, Kujala et al. [19] reported the analysis of 6′-*O*-feruloylsucrose and feruloylglucose in red beetroot by HPLC. But few reports described simultaneous determination of 6′-*O*-feruloylsucrose, 6′-*O*-sinapoylsucrose, hydroxycinnamic acids, and hydroxybenzoic acids. And while some research focuses on the separation and purification of 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose from natural products [20–22], few studies concentrate on developing analytical methodology. The goal of this study was the development of an HPLC method for the simultaneous determination of 6′-*O*-feruloylsucrose, 6′-*O*-sinapoylsucrose, ferulic, sinapinic, *p*-coumaric, chlorogenic (3-caffeoylquinic), caffeic, protocatechuic, hydroxybenzoic, vanillic, and syringic acid (Fig. 1) in rice. In addition, the method was applied to the analysis of these phenolic compounds in brown rice and germinated brown rice soaked in 32 °C water for varying durations.

2. Experimental

2.1. Reagents and standards

6′-*O*-Feruloylsucrose and 6′-*O*-sinapoylsucrose were separated from a methanol extract of rice bran and their structures were confirmed by UV, IR, MS, and NMR spectroscopy [10]. The phenolic acid standards, ferulic, sinapinic, *p*-coumaric, caffeic, protocatechuic, hydroxybenzoic, vanillic, and syringic acid, were purchased from Wako (Osaka, Japan). Chlorogenic acid was obtained from Sigma (MO, USA). Acetonitrile (Nacalai Tesque, Kyoto, Japan) was HPLC grade. Water was purified using an Arium 611 VF water purification system from Sartorius (Tokyo, Japan). Trifluoroacetic acid (TFA) was obtained from Wako (Osaka, Japan). Other chemicals and solvents were of analytical grade.

Stock standard solutions of 6′-*O*-feruloylsucrose, 6′-*O*-sinapoylsucrose, ferulic acid, sinapinic acid, *p*-coumaric acid, chlorogenic acid, caffeic acid, and hydroxybenzoic acid at a concentration of 3 mg/ml, and protocatechuic acid, vanillic acid, and syringic acid at 4 mg/ml, were prepared in methanol and stored at 4 °C in darkness. Working solutions were prepared by successive dilutions with purified water.

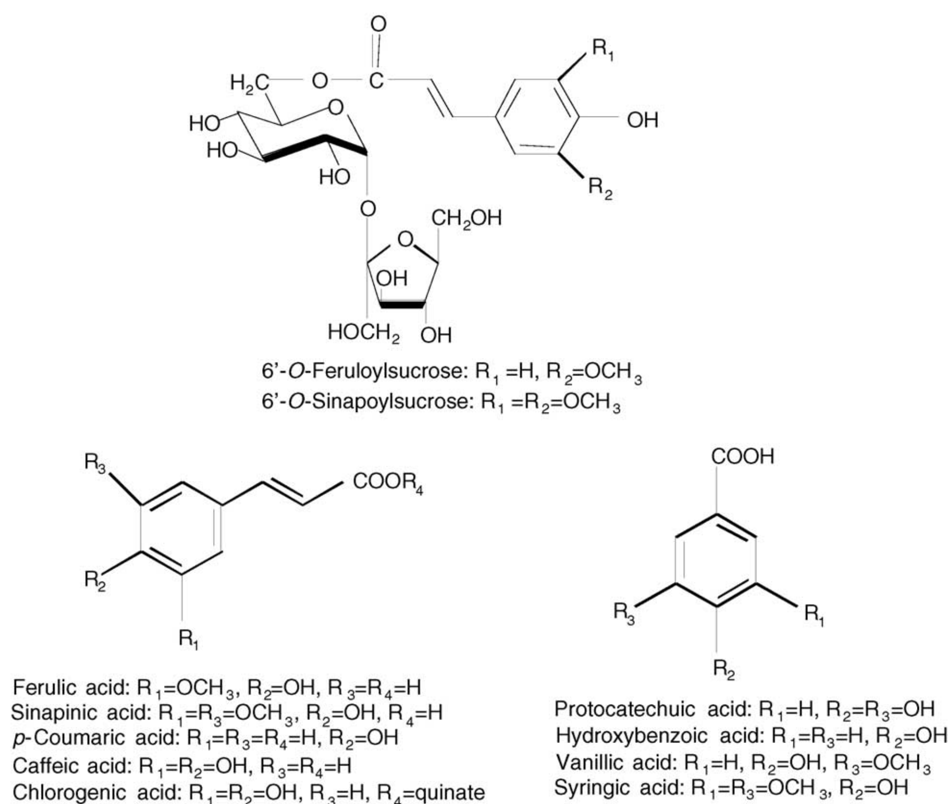

Fig. 1. Chemical structures of phenolic compounds in rice.

