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Short communication

Characterization of increased phenolic compounds from fermented Bokbunja (*Rubus coreanus* Miq.) and related antioxidant activity

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ABSTRACT

This study examined the changes in the phenolic acid-content and antioxidant activity of Rubi Fructus (RF), the fruit of *Rubus coreanus* Miq., after fermentation with yeast (*Saccharomyces cerevisiae*). The phenolic acids were fractionated into three forms, free (Fr. A), ester (Fr. B), and insoluble-bound phenolic acids (Fr. C) and quantified by high performance liquid chromatography with a diode array detector (HPLC-DAD). This method was validated and allowed the successful identification of 11 phenolic acids in the RF extracts. HPLC-DAD analysis of the samples showed substantial increases in the levels of protocatechuic, vanillic and p-coumaric acid as the result of yeast fermentation. The total phenolic content (TPH) was also increased by fermentation. The total phenolics in Fr. A and Fr. B increased from 117 to 173 mg GAE/100 g and from 488 to 578 mg GAE/100 g, respectively. The total phenolics in Fr. C decreased from 264 to 175 mg GAE/100 g. The antioxidant activity of the fermented RF was measured as the 1,1-diphenoly-2-picrylhydrazyl (DPPH) radical scavenging capacity, which is expressed as the IC_{50}. The IC_{50} for Fr. A and Fr. B decreased from 5.9 to 4.0 mg/ml (mg of dried RF equiv./ml) and from 1.2 to 0.8 mg/ml, respectively. In Fr. C, the IC_{50} value increased from 2.1 to 2.8 mg/ml. In summary, the fermented RF had a higher total phenolic content and better DPPH radical-scavenging activity than the unfermented material.

1. Introduction

Plants belonging to the *Rosaceae* family have been widely used for therapeutic purposes in Asian countries for centuries [1]. It is well known that they contain anthocyanins, flavonoids, tannins and phenolic acids [2,3]. The phenolic acids, which include proanthocyanidins and lignins, contribute extensively to the antioxidant property of these plants, in that phenolic compounds, because of their reducing power, are excellent oxygen-radical scavengers [4,5]. Highly reactive free radicals and oxygen species in biological systems can modify nucleic acids, proteins, lipids or DNA by oxidation and can initiate certain degenerative diseases. As a result, antioxidant compounds such as phenolic acids, polyphenols and flavonoids in food can play important roles in protecting our overall health [6].

Rubi Fructus (RF) is the fruit of *Rubus coreanum* Miquel, a perennial shrub belonging to the *Rosaceae* family that is cultivated in Korea [7]. Fresh RF has been used to treat of impotence, spermatorrhea, enuresis, asthma, allergic disease and fatigue, and also as a general tonic [8]. RF wine was recently developed, and its consumption is growing rapidly in Korea. The previously reported chemical constituents of RF include flavonoids, tannins, triterpenosides, and phenolic compounds, and it has been reported to exhibit anti-inflammatory, anti-fatigue, anti-gastropathic, anti-rheumatic, and antioxidant activities [9–12]. Park and Jang [8] reported that the lactic acid fermentation of RF increases its electron donating ability, nitrite scavenging ability, superoxide dismutase-like activity, and xanthine oxidase inhibitory activity. Ku and Mun reported changes in the chemical-constituents of RF during the wine-making process [13,14]. Choi *et al.* examined the changes in the organic acid content in RF as a result of fermentation [15]. However, to the present time, fermentation-induced phenolic acid changes remain unreported.

The aim of this study, therefore, was to perform qualitative and quantitative analyses of the phenolic acids that result from fermentation. In addition, the antioxidant activity associated with the total

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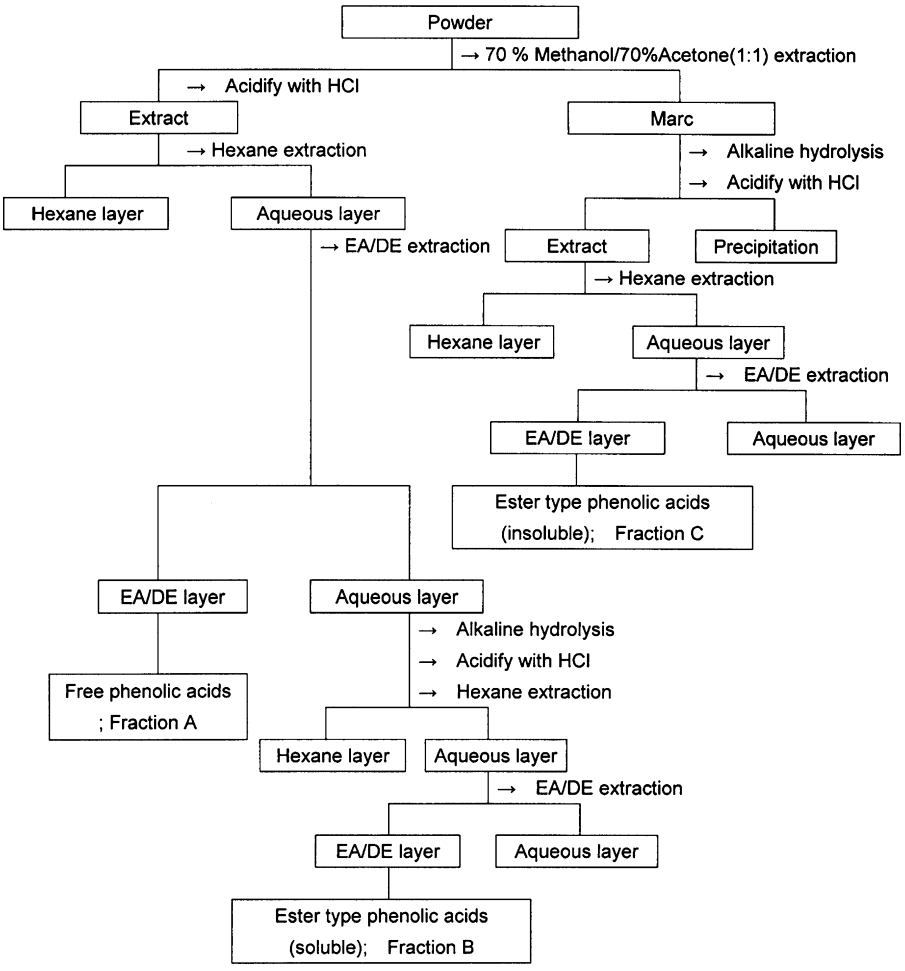

