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# Effects of storage period and heat treatment on phenolic compound composition in dried *Citrus* peels (Chenpi) and discrimination of Chenpi with different storage periods through targeted metabolomic study using HPLC-DAD analysis

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ABSTRACT

Dried *Citrus* peels, known as Chenpi in Chinese medicine, are a traditional medicine for the treatment of indigestion and inflammatory syndromes. In this study, we evaluated the effect of storage periods (1-year vs. 3-year) and heat treatment (90 min vs. 3 h at 120 °C) on the total phenolic content (TPC) and bioactivity (anti-oxidant activity) of Chenpi. It was found that the long-term stored Chenpi had a higher TPC and superior 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity levels compared to the regular stored Chenpi, and that heat treatment increased both TPC and bioactivity. Subsequently, we developed and validated a high performance liquid chromatography with diode-array detection (HPLC-DAD) method to determine individual phenolic acids. Eleven phenolic compounds were determined in different Chenpi samples. Concentrations of total phenolic compounds were higher in long-term stored Chenpi and heat treatment raised the levels of those in regular stored Chenpi. In further study, a targeted metabolomic approach was applied to discriminate Chenpi with different storage periods. Two different phenolic acid fractions (free and ester) from the regular and long-term stored Chenpi were analyzed using the developed HPLC-DAD and the data were used in principal component analysis (PCA) on the HPLC-DAD peak areas of the 11 phenolic acids. Two principal components (PC1 and PC2) accounted for 87.1% of the variation between the regular and long-term stored Chenpi. In a two-dimensional plot of PC1 and PC2, the samples divided into four groups: two storage periods and two fractions.



## 1. Introduction

Dried fruit peels of *Citrus unshiu* Markovich, *C. reticulate* Blanco, and *C. tachibana* Makino Tanaka, which are collectively known as Chenpi, have been widely used as a traditional medicine in Korea, China, and Japan. Chenpi extracts are used to treat indigestion and inflammatory syndromes of the respiratory tract such as bronchitis and asthma [1].

It is known that Chenpi contains more bioactive compounds, such as flavonoids, phenolic acids, and limonoids, than the citrus juice [2,3]. Like flavonoids, which are probably the major anti-oxidants in Chenpi, phenolic compounds are regarded as significant bioactive compounds and have been reported as anti-oxidative agents in various plants [4–6]. The total phenolic content of Chenpi has been correlated with the anti-oxidizing activity of Chenpi [7–9]. In biological systems, highly reactive free radicals and oxygen species modify nucleic acids, proteins, lipids, and DNA through oxidation, modifications that may initiate degenerative diseases [10–17]. Thus, Chenpi has potential as a health-promoting substance.

Phenolic compounds naturally exist in a phenolic acid form as hydroxybenzoic acid or hydroxycinnamic acid derivatives (Fig. 1), and are usually covalently bound to insoluble polymers. This limits the activity of bound phenolic compounds as natural anti-oxidative agents and there have been attempts to liberate bound phenolic acids. For example, bound low molecular weight phenolic compounds in citrus peels have been transformed into free phenolic acids through heat treatment; thereby increasing the anti-oxidant activity of the citrus peel extract [9].

In the current study, Chenpi samples with different storage periods (1 year vs. 3 years) and different heat treatments (90 min vs. 3 h at 120 °C) were assessed for total phenolic content. Eleven individual phenolic acid compounds were determined using an optimized high performance liquid chromatography with diode-array detection (HPLC-DAD) method, which is the first simultaneous analysis of such a large number of phenolic compounds. This study allowed us to explain correlation between antioxidant activity and phenolic content of individual Chenpi samples.

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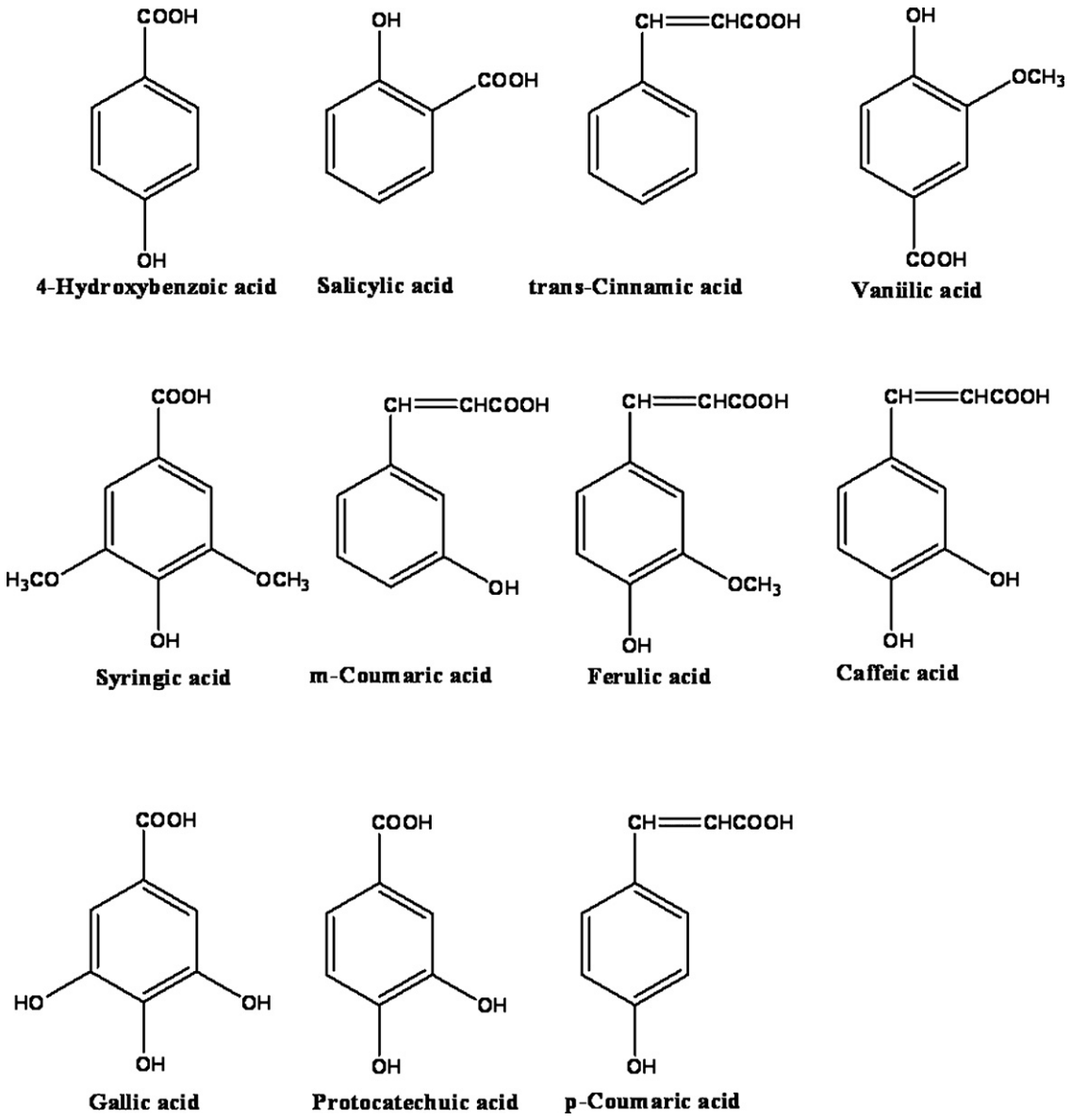


