*Food Sci. Biotechnol. Vol. 18, No. 3, pp. 793 ~ 798 (2009)*

**RESEARCH NOTE**

Food Science and Biotechnology



# Optimization of Extraction Conditions and Comparison of Rosmarinic and Caffeic Acids from Leaves of *Perilla frutescens* Varieties

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**Abstract** The objectives of this present study were to compare the contents and determine optimum extraction conditions for the rosmarinic acid (RA) and caffeic acid (CA) from leaves of Korean *Perilla frutescens* varieties. RA and CA from leaves of cv. Bora, a breeding line of *P. frutescens* were isolated and elucidated using various spectroscopic data. On the basis of 2 phenolic acids, optimum extraction conditions were obtained by employing 50% EtOH for 60 min at 25°C. We reported for the first time on the contents of RA and CA from leaves of 32 Korean varieties. Among them, leaves of *P. frutescens* Brit. var. *acuta* Kudo I exhibited the highest RA content (8.53±0.57 mg/g) and CA content (2.33±0.11 mg/g) showed the highest in the *P. frutescens* Brit. var. *viridis* Makino. Interestingly, average RA content (2.66±0.17 mg/g) showed a markedly higher than that of CA (1.98±0.16 mg/g) in Korean varieties. These results suggest that concentrations of the RA and CA in *P. frutescens* leaves could be a key factor in the selection process of a high quality species.

**Keywords:** *Perilla frutescens* leaf, rosmarinic acid, caffeic acid, high performance liquid chromatography (HPLC)

## Introduction

Phenolic acids, known as a kind of multipurpose bioactive agent, frequently occur in herbal plants, which are important constituents of the human diet (1-5). Recently, these compounds have unprecedented attention due to various diseases such as cancer, heart disease as well as antioxidant activity (6-9). Moreover, the research regarding their effects on signal transduction and gene expression have progressed (10) and focused on the analytical methods for their contents in various foods and natural plants (11-13).

*Perilla frutescens* belonging to the Labiatae family is widely cultivated crop plant as well as edible plant and medicinal herb in East Asia (14,15) such as Korea, China, and Japan. Especially, the leaves of this species are important in Korean cooking, as one of the popular garnish and food colorant. Perilla leaves are commonly conducted on treat various diseases including antioxidant, antitussive, antibiotic, antipyretic, and antiallergic, respectively (16-19). It is well established that flavonoids and phenolic acids including rosmarinic acid (RA), caffeic acid (CA), ferulic acid, catechin, apigenin, and luteolin found in the leaves and seeds of *P. frutescens* (20,21). Especially, caffeonyl ester of RA and CA appears to be a substance of considerable interest, which has led to a broad range of applications, from food preservatives to cosmetics (22). Thus, we exhibited RA and CA among various components in perilla leaves and determined the contents in Korean perilla varieties. RA was reported to anti-inflammatory effects such as inhibitory effects on a complement-dependent inflammatory process, 5-lipoxygenase, allergic reaction, and histamine releases from mast cells (23-26). Moreover, CA could act as antioxidants (27) as well as α-tocopherol protectant in low-density lipoprotein (LDL) (28) and demonstrated good substrates of polyphenol oxidases (29,30). Although the biological activities of perilla leaves as well as their superior safety were well documented (17,31-33), few studies have been published on the determination of RA and CA. Also, no study has evaluated the comparison of these compounds and reported on the effect of extractions in various perilla varieties. In this study, RA and CA from perilla leaves were isolated and identified their structures through spectroscopic methods. Furthermore, RA and CA contents as well as optimal extraction conditions were investigated using high performance liquid chromatography (HPLC). We also reported that the variation of 2 compound contents from 32 Korean perilla varieties.