Fig. 1. Procedure used for the extraction and separation of the free, ester type (soluble) and ester type (insoluble) phenolic compounds (DE/EA = diethyl ether/ethyl acetate, 1:1).

phenolic content of fermented Bokbunja (*Rubus coreanus* Miq.) was evaluated.

2. Materials and methods

2.1. Chemicals and reagents

RF was purchased from a local market in Gochang, Korea. β-Hydroxybenzoic acid, salicylic acid, trans-cinnamic acid, vanillic acid, gentisic acid, syringic acid, m-coumaric acid, trans-ferulic acid, caffeic acid, gallic acid, protocatechuic acid, p-coumaric acid, chlorogenic acid, and *Saccharomyces cerevisiae* were obtained from Sigma–Aldrich (St. Louis, MO, USA). Purity of phenolic acids was confirmed by multi-wavelength-based HPLC-DAD chromatogram. Folin–Coicalteu's phenol reagent, and 1,1-diphenyl-2-picrylhydrazyl were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ortho-phosphoric acid (85%) was acquired from Fluka. HPLC-grade water and acetonitrile were purchased from Duksan (Kyungki-Do, Korea).

2.2. Sample preparation

Dried RF was crushed with a pulverizer (Shinil model SFM-555SP, Hwasung, Korea) and sifted in powder form using a standard sieve (425 μm). Three hundred milligrams of yeast (*Saccharomyces cerevisiae*) and 150 ml of water were added to 30 g of the pulverized RF. The fermentation followed the procedure that was previously reported by Tabera et al. [16]. The fermentation experiments were performed in triplicate.

2.3. Fractionation of phenolic acids

The phenolic acids were fractionated according to the reported method, as shown in Fig. 1 [17]. The samples were extracted three times with a mixture of 70% methanol–70% acetone (1:1, v/v) at room temperature and then centrifuged to remove insolubles, marc. The organic solvent in the supernatant was removed under reduced pressure, the resulting solution acidified with HCl (pH 2; 6N), and extracted three times with hexane. The remaining aqueous layer was extracted with a mixture of diethyl ether and ethyl acetate (1:1, v/v). After collecting the organic phase, the solvent was removed under reduced pressure to give the soluble phenolic fraction (Fr. A). The remaining aqueous layer was hydrolyzed with 4 M NaOH for 4 h under a nitrogen atmosphere at Rt. After acidification and centrifugation, the supernatant was extracted with hexane and then with DE/EA, as described above, to give the soluble ester-type phenolic fraction (Fr. B). The insoluble ester-type phenolic fraction (Fr. C) was obtained from the insoluble fraction, marc, after alkaline hydrolysis and subsequent solvent extraction, as illustrated in Fig. 1.

2.4. Chromatographic conditions

An HPLC system was equipped with two L-7100 Hitachi pumps (Tokyo, Japan) coupled with a Rheodyne model 7125 injector

Table 1
Total phenolic content (TPH) and DPPH radical scavenging activity of each phenolic fraction extracted from RF.

Fraction	Raw		Fermented	
	TPH (GAE mg/100 g)[a]	IC_{50} (mg/ml)[b]	TPH (GAE mg/100 g)[a]	IC_{50} (mg/ml)[b]
A	117 ± 15	5.9 ± 0.01	173 ± 5.6***	4.0 ± 0.02****
B	488 ± 2.6	1.2 ± 0.01	578 ± 8.2****	0.8 ± 0.00***
C	264 ± 2.6	2.1 ± 0.01	175 ± 17***	2.8 ± 0.01***

Values are expressed as means ± S.D. ($n = 3$).
[a] Gallic acid equivalent mg per 100 g of RF equivalent.
[b] Milligram of dry weight of RF in methanol solution.
*** $p < 0.005$ significantly different from raw sample.
**** $p < 0.001$ significantly different from raw sample.

(Cotati, CA, USA), a Hitachi photodiode array detector model L-7450A, a Hitachi column oven model L-7300, and a Phenomenex Degassex™ Model DF-4400 degasser (Torrance, CA, USA). The phenolic acids were separated using a reverse-phase column (Synergi Hydro-RP, 250 mm × 4.60 mm, i.d. 4 μ, Phenomenex, Torrance, CA). The mobile phase consisted of 50 mM aqueous phosphoric acid solution (solvent A) and 100% acetonitrile (solvent B). The following gradient elution program was used: 0–15% B (30 min), 50% B (60 min), 100% B (70 min). The flow rate was 0.7 ml/min.