**Fig. 1.** Eleven phenolic acids analyzed in this study.

Metabolomic research methods offer high-throughput qualitative and quantitative measurement that can be used to monitor sample quality. It also provides a powerful solution in differentiating small-molecule profiles. In this study, a targeted metabolomic analysis of specific phenolic compounds in Chenpi was performed. Data from the HPLC-DAD analysis of phenolic acids of Chenpi underwent principal component analysis (PCA). To our knowledge, this is the first report of determination of principal components (PCs) that would be useful when discriminating storage periods in Chenpi.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Regular (1-year) stored Chenpi and long-term (3-year) stored Chenpi were purchased at the Kyoungdong Market in Seoul, Korea. A pig kidney cell line (LLC-PK1) was obtained from the Korean Cell Line Bank (KCLB; Cancer Research Institute, Seoul, Korea). Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum, and 100 μg/mL Streptomycin antibiotics were purchased from Gibco-BRL, New York, USA and *o*-phosphoric acid (85%) was acquired from Fluka (Steinheim, Germany). HPLC-grade acetonitrile was supplied by Duksan (Kyoungkido, Korea). All the other chemical reagents, including phenolic acid standards (i.e., β-hydroxybenzoic acid, salicylic acid, *trans*-cinnamic acid, vanillic acid, syringic acid, *m*-coumaric acid, *trans*-ferulic acid, caffeic acid, gallic acid, protocatechuic acid, *p*-coumaric acid, and chlorogenic acid) were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless specified elsewhere.

### 2.2. Sample preparation

Regular and long-term stored Chenpi were powdered using an electric mill (Shinil SFM-555SP, Hwasung, Korea) and sieved through a 300 μm sieve. Before powdering, portions of the Chenpi samples were held at 120 °C for either 90 min or 3 h to evaluate the effect of heat treatment on the composition of phenolic compounds.