2.2. Sample preparation

2.2.1. Germination

Brown rice was the ‘koshihikari’ variety, harvested in 2003 in Nagano, Japan. Germination was conducted in a HP-100 Hatsuga Bijin microcomputer electric germination appliance (Takekoshi, Niigata, Japan) according to the following procedure: 250 g of brown rice were cleaned and soaked in 1000 ml of water at 32 °C for 1, 6, 12, or 24 h; a 0.5- to 1-mm bud developed after soaking for 12 h. Germinated brown rice was freeze-dried by using a VD-400F freeze dryer (Taitec, Kosigaya, Japan), and was ground with an ultracentrifugal mill. The powder was sieved (355 μm; 42 mesh), and stored at −20 °C prior to analysis. Brown rice also was cleaned, freeze-dried, powdered, sieved, and stored at −20 °C.

2.2.2. Sample preparation

Rice flour (10 g) was extracted by stirring for 30 min with 50 ml of 70% aqueous ethanol according to the method of Harukaze et al. [23]. The mixture was centrifuged at 2500 × *g* for 10 min, and the supernatant was collected. The residue was re-extracted twice in a similar way. The combined extracts were then filtered through a 0.45-μm Type JH membrane filter (Ø 47 mm, Millipore, Billerica, USA) and evaporated to less than 5 ml at 40 °C under reduced pressure. After addition of 5 ml acidified water (pH 2.45 with 0.025% TFA), the residual aqueous solution was defatted with 10 ml *n*-hexane three times. After concentrated to remove *n*-hexane using a rotary evaporator in vacuo, the aqueous solution was subjected to solid-phase extraction (SPE) using a Sep-Pak® Vac 6 ml (1 g) C_{18} cartridge (Waters, Milford, MA, USA) previously activated with methanol (6 ml) and conditioned with acidified water (12 ml). The cartridge was washed first with 6 ml of acidic water-methanol (9:1, v/v) and then the phenolic compounds eluted using 3 ml of acidic water-methanol (3:7, v/v) at a rate of 1–2 ml/min. The latter 1.5 ml was collected and brought up to 2 ml in volume with pure water for HPLC and HPLC-ESI-MS (electrospray ionization mass) analyses.

Samples of the water from different soaking periods were filtered through a 0.45-μm Type JH membrane filter (Ø 47 mm, Millipore, Billerica, USA) and adjusted to pH 2 with HC1, then extracted directly using SPE prior to analysis by HPLC.

2.3. HPLC analysis

The HPLC system consisted of an SCL-10Avp system controller, two LC-6AD solvent delivery units, a CTO-10Avp column oven, an SPD-M10Avp UV–vis photodiode array detector, and a Multi-PDA Class-VP workstation (Shimadzu, Kyoto, Japan). Separations were conducted with a Waters Cosmosil® $5C_{18}$-MS-II reversed-phase column (150 mm × 4.6 mm i.d., Milford, MA, USA). Gradient elution was performed with a mobile phase of acetonitrile (solvent B) and 0.025% TFA in purified water (solvent A): 0 min, 5% solvent B; 5 min, 9% solvent B; 15 min, 9% solvent B; 22 min, 11% solvent B; and 38 min, 18% solvent B. A washing period of 8 min with 80% solvent B and re-equilibration period of 15 min with 5% solvent B were used between individual runs. Chromatography was performed at 38 °C with a flow rate of 0.8 ml/min and injection volume of 5 μl. Hydroxybenzoic acids were detected at a wavelength of 280 nm and hydroxycinnamic acids and their derivatives detected at 325 nm. Phenolic compounds in the samples were identified by comparing their relative retention times, and UV and ESI-MS spectra with authentic compounds. An external standard method was used for quantification.