2.5. Determination of total phenolics

Total phenolic content in each fraction was measured according to the Folin–Ciocalteu colorimetric method described by Singleton [18]. Gallic acid was used as a standard and prepared by dissolving in methanol at a concentration range from 80 to 400 μg/ml.

One-tenth of each extract was dissolved in 1 ml of methanol, which was then diluted 32-fold with methanol. Forty microliters of the each diluted solution was transferred to a 5 ml vial, to

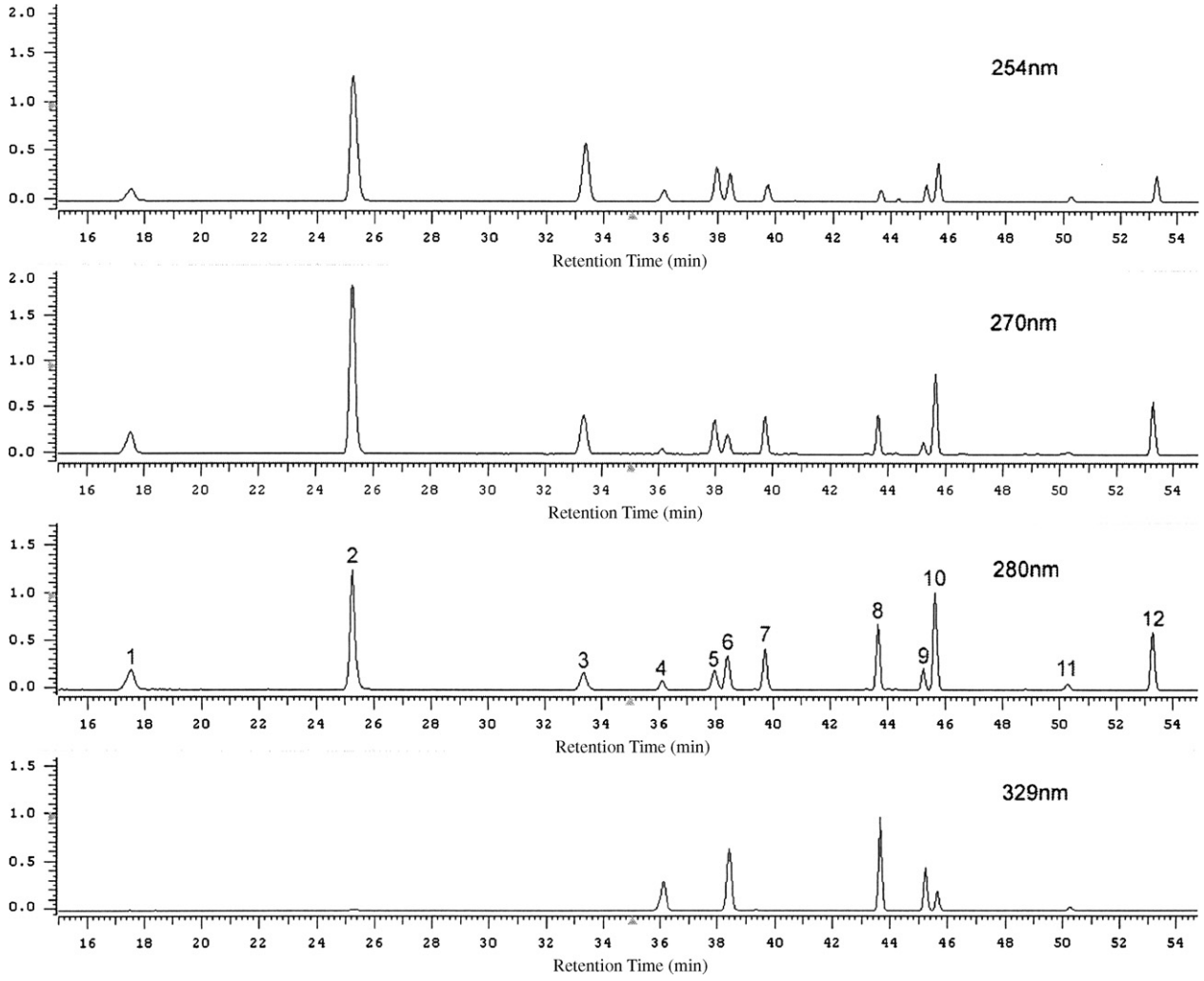

Fig. 2. The chromatogram of 11 phenolic acids and internal standard at different wavelengths; 254, 270, 280, 329 nm (1) gallic acid; (2) protocatechuic acid; (3) p-hydroxybenzoic acid; (4) chlorogenic acid (ISTD); (5) vanillic acid; (6) caffeic acid; (7) syringic acid; (8) p-coumaric acid; (9) ferulic acid; (10) m-coumaric acid; (11) salicylic acid; (12) cinnamic acid; *y*-axis is abundance (AU).

which 1.8 ml of diluted Folin–Ciocalteu's phenol reagent (1:10 with water, v/v) was added, after which it was maintained for 5 min at room temperature. This was followed by the addition of 1.2 ml of a 15% Na_2CO_3 solution and incubation for 90 min at room temperature, after which the absorbance was measured at 765 nm using a microplate reader (Spectramax 340PC, Molecular Devices, Sunnyvale CA, USA). The results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of dry weight.