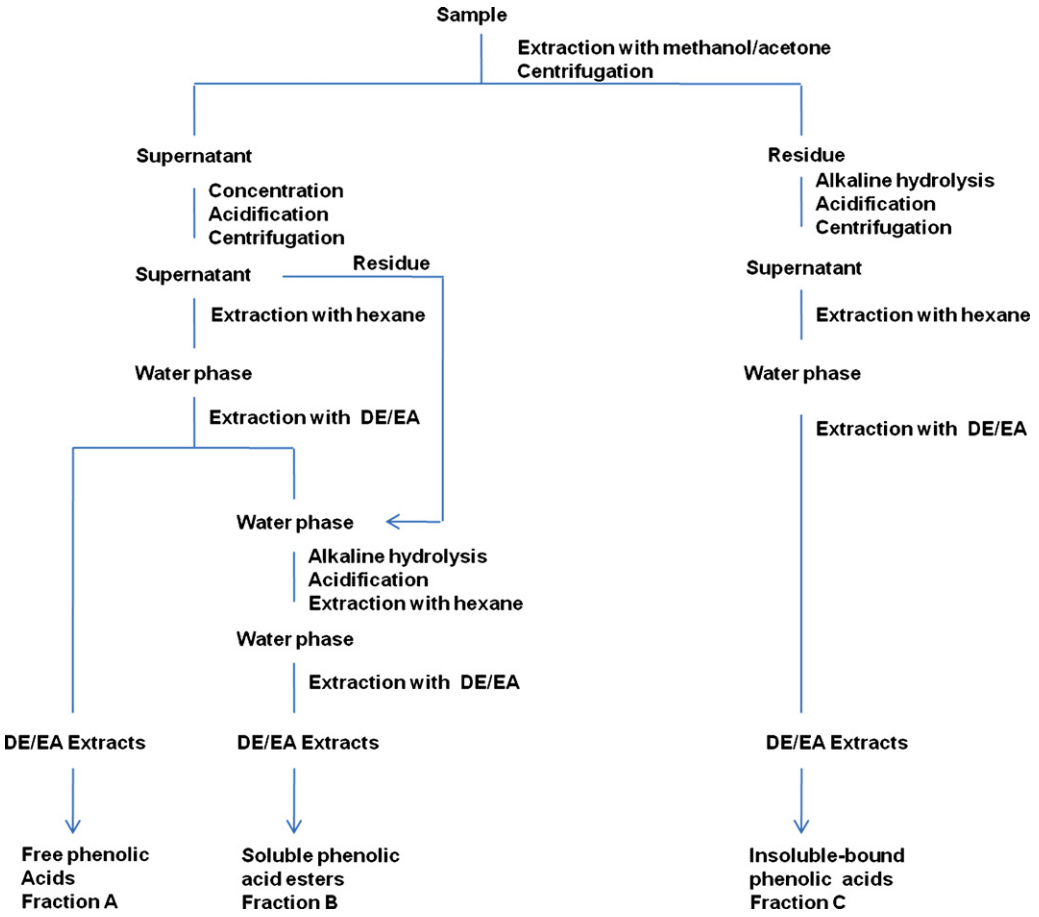


**Fig. 2.** Procedure used to extract and separate the free acid (fraction A), soluble ester (fraction B) and insoluble bound (fraction C) fractions from Chenpi using methanol/acetone (70% methanol–70% acetone mixture, 1:1, v/v) and DE/EA (diethyl ether–ethyl acetate, 1:1, v/v).

Free phenolic acids (fraction A), soluble phenolic acid esters (fraction B), and insoluble-bound phenolic acids (fraction C) were prepared according to the Krygier et al.'s method [18] as described below and as schematically shown in (Fig. 2). Briefly, 30 g of powdered sample was extracted in 20 mL of a 70% methanol/70% acetone (1:1, v/v) mixture at room temperature. The supernatant was concentrated under reduced pressure, acidified to pH 2 with 6 N HCl, and then extracted in hexane. The aqueous phase was extracted in the diethyl ether/ethyl acetate mixture (DE/EA, 1:1, v/v). The organic layer was dehydrated with sodium sulfate, followed by evaporation, to yield fraction A. The aqueous phase extract in DE/EA was alkali hydrolyzed in 4 N NaOH, acidified, and then extracted with hexane. The resulting aqueous layer underwent extractions with hexane and DE/EA (as described for fraction A) resulting in fraction B. Fraction C was obtained from the 70% methanol/70% acetone extraction residue which was then treated with alkali hydrolysis followed by extraction with hexane and DE/EA as described for fraction B.

### 2.3. HPLC analysis and method validation

A Waters-510 HPLC system (Milford, MA, USA) equipped with a DAD detector (210–400 nm range) was used. A $C_{18}$ column (Synergi Hydro-RP, 250 mm × 4.60 mm, 4 μm particle size; Phenomenex, Torrance, CA, USA) was used for phenolic acid analysis. Mobile phases consisted of 50 mM phosphoric acid in water (A) and acetonitrile (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 1 mL/min and the phenolic acids were detected at 280 nm [19].

Method validation was performed according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formula in ICH guideline Q2B. Chenpi samples and 11 phenolic acid standards were analyzed 3 times by HPLC-DAD. A standard stock solution of 11 phenolic acids was prepared at a concentration of 2 mg/mL in methanol and then diluted to various concentrations for method validation. Sample solutions were prepared at a concentration of 5 mg/mL in methanol.

### 2.4. Determination of total phenolic acid content

Total phenolic acid content of each fraction was measured according to the Payet et al.'s method [20] using gallic acid (dissolved in methanol at concentrations ranging from 0.125 to 2 mg/mL) as a standard. In brief, Chenpi extracts were diluted to 1 mg/mL in methanol and 30 μL of each diluted extract with 150 μL of Folin-Ciocalteu reagent were transferred to a 96-well plate. An aqueous 7.5% $Na_2CO_3$ solution (120 μL) was added and the absorbance of solutions was measured at 765 nm after 1 h of incubation.

### 2.5. Cell culture

LLC-PK1 cell lines were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 100 μg/mL streptomycin. Cell cultures were maintained at 37 °C in a humidified incubator (95% air–5% $CO_2$). The culture medium was changed every 2–3

**Table 1**
Total phenolic content (TPC) of phenolic fractions from Chenpi ($n = 3$).

| Fraction | TPC (GAE g/100 g)[a] | | | | | |
|---|---|---|---|---|---|---|
| | CP | 3CP | HCP9m | 3HCP9m | HCP3h | 3HCP3h |
| A | 1.77 ± 0.02 | 3.22 ± 0.58* | 3.02 ± 1.18* | 2.61 ± 0.28* | 3.88 ± 0.78* | 3.43 ± 0.13* |
| B | 1.71 ± 0.73 | 2.21 ± 0.15 | 0.88 ± 0.28 | 1.69 ± 0.10 | 0.90 ± 0.07 | 1.95 ± 0.10 |
| C | 0.64 ± 0.09 | 0.60 ± 0.06 | 0.63 ± 0.08 | 0.66 ± 0.03 | 0.74 ± 0.07 | 0.68 ± 0.03 |

CP, regular stored Chenpi; 3CP, long-term stored Chenpi; HCP9m, regular stored Chenpi with 90 min heat treatment; 3HCP9m, long-term stored Chenpi with 90 min heat treatment; HCP3h, regular stored Chenpi with 3 h heat treatment; 3HCP3h, long-term stored Chenpi with 3 h heat treatment. Fraction A, free phenolic acid fraction; fraction B, soluble phenolic acid ester fraction; fraction C, insoluble-bound phenolic acid fraction.