2.4. HPLC-ESI-MS analysis

HPLC-MS analysis was performed with the HPLC analytical conditions described above using an Agilent (Palo Alto, CA, USA) 1100 Series capillary liquid chromatography/mass selective detector (LC/MSD) Trap system composed of a capillary pump, micro vacuum degasser, thermostatted micro autosampler, thermostatted column compartment, diode-array detector, and LC/MSD Trap equipped with an ESI source. Complete system control and data evaluation were accomplished with Agilent LC/MSD Trap SW software Version 4.1. The mass spectrometer was operated in the positive ion mode in a scan range from 100 to 800 *m*/*z* using 325 °C drying gas (N_2) temperature, 10 1/min drying gas flow, and 50 psi nebulizer gas (N_2) pressure.

3. Results and discussion

3.1. Optimization of solid-phase extraction

Liquid–liquid extraction and column chromatography on different sorbents play important roles in the isolation and purification of phenolic compounds [24]. However, recent research has demonstrated that liquid–liquid extraction with weak-polar organic solvents is less acceptable than SPE, mainly due to low recoveries and repeatability [25]. In preliminary analyses, liquid–liquid extraction with ethyl acetate gave low recoveries of 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose. As a result, a traditional C_{18}-bonded silica reversed-phase cartridge was employed. SPE was evaluated using standard solutions containing 300 μg 6′-*O*-feruloylsucrose, 80 μg 6′-*O*-sinapoylsucrose, and 15 μg each of the other free phenolics corresponding to their amounts in 10 g of brown rice. Methanol and aqueous acid were chosen as the elution solvents, and the methanol:water ratio sufficient to clean the sample and elute the analyte was tested from 0 to 100% in increments of 10% for the rinsing step. Fractions were monitored with HPLC. Protocatechuic acid was the first analyte, observed in the 20% fraction; 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose eluted with 40% methanol, followed by ferulic acid and sinapinic acid in 50% methanol. Thus, 10% acidic methanol eliminates possible polar interference, then 70% acidic methanol was used for elution of all

phenolic compounds and the recovery was greatest in the latter 1.5 ml of the 3 ml elution volume. The solution collected was brought up to a volume of 2 ml with pure water prior to HPLC analysis. Recovery and precision (expressed as relative standard deviation, RSD %) of the SPE process were calculated using five standard replicates and yielded values of 94% (1.75%) for 6′-*O*-feruloylsucrose; 92% (1.72%) for 6′-*O*-sinapoylsucrose; 96% (2.09%) for ferulic acid; 100% (1.66%) for sinapinic acid; 99% (2.51%) for *p*-coumaric acid; 94% (5.98%) for chlorogenic acid; 93% (1.67%) for caffeic acid; 97% (3.03%) for protocatechuic acid; 94% (4.51%) for hydroxybenzoic acid; 92% (2.30%) for vanillic acid; and 93% (3.13%) for syringic acid. Reproducibility and accuracy values confirmed that no analyte was lost during this procedure.

3.2. Optimization of chromatographic conditions

Research work have indicated that chromatographic gradient systems composed of acetonitrile and water in a C_{18} column sharpen peak shapes and improve analytical sensitivity and resolution for the HPLC analysis of hydroxycinnamic acids [26]. Therefore, acetonitrile and water were used as the mobile phase in this study. As acetonitrile concentration was increased from 7 to 15%, retention factor (k) decreased significantly, with all of the phenolic compounds following a similar tendency. This resulted in the co-elution of most of the phenolics with isocratic elution using 15% acetonitrile. Isocratic elution at lower concentrations such as 7% acetonitrile also resulted in poor chromatographic separation of chlorogenic acid, vanillic acid and caffeic acid, while sinapinic acid was eluted after approximately 78 min. This information was used to create an optimal gradient. The segmented gradient consisted of a shallow linear gradient from 5 to 9% acetonitrile, followed by isocratic elution of 9% acetonitrile for 10 min to improve the resolution of phenolic compounds eluting early; then the concentration of acetonitrile in the mobile phase was gradually raised to 18% to produce reasonable retention time for the components eluting later. The resulting gradient program permitted resolution of all of the phenolic compounds within 38 min. It has been known that temperature can have a profound effect on reversed phase chromatography. Under these gradient conditions, an increase from 25 to 50 °C in column temperature decreased analysis time, which was more dramatic for *p*-coumaric acid and caffeic acid compared to the other phenolics. A significant decrease in the retention times of *p*-coumaric acid and caffeic acid resulted in the overlaps of *p*-coumaric acid and 6′-*O*-sinapoylsucrose at 30 and 35 °C, *p*-coumaric acid and 6′-*O*-feruloylsucrose above 40 °C,