2.6. Antioxidant activity assay

The antioxidant activity assay was performed by measuring the DPPH radical scavenging activity. The DPPH radical scavenging activity of the each fraction was measured by Blois's method [19] with minor modifications. In brief, each fraction was dissolved in methanol and was diluted to yield a final concentration of 0.1–10 mg/ml (mg of dried RF equiv./ml). One hundred microliters of diluted solution was then added to a 96-well plate, into which

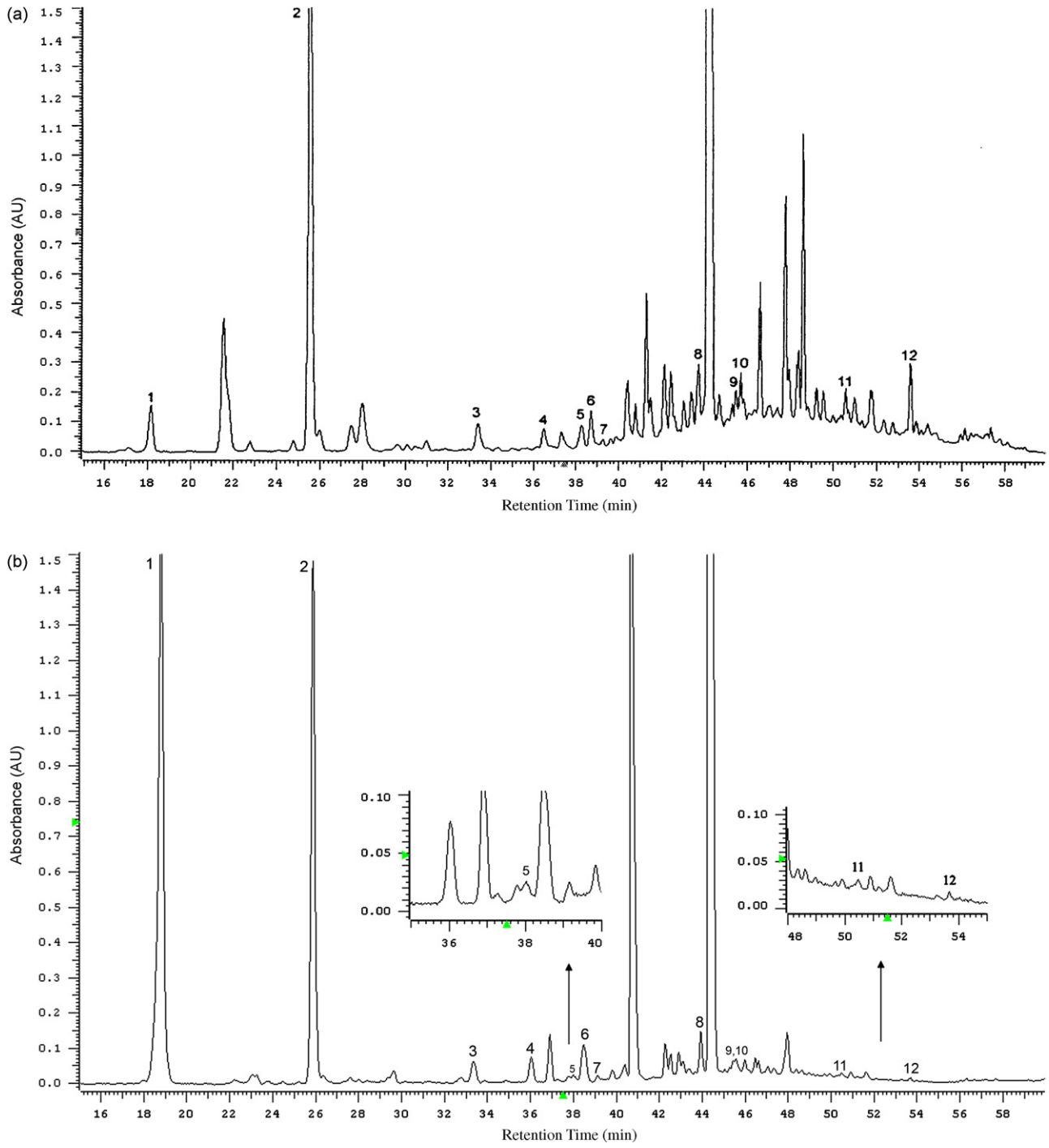

Fig. 3. HPLC chromatograms of phenolic acids in unfermented RF: free phenolic acid fraction (a), ester type (soluble) phenolic acid fraction (b), ester type (insoluble) phenolic acid fraction (c). Marked peaks (no. 1–12) are identified in Fig. 2 and the small peaks are magnified in order to show them more clearly. The concentration of (a)–(c) were 13, 47, and 30 mg/ml, respectively; milligram of extract of RF in methanol solution.