[a] Gallic acid equivalent g per 100 g of citrus equivalent.

* Significantly different from the regular stored Chenpi ($p < 0.05$).

days. Cells grown to confluence in 75 $cm^2$ flasks over 6–7 days were trypsinized prior to use.

### 2.6. Antioxidant activity assays (DPPH and AAPH assays)

Antioxidant activity was measured by determining the amounts of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azobis-2-methyl-propanimidamide (AAPH) radical scavenging activity in LLC-PK1 cells. The DPPH radical scavenging activity was measured using Blois's method with slight modifications [21]. Samples of fractions A, B, and C were diluted to concentrations from 0.3125 to 5 mg/mL, mixed with the same volume of 60 μM DPPH methanolic solution in a 96-well plate, and incubated in the dark for 30 min at room temperature. Absorbance was measured at 517 nm.

The AAPH radical scavenging activity was measured using LLC-PK1 cells [22]. Stock solutions (50 mg/mL) of samples were prepared in dimethyl sulfoxide (DMSO) and serially diluted in 0.4% DMSO/PBS to make test solutions. A single cell suspension (100 μL) containing $1 \times 10^5$ cells/mL was seeded into 96-well microtiter plates (NUNC, Roskilde, Denmark), which were incubated for 2 h. Subsequently, 50 μL of the sample solution and 50 μL of the AAPH toxicant (4 mM in media) were added to the plates, followed by 24 h of incubation. Cell viability was measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [23].

### 2.7. Statistical analysis

Data are expressed as means ± standard deviation (SD) of triplicate samples. Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. A $P$ value <0.05 was considered significant. Principal component analysis (PCA) was performed by SAS 9.1 software (SAS Institute, Cary, NC, USA) using the peak areas of 11 phenolic compounds as variables. The component information for each Chenpi sample was determined by its chemical descriptors. Calculated peak areas were applied to assort the data matrix, which was composed of 11 columns (variables) and 4 rows (samples).

## 3. Results and discussion

### 3.1. Total phenolic content and radical-scavenging activities of Chenpi samples

Phenolic acids were prepared in three different fractions (A, free phenolic acids; B, soluble phenolic acid esters; and C, insoluble-bound phenolic acids) from Chenpi as shown in Fig. 2. TPC values determined from fractions A, B, and C of various Chenpi samples are summarized in Table 1. The TPC of fraction A in the long-term stored Chenpi (3.22 g gallic acid equivalents (GAE) per 100 g of citrus equivalent) was significantly higher than that of fraction A in regular stored Chenpi (1.77 g GAE/100 g), but was comparable to the highest TPC value (3.88 g GAE/100 g) found in fraction A of the 3 h heat-treated regular stored Chenpi samples. Similarly, the TPC of fraction B increased from 1.71 to 2.21 g GAE/100 g with the increase in storage time. The higher level of TPC found in fractions A and B of the long-term stored Chenpi might have resulted from enzymatic hydrolysis or from biodegradation of previously unextractable bound phenolic compounds over the extended storage period.

In the regular stored Chenpi, the TPC values in fraction A increased from 1.77 to 3.02 and 1.77 to 3.88 g GAE/100 g with heat treatment periods of 90 min and 3 h, respectively. However, the TPC of fraction A from the long-term stored Chenpi did not change markedly with heat treatment. The TPC of fraction B in heat treatment was about 2-fold lower than the regular stored Chenpi whereas the TPC of fraction B from the long-term stored Chenpi did not decrease as markedly. The TPC of fraction C remained mostly unchanged regardless of the duration of the heat treatment. Similarly, fraction C from the long-term stored Chenpi remained mostly unchanged with heat treatment.

Table 2 summarizes the DPPH radical-scavenging activity [as half maximal inhibitory concentration ($IC_{50}$)] of each fraction. Overall, the $IC_{50}$ values changed in a similar fashion to TPC values. In the regular stored Chenpi, the anti-oxidizing activity increased with longer heat treatment in fraction A, in which the DPPH activity also increased with extended storage period. However, no big changes were associated with extended storage period or heat treatment in fraction B, and the DPPH activity was reduced with longer heat

**Table 2**
DPPH radical-scavenging activities of phenolic fractions from Chenpi ($n = 3$).

| Fraction | IC50 (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | CP | 3CP | HCP9m | 3HCP9m | HCP3h | 3HCP3h |
| A | 105.74 ± 3.92 | 77.66 ± 2.88* | 92.81 ± 4.94* | 94.89 ± 8.03* | 45.02 ± 2.20* | 65.21 ± 4.08* |
| B | 106.57 ± 9.42 | 98.37 ± 5.60 | 152.32 ± 7.89 | 107.88 ± 8.21 | 113.07 ± 7.94 | 101.43 ± 7.90 |
| C | 183.25 ± 9.32 | 232.98 ± 4.79 | 236.55 ± 10.96 | 269.53 ± 1089 | 456.88 ± 5.30 | 283.42 ± 7.61 |

CP, regular stored Chenpi; 3CP, long-term stored Chenpi; HCP9m, regular stored Chenpi with heat treatment for 90 min; 3HCP9m, long-term stored Chenpi with heat treatment for 90 min; HCP3h, regular stored Chenpi with heat treatment for 3 h; 3HCP3h, long-term stored Chenpi with heat treatment for 3 h. Fraction A, free phenolic acid fraction; fraction B, soluble phenolic acid ester fraction; fraction C, insoluble-bound phenolic acid fraction.