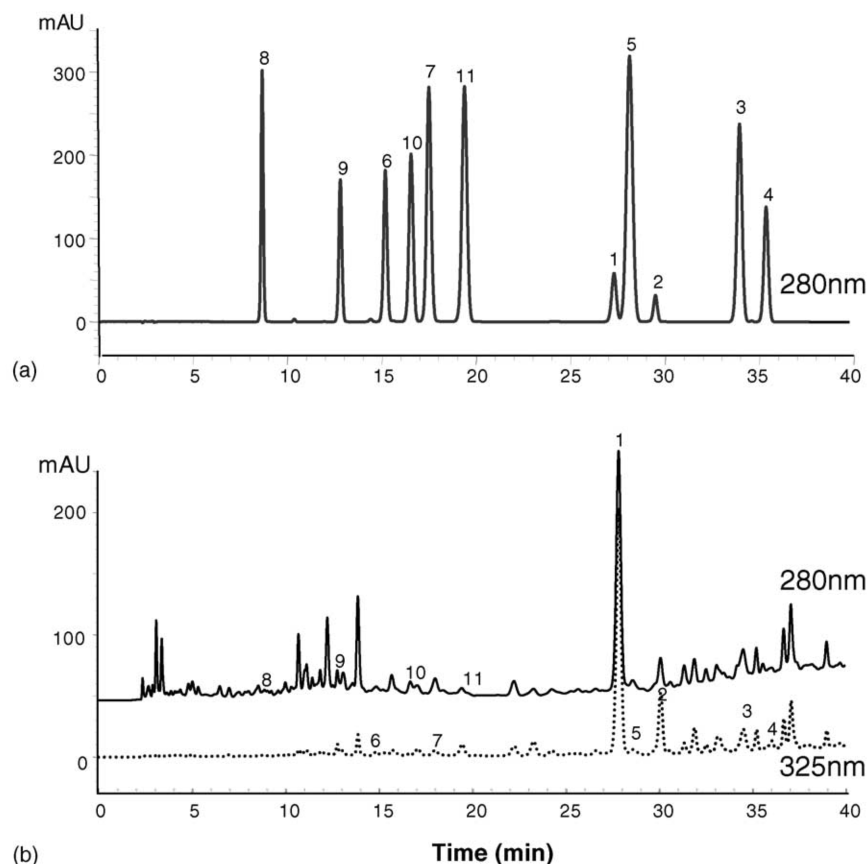

Fig. 2. HPLC chromatograms of phenolic standards at the wavelength of 280 nm (a) and brown rice sample (b) at 280 and 325 nm. For conditions of analysis see Section 2. (1) 6′-*O*-Feruloylsucrose; (2) 6′-*O*-sinapoylsucrose; (3) ferulic acid; (4) sinapinic acid; (5) *p*-coumaric acid; (6) chlorogenic acid; (7) caffeic acid; (8) protocatechuic acid; (9) hydroxybenzoic acid; (10) vanillic acid; (11) syringic acid.