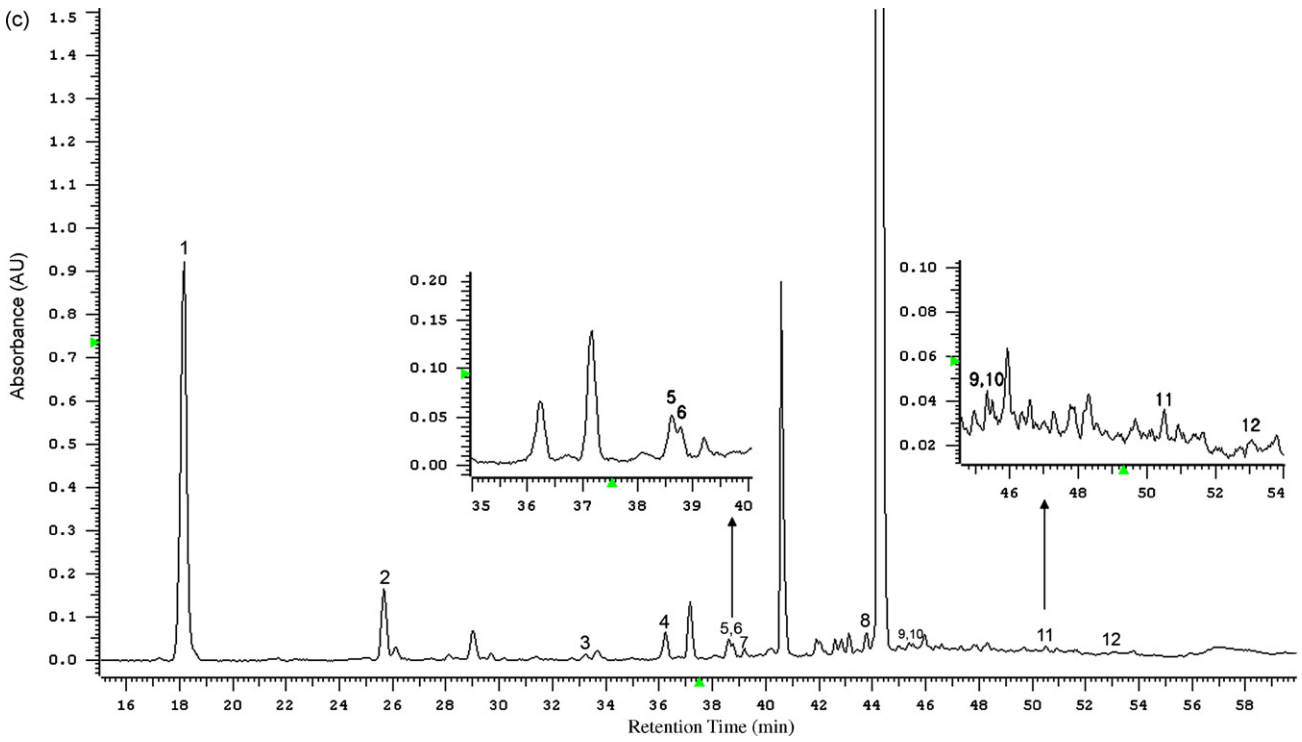

Fig. 3. (*Continued*).

solution the same volume of 200 μM DPPH methanolic solution (100 μl) was added. The plate was incubated for 30 min in the dark at Rt. After incubation, the plate was gently agitated for 5 min, and the absorbance of each well was measured at 517 nm.

2.7. Method validation

Validation was performed in terms of linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). The analytical procedures for validation were performed by ICH guideline and LOD and LOQ were calculated by formula in ICH guideline Q2B [20]. Each solution was subjected to the HPLC-DAD analysis three times. A standard stock solution containing 11 phenolic acids was prepared at a concentration of 1 mg/ml in methanol and diluted to various concentrations for the method validation.

2.8. Statistical analysis

All data are expressed as the mean ± S.D. (standard deviation) corresponding to three replicates. The experimental data were analyzed by the one-way analysis of variance (ANOVA) and statistically, p values of less than 0.05 denoted significant differences among the samples.

3. Results and discussion

3.1. Total phenolic content (TPH) and radical-scavenging activity

The TPH of the RF was 1.7 g GAE/100 g (gallic acid equivalent per 100 g of dried RF equivalent), and the DPPH radical-scavenging

Table 2
Phenolic acids content of raw and fermented RFs (mg/30 g of dried RF).

	Raw				Fermented			
	Fr. A	Fr. B	Fr. C	Total	Fr. A	Fr. B	Fr. C	Total
Hydroxybenzoic acid derivatives								
Gallic acid	15.8 ± 2.29	611 ± 20.5	442 ± 4.50	1069	115 ± 12.8**	478 ± 53.2*	418 ± 15.8	1011
Protocatechuic acid	302 ± 5.57	392 ± 61.9	135 ± 1.66	829	244 ± 13.6*	982 ± 10.8**	116 ± 9.29	1342
p-hydroxybenzoic acid	14.2 ± 0.06	21.0 ± 0.91	5.71 ± 0.06	40.9	17.9 ± 1.91	34.5 ± 2.54*	5.08 ± 0.53	57.5
Vanillic acid	12.6 ± 0.46	10.4 ± 1.70	14.7 ± 4.87	37.6	16.6 ± 2.96	11.8 ± 3.51	20.5 ± 2.81	48.9
Syringic acid	0.94 ± 0.02	4.52 ± 0.12	1.07 ± 0.13	6.54	1.85 ± 0.81	5.31 ± 0.23*	1.00 ± 0.06	8.17
Salicylic acid	42.1 ± 0.28	nd	13.5 ± 2.41	55.6	17.7 ± 3.79*	nd	nd	17.7
Hydroxycinnamic acid derivatives								
Caffeic acid	12.1 ± 0.30	39.5 ± 7.57	8.12 ± 0.52	59.7	16.4 ± 0.93*	34.9 ± 1.64	2.92 ± 0.24**	54.2
p-coumaric acid	8.76 ± 0.94	17.8 ± 4.35	7.81 ± 2.48	34.4	13.8 ± 2.30	26.7 ± 1.77*	9.66 ± 0.01	50.1
Ferulic acid	8.69 ± 0.04	1.39 ± 1.07	7.49 ± 1.78	17.6	3.88 ± 0.60**	5.91 ± 2.10	12.7 ± 1.53	22.5
m-coumaric acid	2.37 ± 0.36	4.35 ± 2.27	1.27 ± 0.75	7.99	1.72 ± 0.10	1.59 ± 0.05	1.42 ± 0.79	4.73
Cinnamic acid	4.12 ± 0.48	0.56 ± 0.21	0.50 ± 0.28	5.18	1.44 ± 0.47*	0.26 ± 0.06	0.37 ± 0.13	2.07
Total	423.7	1102.5	637.2		450.3	1581	587.7	

Values are expressed as mean ± S.D. (n = 3). Fraction A; free phenolic acids, fraction B; ester type phenolic acids (soluble), fraction C; ester type phenolic acids (insoluble) nd: not determined.