* Significantly different from regular stored Chenpi ($p < 0.05$).

treatment in fraction C. Heat treatment had minimal effect on the activities of all three fractions in the long-term stored Chenpi. The antioxidant activity of the three fractions was also measured using AAPH toxicant but the activity was weak (data not shown). Taken together, the long-term stored Chenpi was almost as effective as the heat-treated regular stored Chenpi in terms of free phenolic acid contents and anti-oxidizing activity.

### 3.2. HPLC-DAD method validation

We examined the levels of individual phenolic acid compounds in various Chenpi samples by HPLC analysis and chose the most abundant 11 phenolic acids in Chenpi for further study. This is the first simultaneous analysis of such a large number of phenolic compounds in this type of research. The analyzed compounds were: gallic, protecatechuic, β-hydroxybenzoic, vanillic, syringic, and salicylic acids as hydroxybenzoic acid derivatives; and caffeic, *p*-coumaric, ferulic, *m*-coumaric, and cinnamic acids as hydrocinnamic acid derivatives. HPLC-DAD was established to determine the 11 phenolic acids. For method validation, a gradient elution consisting of 50 mM phosphoric acid in water and acetonitrile was applied and chlorogenic acid was the internal standard. A chromatogram of the standards is shown in Fig. 3.

Linearity, accuracy, precision, and the limit of quantification (LOQ) were examined for the method validation. The calibration curves showed good linearity (correlation coefficient ($r^2$) ≥ 0.9941) within a range of 1–200 μg/mL. The inter- and intra-day precisions, expressed as relative standard deviations (RSD), were determined at a concentration of 60 μg/mL three times a day for 3 days. The intra-day precisions were ≤8.0% and the inter-day precisions were ≤9.6%. LOD ranged from 0.08 to 1.72 μg/mL and LOQ was between 0.25 and 5.22 μg/mL. Accuracy measured at 60 μg/mL ranged from 96 to 118%. The validation results are summarized in Table 3.

### 3.3. Analysis of the phenolic acid compositions in Chenpi samples

The HPLC-DAD method was applied to assess changes in the composition of phenolic compounds in three fractions from regular and long-term stored Chenpi samples with or without heat treatment for either 90 min or 3 h at 120 °C.

In fraction A samples, the amounts of 8 phenolic acids (vanillic, caffeic, syringic, *p*-coumaric, ferulic, *m*-coumaric, salicylic, and *trans*-cinnamic) increased with extended storage time (Table 4). Gallic, protocatechuic, and β-hydroxybenzoic acids, not detected in regular stored Chenpi, were present in long-term stored Chenpi. Gallic acid exists in either free or bound form as gallotannins and ellagitannins in plants and the bound form of gallic acid is easily hydrolyzed by acid or enzymes [24]. Protocatechuic acid and protocatechuic aldehyde, which are usually bound to polysaccharidic material of cell wall in covalent form, are degraded by extracellular enzymatic activities (cellulases, laccases, peroxidases) [25,26]. Thus, it is possible that the reactions above occurred naturally during 2 years of extended storage.

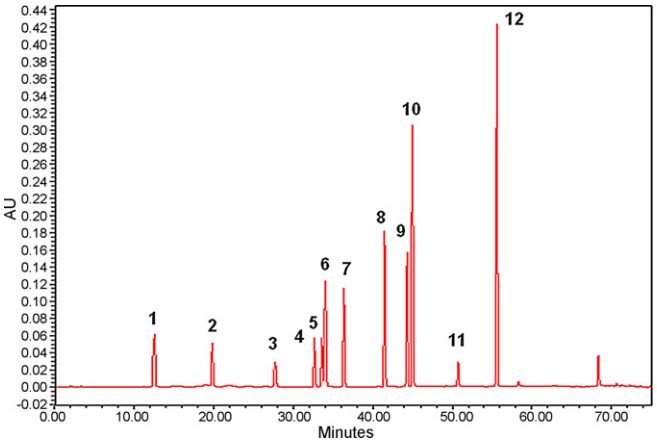


**Fig. 3.** Chromatogram of 11 phenolic acid standards along with the internal standard at 280 nm. (1) Gallic acid, (2) Protocatechuic acid, (3) β-Hyroxybenzoic acid, (4) Chlorogenic acid (internal standard), (5) Vanillic aicd, (6) Caffeic acid, (7) Syringic acid, (8) *p*-Coumaric acid, (9) *trans*-Ferulic acid, (10) *m*-Coumaric acid, (11) Salicylic acid, and (12) *trans*-Cinnamic acid.

We also examined the effect of heat treatment and found that heat treatment increases the amount of phenolic acids in fraction A of regular stored Chenpi. In particular, 3 h of heating was more effective than 90 min of heating in increasing the amount of all phenolic acids, except for gallic and syringic acids, in fraction A.