Table 1
Standard calibration curves, detection limits and method validation data

	Calibration curves			LOD (ng)	RSD ($n=9$) (%)	Recovery ($n=5$) (%)
	Linear range (μg/ml)	Regression equation[a]	R^2			
6′-*O*-Feruloylsucrose	3.9–300	$y=(1.3x-0.8)10^4$	0.9998	0.30	1.52	103
6′-*O*-Sinapoylsucrose	3.9–300	$y=(1.2x-2.3)10^4$	0.9996	0.35	3.34	99
Ferulic acid	1.6–600	$y=(3.3x-2.6)10^4$	0.9999	0.13	1.39	104
Sinapinic acid	1.6–600	$y=(3.2x-1.1)10^4$	0.9997	0.15	1.06	100
p-Coumaric acid	1.6–600	$y=(3.1x-2.6)10^4$	0.9995	0.14	3.34	102
Chlorogenic acid	1.6–600	$y=(1.9x-1.7)10^4$	1	0.13	1.72	108
Caffeic acid	1.6–600	$y=(3.3x-1.1)10^4$	0.9996	0.10	3.95	100
Protocatechuic acid	2.4–800	$y=(8.5x+3.0)10^3$	0.9998	0.16	0.43	102
Hydroxybenzoic acid	1.6–600	$y=(8.2x+3.9)10^3$	1	0.23	0.22	102
Vanillic acid	2.4–800	$y=(10.6x-5.1)10^3$	1	0.23	1.42	106
Syringic acid	2.4–800	$y=(17.7x-1.9)10^3$	0.9999	0.18	0.53	104

[a] *y*, Peak area; *x*, concentration.

respectively, as well as the co-elution of caffeic acid and vanillic acid at column temperatures above 40 °C. In addition, a column temperature of 25 °C also yielded poor selectivity of caffeic acid and syringic acid, ferulic acid and sinapinic acid. These results indicate that temperature exerts a significant effect on selectivity in this analysis, especially for *p*-coumaric acid, 6′-*O*-feruloylsucrose, and 6′-*O*-sinapoylsucrose. The optimal column temperature of 38 °C was chosen.

To obtain the optimum analytical conditions, the influence of mobile phase pH was also investigated. Varying amounts of phosphoric acid were added to 50 mM phosphoric buffer to adjust the pH from 2.1 to 4.0. The retention