* $p < 0.05$ significantly different from raw sample.

** $p < 0.01$ significantly different from raw sample.

activity, expressed as the IC_{50} was 0.06 mg/ml (mg of dried RF equiv./ml) before fermentation. After fermentation, the TPH increased to 2.7 g GAE/100 g, and the radical-scavenging activity, expressed as the IC_{50} decreased to 0.04 mg/ml. The phenolic acids were fractionated by the soluble free type (Fr. A), the soluble ester type (Fr. B), and the insoluble ester type (Fr. C). The TPH in each fraction was determined, and the results are summarized in Table 1. The highest TPH value in both the unfermented and fermented RF samples was found in Fr. B. Fermentation increased the TPH from 117 to 173 mg GAE/100 g in Fr. A, and from 488 to 578 mg GAE/100 g in Fr. B. However, the TPH in Fr. C decreased from 264 to 175 mg GAE/100 g. The increase in the TPH in Fr. A might have been due to

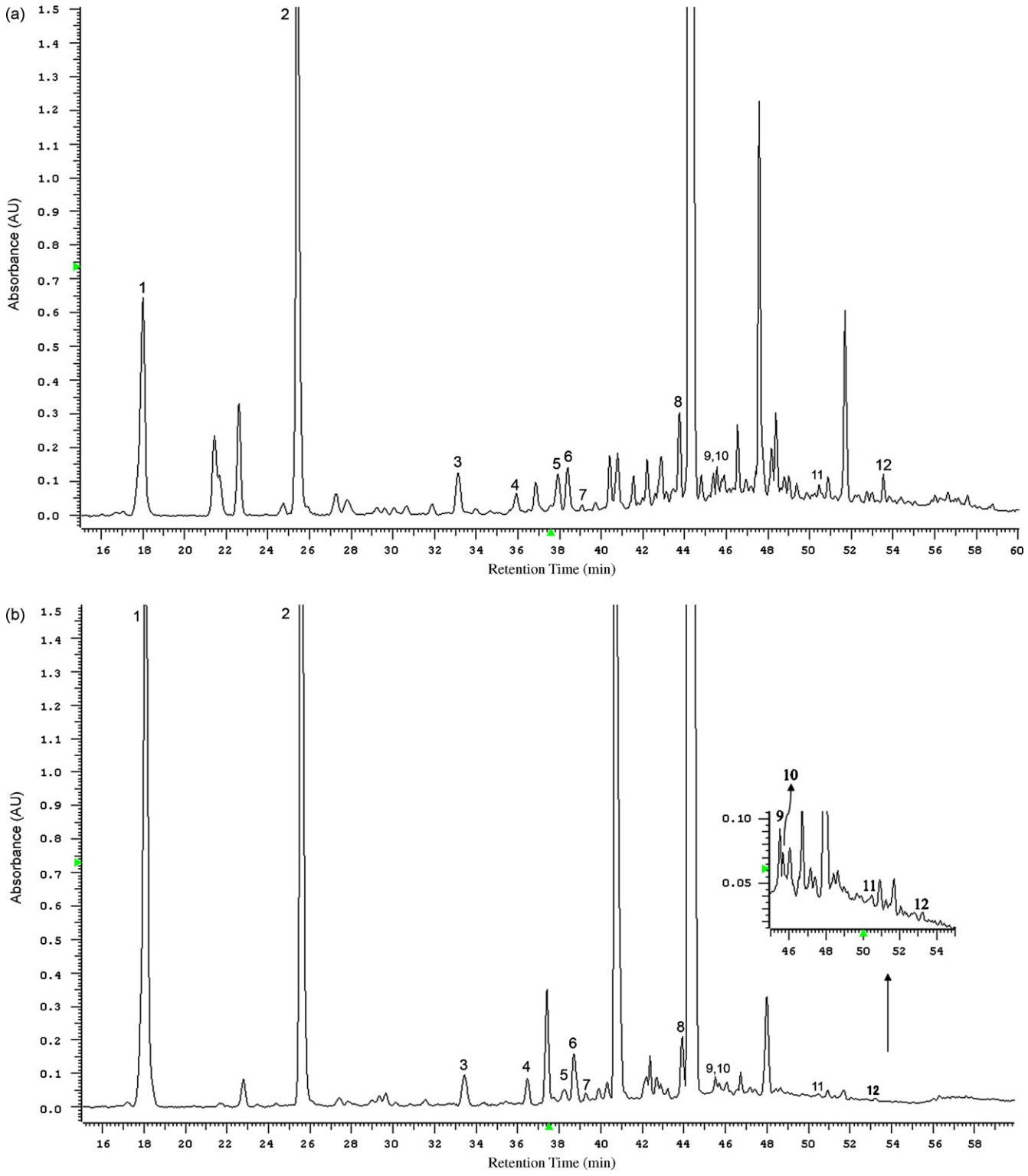

Fig. 4. HPLC chromatograms of phenolic acids in fermented RF: free phenolic acid fraction (a), ester type (soluble) phenolic acid fraction (b), ester type (insoluble) phenolic acid fraction (c). Marked peaks (no. 1–12) are identified in Fig. 2 and the small peaks are magnified in order to show them more clearly. The concentration of (a)–(c) were 13, 37, and 45 mg/ml, respectively; milligram of extract of RF in methanol solution.