In fraction A of long-term stored Chenpi, the amount of protocatechuic, β-hydroxybenzoic, caffeic, and *p*-coumaric acids increased after heat treatment, with a greater increase following the 3 h treatment compared to the 90 min treatment. In fraction B the concentrations of protocatechuic, β-hydroxybenzoic, vanillic, *p*-coumaric, *m*-coumaric, salicylic acids increased when the long-term stored Chenpi was heated for 3 h, and they showed a greater increase after the longer treatment. However, the amounts of the

**Table 3**
HPLC-DAD method validation: precision, accuracy, and limit of quantification for 11 phenolic acids.

| | λ (nm) | Retention time (min) | Regression equation[a] | $r^2$ | LOD[b] (μg/mL) | LOQ[b] (μg/mL) | Precision (RSD %[c]) | | Accuracy (%)[d] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Intra | Inter | |
| Hydroxybenzoic acid derivatives | | | | | | | | | |
| Gallic acid | 280 | 12.46 | $Y = 0.0432x + 0.0110$ | 0.9980 | 0.08 | 0.25 | 2.46 | 2.76 | 100.6 ± 3.71 |
| Protocatechuic acid | 280 | 20.03 | $Y = 0.0438x + 0.0602$ | 0.9951 | 0.57 | 1.72 | 1.72 | 5.60 | 99.9 ± 1.53 |
| β-Hydroxybenzoic acid | 280 | 27.42 | $Y = 0.0221x + 0.0031$ | 0.9945 | 1.72 | 5.22 | 5.22 | 1.22 | 117.7 ± 1.96 |
| Vanillic acid | 280 | 33.68 | $Y = 0.0344x + 0.0085$ | 0.9941 | 0.37 | 1.11 | 1.11 | 9.58 | 104.2 ± 0.31 |
| Syringic acid | 280 | 36.16 | $Y = 0.0358x + 0.0119$ | 0.9952 | 1.16 | 3.51 | 3.51 | 9.40 | 109.0 ± 2.47 |
| Salicylic acid | 280 | 49.79 | $Y = 0.0254x + 0.0020$ | 0.9957 | 0.21 | 0.64 | 6.43 | 2.84 | 95.6 ± 8.63 |
| Hydroxycinnamic acid derivatives | | | | | | | | | |
| Caffeic acid | 280 | 34.06 | $Y = 0.0384x + 0.0272$ | 0.9955 | 0.46 | 1.40 | 1.40 | 2.14 | 105.3 ± 1.00 |
| *p*-Coumaric acid | 280 | 41.3 | $Y = 0.0493x + 0.0112$ | 0.9943 | 0.61 | 1.85 | 1.85 | 4.42 | 102.4 ± 1.46 |
| Ferulic acid | 280 | 44.18 | $Y = 0.0168x + 0.0143$ | 0.9970 | 0.47 | 1.43 | 1.43 | 1.58 | 99.9 ± 0.54 |
| *m*-Coumaric acid | 280 | 44.76 | $Y = 0.0293x + 0.0448$ | 0.9955 | 0.58 | 1.74 | 1.74 | 2.39 | 100.3 ± 1.53 |
| Cinnamic acid | 280 | 54.93 | $Y = 0.0387x + 0.0409$ | 0.9962 | 0.26 | 0.80 | 8.03 | 3.38 | 109.0 ± 3.71 |

[a] $x$, ratio of sample peak area and internal standard area; $Y$, concentration (μg/mL).
[b] Values were expressed as 3.3 $\sigma/s$ and 10 $\sigma/s$ for LOD and LOQ, respectively, where $s$ is the slope and $\sigma$ is the standard deviation of the regression line (μg/mL).
[c] Relative standard deviation (%) = (standard deviation/mean) × 100.
[d] Accuracy mean [(found/nominal) × 100%] ± SD ($n$ = 3) at a concentration of 60 μg/mL.