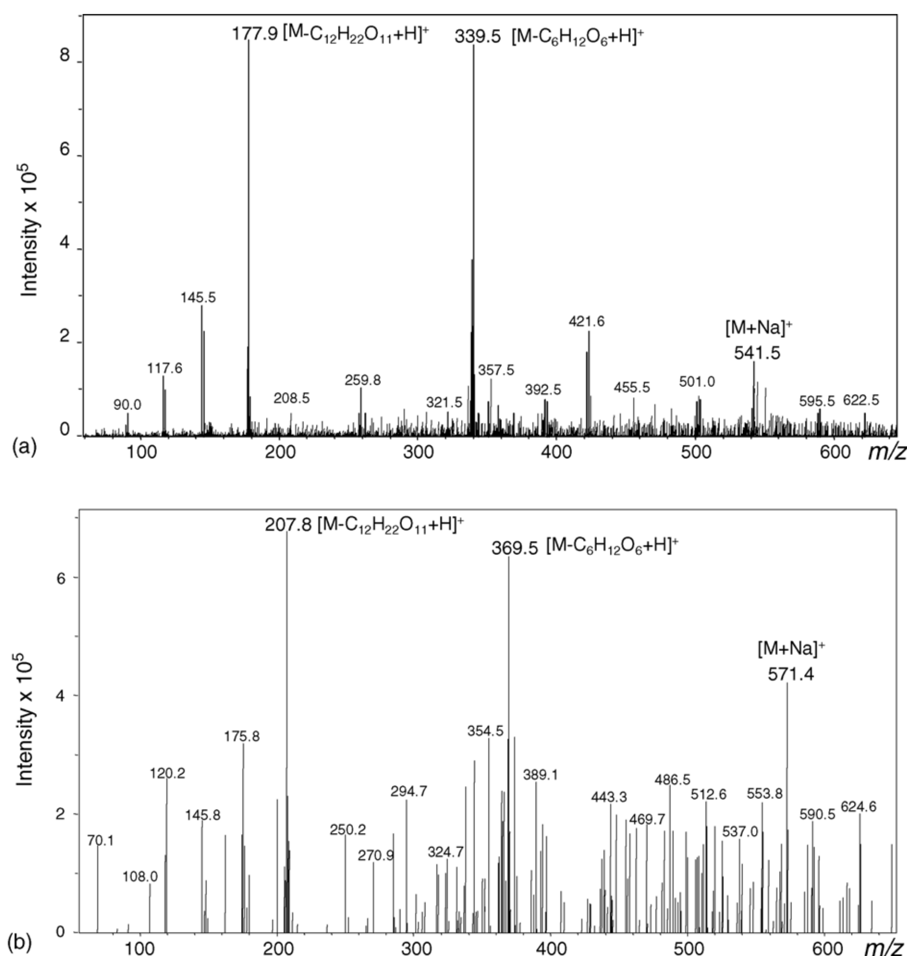

Fig. 3. Positive-ion ESI mass spectra of 6′-*O*-feruloylsucrose (a) and 6′-*O*-sinapoylsucrose (b) in brown rice sample.

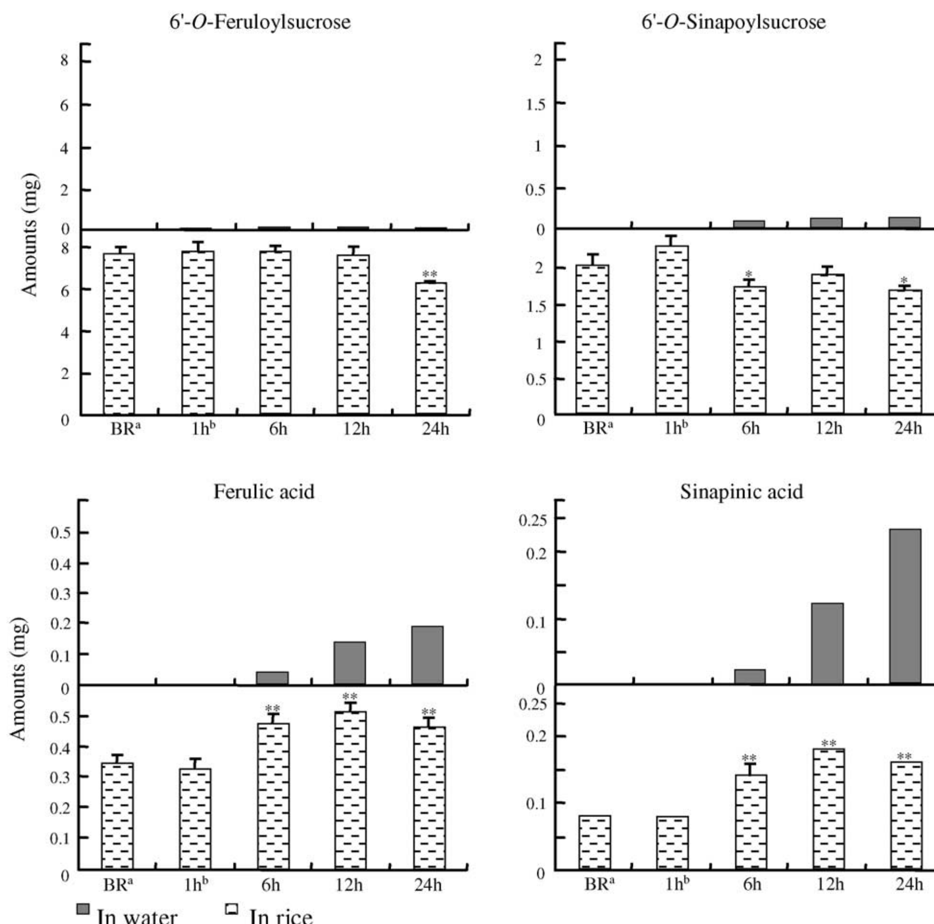

Fig. 4. The amounts of 6′-*O*-feruloylsucrose, 6′-*O*-sinapoylsucrose, ferulic acid and sinapinic acid in brown rice (250 g) and germinated brown rice soaked in 32 °C water for varying durations as well as in the water (1000 ml) from different soaking periods based on the procedure of germination described in Section 2. Superscript (a) means brown rice. Superscript (b) means germinated brown rice soaked for 1 h. $^{*}P < 0.05$; $^{**}P < 0.01$.

factors of 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose are not pH-dependent, since 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose are not as readily ionized as carboxylic acids. The other free phenolic compounds with a carboxylic acid moiety are partially ionized as the pH increases from 2.7 to 4.0. This ionization reduces the retention times of these free phenolic acids on a C_{18} column. When the pH of the mobile phase is 2.1–2.7, the stable k values and good chromatographic separation can be obtained for all of the phenolic compounds. Because a very low pH could damage the silica-based column, a pH value near 2.5 was chosen as the optimal value. For convenience, 0.025% TFA aqueous solution (pH 2.45) was used instead of phosphoric buffer, which yielded similar separation results.