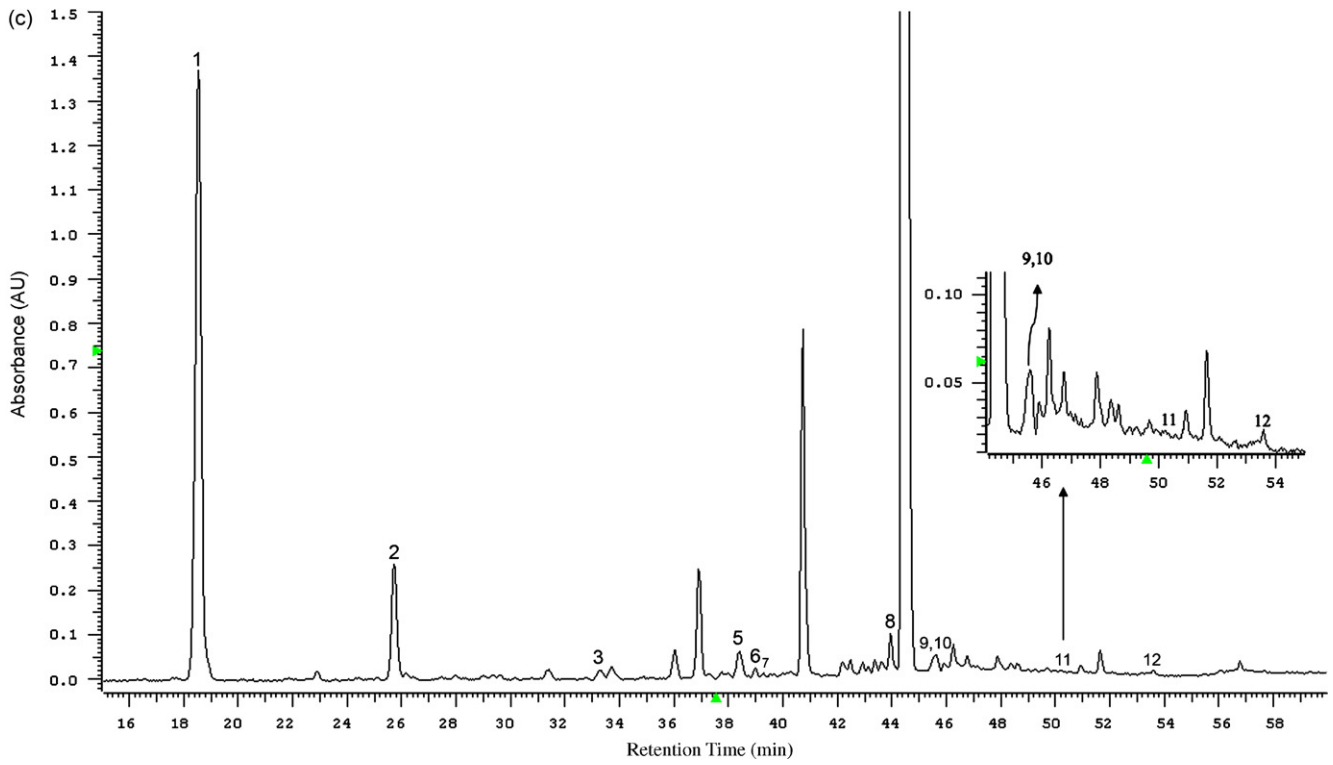

Fig. 4. (*Continued*).

enzymatic hydrolysis by a glycoside hydrolase or the biodegradation of ester-type phenolic acids in Fr. C during yeast fermentation. The increased TPH in Fr. B, meanwhile, might have been due to RF's larger molecules, such as glycosides or anthocyanins, which can lead to the production of ester-type phenolic acids during the fermentation process.

Table 1 summarizes the DPPH radical-scavenging activity (IC_{50}) of each fraction. As the result of fermentation, the IC_{50} value decreased from 5.9 to 4.0 mg/ml (mg of dried RF equiv./ml) in Fr. A, and from 1.2 to 0.8 mg/ml in Fr. B. In Fr. C, the value increased from 2.1 to 2.8 mg/ml. The highest radical-scavenging activity was observed in Fr. B, from the fermented sample, and the lowest activity was observed in Fr. A, from the unfermented sample.

The increase in radical-scavenging activity of the fractions as a result of fermentation was proportional to the total phenolic content in the fractions, suggesting that phenolic compounds appear to contribute antioxidant activity.

3.2. Analysis of phenolic acids in RF

To determine the free phenolic acids that are changed by fermentation, 11 phenolic acids (gallic, protocatechuic, p-hydroxybenzoic, vanillic, syringic, and salicylic acid as hydroxybenzoic acid derivatives; caffeic, p-coumaric, ferulic, m-coumaric, and cinnamic acid as hydrocinnamic acid derivatives) were analyzed by HPLC using chlorogenic acid as internal standard. To identify the phenolic compounds, HPLC-DAD was used at the following specific wavelengths,

Table 3
Results of HPLC method validation; precision, accuracy, limit of quantification and recovery of 11 phenolic acids.