**Table 4**
Compositions of phenolic acids in fractions from Chenpi (mg/30 g of samples) ($n = 3$).

| Phenolic acid | CP | 3CP | HCP9m | 3HCP9m | HCP3h | 3HCP3h |
|---|---|---|---|---|---|---|
| Fraction A (mg/30 g) | | | | | | |
| Gallic acid | nd | 25.29 ± 0.56 | 35.75 ± 0.34 | 9.46 ± 0.28 | 14.01 ± 0.56 | 4.04 ± 0.28 |
| Protocatechuic acid | nd | 126.40 ± 0.33 | 19.32 ± 0.63 | 47.12 ± 0.38 | 77.88 ± 0.65 | 141.80 ± 0.56 |
| β-Hydroxybenzoic acid | nd | 14.71 ± 0.57 | 17.68 ± 0.63 | 22.44 ± 0.33 | 80.50 ± 0.63 | 92.80 ± 0.51 |
| Vanillic acid | 2.07 ± 0.18 | 14.11* ± 0.43 | nd | nd | nd | nd |
| Caffeic acid | 0.75 ± 0.10 | 10.90* ± 0.26 | 51.28* ± 0.52 | 35.83* ± 0.73 | 214.75* ± 0.46 | 156.34* ± 0.54 |
| Syringic acid | 17.34 ± 0.17 | 36.27 ± 0.32 | 4.97* ± 0.28 | 26.95 ± 0.38 | 4.59* ± 0.16 | 5.89* ± 0.22 |
| *p*-Coumaric acid | 11.00 ± 0.12 | 20.21 ± 0.37 | 36.91 ± 0.43 | 22.87 ± 0.53 | 72.18* ± 0.54 | 72.40* ± 0.31 |
| Ferulic acid | 48.21 ± 0.69 | 54.99 ± 0.44 | nd | nd | nd | nd |
| *m*-Coumaric acid | 17.66 ± 0.25 | 50.82 ± 0.58 | 2.48* ± 0.34 | nd | 32.69 ± 0.47 | nd |
| Salicylic acid | 17.59 ± 0.60 | 69.07 ± 0.49 | 17.86 ± 0.43 | 5.85* ± 0.17 | 40.28 ± 0.51 | 30.26 ± 0.47 |
| *trans*-Cinnamic acid | 0.16 ± 0.06 | 14.58* ± 0.31 | 5.43* ± 0.32 | nd | 10.71* ± 0.12 | nd |
| Total | 114.79 | 437.34 | 191.69 | 170.51 | 547.58 | 503.53 |
| Fraction B (mg/30 g) | | | | | | |
| Gallic acid | nd | 0.24 ± 0.02 | nd | nd | nd | nd |
| Protocatechuic acid | nd | nd | nd | nd | 4.92 ± 0.08 | 9.74 ± 0.29 |
| β-Hydroxybenzoic acid | nd | 1.25 ± 0.09 | nd | nd | 7.84 ± 0.04 | 3.36 ± 0.29 |
| Vanillic acid | 1.21 ± 0.05 | 2.67 ± 0.07 | nd | 1.33 ± 0.04 | 19.91* ± 0.02 | 24.35* ± 0.19 |
| Caffeic acid | 4.42 ± 0.04 | 13.95 ± 0.05 | 2.99 ± 0.06 | 7.08 ± 0.08 | 4.40 ± 0.27 | 8.01 ± 0.05 |
| Syringic acid | nd | nd | nd | 1.58 ± 0.12 | 2.62 ± 0.07 | nd |
| *p*-Coumaric acid | 7.16 ± 0.10 | 15.90 ± 0.04 | 5.36 ± 0.05 | 8.90 ± 0.40 | 27.01* ± 0.10 | 28.72* ± 0.32 |
| Ferulic acid | 149.87 ± 0.11 | 334.49* ± 0.14 | 80.28 ± 0.23 | 129.48 ± 0.03 | 48.36* ± 0.07 | 100.91 ± 1.03 |
| *m*-Coumaric acid | 7.43 ± 0.08 | 6.27 ± 0.07 | nd | 3.52 ± 0.09 | 29.40* ± 0.08 | 28.60* ± 0.13 |
| Salicylic acid | 5.61 ± 0.09 | 4.83 ± 0.18 | 1.36* ± 0.07 | 7.83 ± 0.16 | nd | 18.53* ± 0.47 |
| *trans*-Cinnamic acid | 5.17 ± 0.10 | 9.68 ± 0.02 | 7.25 ± 0.33 | 19.67* ± 0.33 | 2.57 ± 0.16 | 5.61 ± 0.15 |
| Total | 180.87 | 389.28 | 97.23 | 179.40 | 147.02 | 227.84 |
| Fraction C (mg/30 g) | | | | | | |
| Gallic acid | 6.73 ± 0.01 | nd | 0.15 ± 0.01 | nd | nd | 2.86 ± 0.05 |
| Protocatechuic acid | 79.54 ± 0.04 | 19.11* ± 0.08 | 5.60 ± 0.05 | 0.25* ± 0.02 | nd | 1.42* ± 0.02 |
| β-Hydroxybenzoic acid | 31.90 ± 0.14 | 15.73 ± 0.15 | 6.99* ± 0.11 | 3.81* ± 0.06 | 0.72* ± 0.14 | 2.14* ± 0.01 |
| Vanillic acid | 29.90 ± 0.04 | 17.01 ± 0.12 | 7.51* ± 0.10 | 1.56* ± 0.02 | 6.50* ± 0.04 | nd |
| Caffeic acid | 50.55 ± 0.04 | 61.14 ± 0.07 | 22.94 ± 0.05 | 14.16 ± 0.09 | 8.70* ± 0.05 | 6.31* ± 0.34 |
| Syringic acid | 4.90 ± 0.04 | 3.41 ± 0.04 | 0.81* ± 0.11 | 5.71 ± 0.08 | nd | 0.99* ± 0.05 |
| *p*-Coumaric acid | 98.12 ± 0.02 | nd | 28.61 ± 0.03 | 7.30* ± 0.07 | 3.29* ± 0.04 | 10.79* ± 0.10 |
| Ferulic acid | 1111.65 ± 0.16 | 1007.47 ± 0.07 | 12.95* ± 0.17 | 35.59* ± 0.14 | 3.24* ± 0.15 | 13.12* ± 0.12 |
| *m*-Coumaric acid | 42.53 ± 0.16 | nd | 9.64* ± 0.15 | 3.58* ± 0.03 | nd | 1.59* ± 0.07 |
| Salicylic acid | 43.71 ± 0.10 | 48.43 ± 0.10 | 13.04 ± 0.04 | 3.69* ± 0.02 | 3.73* ± 0.12 | 9.66* ± 0.13 |
| *trans*-Cinnamic acid | 110.61 ± 0.06 | 66.39 ± 0.06 | 40.53 ± 0.07 | nd | nd | 1.36* ± 0.04 |
| Total | 1610.13 | 1238.69 | 148.78 | 75.65 | 26.20 | 50.25 |

CP, regular stored Chenpi; 3CP, long-term stored Chenpi; HCP9m, regular stored Chenpi with heat treatment for 90 min; 3HCP9m, long-term stored Chenpi with heat treatment for 90 min; HCP3h, regular stored Chenpi with heat treatment for 3 h; 3HCP3h, long-term stored Chenpi with heat treatment for 3 h.
Fraction A, free phenolic acid fraction; fraction B, soluble phenolic acid ester fraction; fraction C, Insoluble-bound phenolic acid fraction. nd, not detected.
* Significantly different from regular (1 year) stored Chenpi ($p < 0.05$).

other phenolic acids in fraction B decreased with the longer heat treatment.

In fraction C, the amount of insoluble bound phenolic acids decreased with storage time. These different results might have been caused by the conversion of insoluble-bound phenolic compounds into free or soluble ester forms following degradation of esters to free forms during heat treatment and prolonged storage.