According to UV spectra obtained with diode array detection, the maximum absorptions of 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose similar to those of the hydroxycinnamic acids occur near 325 nm, and those of hydroxybenzoic acids near 280 nm. As a result, the optimal conditions for HPLC analysis were determined as described in the Section 2. The HPLC standard chromatograms under optimized HPLC conditions are shown in Fig. 2a.

3.3. HPLC method validation

Validation results are summarized in Table 1. Standard linearity was tested using linear regression and the phenolic compounds showed excellent linearity with correlation coefficient greater than 0.9995 in the range studied. The detection limit (LOD) was defined with a signal-to-noise (S/N) ratio of 3:1. Within-run precision was measured using RSD for nine replicate standards of 300 μg 6′-*O*-feruloylsucrose, 80 μg 6′-*O*-sinapoylsucrose, and 15 μg each for the other phenolic compounds. RSD values of the peak areas were within 0.22 and 3.95%. The accuracy of the HPLC method was validated by adding phenolic standards (300 μg of 6′-*O*-feruloylsucrose, 80 μg of 6′-*O*-sinapoylsucrose, and 15 μg each of the other free phenolic acids) to 10 g of brown rice sample extract. Average recoveries of 5 replicates for these phenolic compounds were between 99 and 108%.

3.4. Application

This analytical method was applied to the determinations of 11 phenolic compounds in brown rice and germinated

brown rice. HPLC chromatograms of brown rice samples are shown in Fig. 2b. Phenolic compounds in the samples were identified by comparing their relative retention times and UV spectra with authentic compounds; identities were confirmed by LC-MS analysis. The mass spectra of 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose in brown rice sample are shown in Fig. 3. Each sample was analyzed in triplicate. The amounts of 6′-*O*-feruloylsucrose, 6′-*O*-sinapoyl sucrose, ferulic acid, and sinapinic acid are shown in Fig. 4; content of the other phenolics are shown in Fig. 5. Results showed that 6′-*O*-feruloylsucrose (7.8 mg) and 6′-*O*-sinapoylsucrose (2.0 mg) were the major soluble phenolic compounds in brown rice, and there were significant decreases ($P < 0.01$ or $P < 0.05$) during germination for 24 h, while the levels of free ferulic acid and sinapinic acid increased significantly ($P < 0.01$). This trend was in agreement with our previous analysis using a similar HPLC method, with small differences in the extent of increase and decrease on phenolic content [10]. The differences are due to the use of rice samples different from those used in this study. In addition, the content of phenolic compounds in the water used for soaking the rice was determined and revealed that the decrease in 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose during germination was not due to loss in the soaking water but was probably caused by hydrolysis. The increases in ferulic acid and sinapinic acid, both in the germinated rice and in the soaking water at 12 and 24 h verify that germination causes metabolism of phenolic compounds, especially as the bud appears. The two hydroxycinnamate glycosides may participate in this metabolic conversion.

Previous research has demonstrated that brown rice contains abundant phenolic compounds, most of which are an insoluble bound form found in significant quantities in the rice bran. The beneficial effects of rice consumption have been attributed mainly to these phenolic compounds and their associated antioxidant activity. In this study, an RP-HPLC analytical method was developed for the simultaneous determination of phenolic compounds including 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose in rice. This method not only has the ability to separate free and conjugated forms of phenolic acids, but also is well suited for the analysis of insoluble bound phenolic compounds after alkaline hydrolysis [10]. Moreover, this method also was applied successfully to the determination of these phenolic compounds in brown rice and germinated brown rice products. 6′-*O*-Feruloylsucrose and 6′-*O*-sinapoylsucrose were the main phenolic compounds. However, previous analytical methods were unable to determine them. We expect that this improved method may provide the basis for quantitative analysis of phenolic compounds including 6′-*O*-feruloylsucrose and 6′-*O*-sinapoylsucrose in other plant materials and a means of clarifying the metabolic pathways involving phenolic compounds.