	λ (nm)	Retention time (min)	Regression equation[a]	r^2	LOD[b] (μg/ml)	LOQ[b] (μg/ml)	Precision[c] (R.S.S.D.%)		Accuracy (%)[d]	Recovery (%)[e]
							Intra	Inter		
Hydroxybenzoic acid derivatives										
Gallic acid	280	17.6	$Y = 0.0475x + 0.0012$	0.9958	0.67	2.03	9.21	9.51	104.2 ± 14.9	99.9 ± 8.63
Protocatechuic acid	270	25.2	$Y = 0.0275x + 0.0108$	0.9947	0.48	1.45	5.63	1.75	105.5 ± 1.96	95.6 ± 8.49
p-hydroxybenzoic acid	254	33.4	$Y = 0.0290x + 0.0048$	0.9961	1.42	4.30	4.03	1.52	123.8 ± 2.02	105.3 ± 8.23
Vanillic acid	270	38.0.	$Y = 0.0276x + 0.0103$	0.9946	0.07	0.21	3.88	0.29	112.5 ± 0.31	100.6 ± 3.71
Syringic acid	280	39.7	$Y = 0.0459x + 0.0403$	0.9967	0.17	0.53	1.52	9.99	102.4 ± 17.5	100.3 ± 1.53
Salicylic acid	280	50.3	$Y = 0.0008x - 0.0048$	0.9965	1.58	4.80	2.78	8.76	109.0 ± 9.50	95.6 ± 2.47
Hydroxycinnamic acid derivatives										
Caffeic acid	329	38.4	$Y = 0.0419x + 0.0378$	0.9960	0.25	0.77	1.54	2.28	99.9 ± 2.48	90.8 ± 1.46
p-coumaric acid	329	43.7	$Y = 0.062\ 1x + 0.0363$	0.9989	0.15	0.46	5.23	7.80	102.4 ± 3.60	92.0 ± 4.94
Ferulic acid	329	45.2	$Y = 0.0356x + 0.0082$	0.9984	0.74	2.23	1.40	1.55	99.9 ± 0.54	90.9 ± 9.83
m-coumaric acid	280	45.6	$Y = 0.1053x + 0.0994$	0.9928	0.27	0.81	1.60	0.95	117.7 ± 1.26	105.3 ± 1.73
Cinnamic acid	280	53.3	$Y = 0.1071x - 0.0000$	0.9949	0.22	0.68	5.47	0.82	116.9 ± 1.00	99.0 ± 5.01

[a] x, ratio of sample peak area and internal standard area: Y, concentration (μg/ml).
[b] Values were expressed as LOD = $3.3\sigma/s$, LOQ = $10\sigma/s$, where s is the slope and σ is standard deviation of regression line (μg/ml).
[c] R.S.D. (%) = ((standard deviation/mean) × 100).
[d] Mean [(found/nominal) × 100%] ± S.D. ($n = 3$) at concentration of 60 μg/ml.
[e] Mean ± S.D. ($n = 3$).

which were selected according to the specific phenolic acids to be determined; protocatechuic acid and vanillic acid at 270 nm; p-hydroxybenzoic acid at 254 nm; gallic, syringic, m-coumaric, salicylic, and cinnamic acid at 280 nm; caffeic, p-coumaric, and ferulic acid were detected at 329 nm (Fig. 2).

Protocatechuic acid and gallic acid comprised a significant proportion of all fractions. The gallic and caffeic acid contents in Fr. A increased after fermentation, whereas the protocatechuic, salicylic, ferulic, and cinnamic acid concentrations decreased. The protocatechuic, p-hydroxybenzoic, syringic, and p-coumaric acid contents increased, whereas the gallic acid decreased in Fr. B. The salicylic, caffeic, and cinnamic acid contents decreased in Fr. C; however, no significant increases were observed. The chromatograms corresponding to the fractions are shown in Figs. 3 and 4. Table 2 summarizes the content of each phenolic compound in each fraction.

The vanillic acid content increased from 12.6 to 16.6 mg/30 g of dried RF equivalent, whereas that of ferulic acid decreased from 8.69 to 3.88 mg/30 g of dried RF equivalent, in Fr. A. These results might have been due to the conversion of ferulic acid (present as a free phenolic acid) to vanillic acid by β-oxidation during the fermentation [21]. The p-coumaric acid content increased from 8.76 to 13.8 mg/30 g of dried RF in Fr. A, possibly due to the enzymatic hydrolysis of hydroxycinnamic-ester (fertaric) during the fermentation. Some of the phenolic acid increases in Fr. A can be explained by a hydrolysis mechanism of ester-type phenolic acids. The decreased phenolic acid content in Fr. A can be attributed to either microbial oxidation, reduction, or the degradation of the phenolic compounds by the fermenting microbes. Two compounds that eluted at 40.8 min and 44.5 min were, not identified in this study, but their concentrations was unaltered after fermentation.

3.3. Method validation

The modified HPLC method was validated in terms of linearity, accuracy, precision, LOD, and LOQ. The calibration curve was found to have good linearity in the range of 1–200 μg/ml ($r^2 \geq 0.993$). The inter-day precision and intra-day precision were analyzed at a concentration of 60 μg/ml six times over a 2-day period and the R.S.D. values for the 11 phenolic acids were all less than 10.0%. The recovery and accuracy tests were carried out at the same concentration and the results are summarized in Table 3.

4. Conclusions

Eleven phenolic acids in both unfermented and fermented RF were analyzed by HPLC-DAD. The total phenolic content and DPPH radical-scavenging activity of RF were measured before and after fermentation.

Gallic acid and protocatechuic acid were the most abundant phenolic acids in RF, and their contents were increased as a result of the fermentation process. The total phenolic content and DPPH radical-scavenging activity were both enhanced by fermentation of the RF.

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