### 3.4. Discrimination of Chenpi with different storage periods using targeted metabolomic analysis

The aim of metabolomics is to classify samples and understand the basic principles that contribute to the differences among them. Metabolomics has been applied to the classification of plants with principal component analysis often used as its main statistical approach [27]. PCA, with its general objectives of data reduction and interpretation [28], combines various experimental data and explains the variance–covariance structure of a set of variables through linear combinations of the variables.

In this study, a targeted metabolomics approach was employed to discriminate Chenpi with different storage periods for the first time. Eleven phenolic acids were the target compounds used as variables. Initially, a PCA correlation matrix was calculated. The matrix values were the degrees of association among the 11 variables derived from the peak areas of 11 phenolic acids in the HPLC-DAD analysis. Next, eigenvectors were extracted from the matrix to obtain the principal components (PCs), i.e., components that contain the majority of the available information in the dataset and can provide linear summaries of experimental outcomes [29]. The extracted eigenvectors are presented in Table 5. Based on a

**Table 5**
Eigenvectors of principal components PC1 and PC2 against 11 phenolic acid variables.

| Variables | PC1 | PC2 |
|---|---|---|
| Gallic acid | 0.135385 | 0.494505 |
| Protocatechuic acid | 0.142775 | 0.49319 |
| β-Hydroxybenzoic acid | 0.053577 | 0.493524 |
| Vanillic acid | 0.308212 | −0.306368 |
| Caffeic acid | −0.354458 | −0.033164 |
| Syringic acid | 0.376439 | −0.115984 |
| *p*-Coumaric acid | −0.123351 | −0.371387 |
| Ferulic acid | −0.372949 | 0.029405 |
| *m*-Coumaric acid | 0.386509 | −0.089511 |
| Salicylic acid | 0.381098 | 0.016533 |
| *trans*-Cinnamic acid | −0.383591 | 0.114761 |

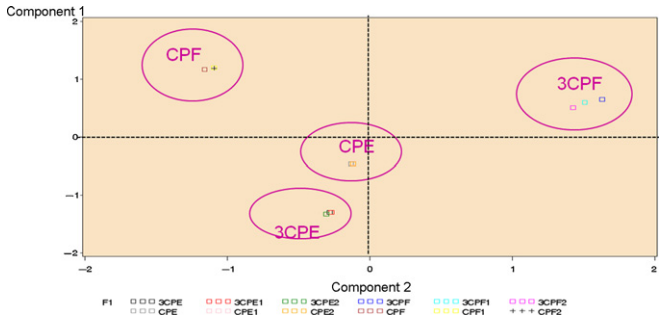


**Fig. 4.** Two-dimensional plot of regular (1-year) stored Chenpi and long-term (3-year) stored Chenpi. CPF and 3CPF are the free phenolic acid fractions (fraction A) of the regular and long-term stored Chenpi, respectively. CPE and 3CPE are the soluble ester phenolic acid fractions (fraction B) of the regular and long-term stored Chenpi, respectively.

scree plot of cumulative eigenvalues, we chose 2 PCs that explained 87.10% of the variations between the regular and long-term stored Chenpi. The two PCs (PC1 and PC2) explained 55.54% and 31.36% of the sample diversity, respectively. In PC1, phenolic acids with double bonds, such as caffeic, ferulic, and *trans*-cinnamic acids, produced negative eigenvectors. On the other hand, in PC2, variables containing a *p*-hydroxybenzoic acid structure, including gallic, protocatechuic, and β-hydroxybenzoic acids, exhibited positive eigenvectors while those with a double bond or a methoxy group, such as vanillic, caffeic, syringic, and *p*-coumaric acids, showed negative vectors.

Lastly, PC scores were used to distinguish Chenpi samples of different storage periods. The calculated PC scores showed that fraction A from long-term stored Chenpi had positive PC1 and PC2 values while fraction A from regular stored Chenpi had positive PC1 and negative PC2 values. On the other hand, fractions B from regular and long-term stored Chenpi displayed negative values for both PC1 and PC2. In particular, phenolic compounds with double bonds possessed negative eigenvectors in PC1. In PC2, variables containing *p*-hydroxybenzoic acid structure exhibited positive vectors, but those with double bond or methoxy group showed negative vectors. When the values for the two PCs were plotted in two dimensions, the Chenpi samples divided into 4 distinct groups: free phenolic acid fractions of regular and long-term stored Chenpi, and phenolic ester fractions of regular and long-term stored Chenpi (Fig. 4).

## 4. Conclusion

Total phenolic content and anti-oxidizing activity measured as DPPH radical-scavenging activity were compared in three phenolic acid fractions from Chenpi samples prepared under different conditions. The TPC and DPPH bioactivity generally increased with extended storage and longer heat treatment in the free phenolic acid fraction, a fraction that is therapeutically important. In particular, the free acid fraction of the long-term stored Chenpi showed levels of TPC and anti-oxidizing activity that were comparable to those of heat-treated regular stored Chenpi. These observations were consistent with the results obtained from HPLC-DAD analysis of 11 phenolic acids from Chenpi samples. Our findings indicate the potential usefulness of long-term stored Chenpi as a health-promoting natural product, a product that would possess almost equivalent bioactivity and active compounds as in heat-treated regular storage period Chenpi. Moreover, such extended storage period requires no additional process to produce this enhancement of activity level.

To differentiate Chenpi samples of different storage periods, targeted metabolomic analysis was performed on 11 phenolic compounds in free acid and ester fractions of the regular and long-term stored Chenpi samples. Peak areas of the compounds derived from HPLC-DAD analysis were measured and subjected to PCA, resulting in the identification of two principal components. Those components described 87.1% of the variation between the regular and long-term stored Chenpi. A two-dimension plot of the two principal components clearly divided the Chenpi samples into four groups based on storage periods and extracted fractions.

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