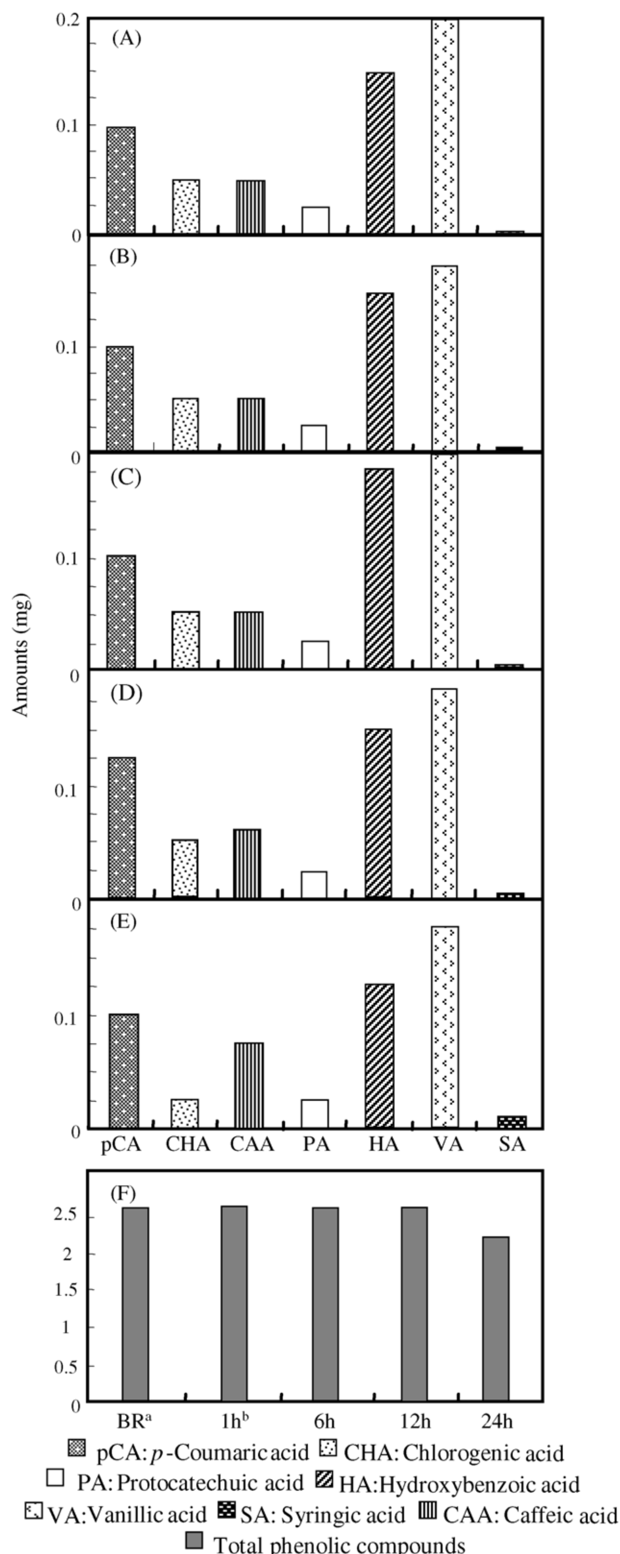

Fig. 5. The amounts of *p*-coumaric, chlorogenic, caffeic, protocatechuic, hydroxybenzoic, vanillic and syringic acid in brown rice (250 g) and germinated brown rice soaked in 32 °C water for varying durations, and the total content of 11 phenolic compounds in each rice samples studied. (A) Brown rice; (B) germinated brown rice soaked for 1 h; (C) germinated brown rice soaked for 6 h; (D) germinated brown rice soaked for 12 h; (E) germinated brown rice soaked for 24 h; (F) the total content of 11 phenolic compounds in brown rice and germinated brown rice soaked for varying durations. Superscript (a) means brown rice. Superscript (b) means germinated brown rice soaked for 1 h.

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