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Received May 5, 2008; Revised December 29, 2008;
Accepted January 22, 2009

## Materials and Methods

**Plant material** To evaluate optimal extraction conditions and isolation of rosmarinic acid (RA), caffeic acid (CA) from perilla leaves, cv. Bora one of the Korean cultivars, were collected on August 15, 2005, in the experimental field of the Yeongnam Agricultural Research Institute (YARI), National Institute of Crop Science, Rural Development Administration (RDA), Miryang, Korea. Also, the perilla

leaves of 32 different varieties were harvested on August 15, 2005, in the same experimental field. All the plants were grown in the same location and conditions to avoid variations of contents due to environmental factors. All samples were freeze-dried and then stored at −80°C before analysis.

**Standard materials and reagents** RA and CA used for the standard materials were isolated from the methanol extracts the dried perilla leaves (cv. Bora) and elucidated by the nuclear magnetic resonance (NMR) spectroscopic data analysis. Acetonitrile and water (HPLC grade, 99.9%, v/v) were purchased from Merck (Darmstadt, Germany). Also, all the reagent grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Instruments** $^{1}$H- and $^{13}$C-NMR along with 2D-NMR data were obtained on a Bruker AM 500 ($^{1}$H-NMR at 500 MHz, $^{13}$C-NMR at 125 MHz) spectrometer (Bruker, Karlsruhe, Germany) in $CD_3OD$ (Sigma-Aldrich). HPLC was performed using an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) quaternary pump, Agilent 1100 series photodiode array detector (PDA), and reversed-phase column (ODS-120T; 250×4.6 mm, 5 μm, Tosoh Corp., Tokyo, Japan). The identify and purity of RA and CA in the samples were confirmed by matching the retention times in liquid chromatography (LC) in combination with the mass spectral analyses of the standards by electron spray impact mass (ESI/MS) using a Bruker, DE/ESQUIRE 4000. The LC-ESI/MS analysis was performed in negative ionization mode and the ion trap was connected to an Agilent 1100 series HPLC instrument. HPLC 2D ChemStation Software with a ChemStation Spectral SW module was used for the process guidance.

**Extraction and isolation** Extraction and isolation methods for RA and CA employed the method of Lee *et al.* (16).

**Extraction conditions** To evaluate optimal extraction conditions of RA and CA, *P. frutescens* cv. Bora and Namchen were selected. The extractions were performed at constant temperature by means of a temperature controller. Three different solvent systems (EtOH, MeCN, and MeOH) with several water percentages (between 10 and 100%) and extraction times (30, 60, 90, and 120 min) were evaluated at 25 and 50°C. After extraction, the extracts were centrifuged for 10 min and filtered though 0.45-μm syringe filter (Millipore, MSI, Westboro, MA, USA) before chromatographic analysis. All experiments were performed in triplate.

**HPLC and LC-ESI/MS analysis conditions** HPLC separation and quantification of RA and CA were performed on a Agilent 1100 series instrument equipped with PDA with the wavelength set at 330 nm, using a reversed-phase column (Tosoh ODS-120T, 250×4.6 mm, 5 μm). The column temperature was set to 30°C and the flow rate was 1.0 mL/min. The gradient elution systems consisted of 0.85%(v/v) $H_3PO_4$ in water (solvent A) and 100% acetonitrile (solvent B). Elution was started with a linear gradient of B from 10 to 30% by 30 min, then to 40% by 35 min, and finished isocratically with 90% of B for 10 min. The sample injection volume was 20 mL. RA and CA were identified using their retention times as well as LC-ESI/MS. Conditions of LC-ESI/MS detection were as follows: target mass (negative ionization mode), 358.9 (RA) and 179.0 (CA); compound stability, 100%; drying gas ($N_2$ from generator) flow rate, 0.2 mL/min; gas temperature, 350°C; nebulizer pressure, 30 psi; and collision gas He pressure, $6\times10^{-6}$ mbar. Data were acquired in the electronspray impact mode at 1.10 kV with a scan range of 100-400 *m/z*.

**Preparation of calibration curve** Approximately 2 mg each of isolated RA and CA were weighted and dissolved in a 10-mL volumetric flask containing 50% EtOH to obtain stock solution. Each stock solution was diluted with 50% EtOH to obtain the concentration sequence. The linear range and the equations of linear regression were obtained through a sequence of 0.75, 1.5, 3, 6, 8, 10, 20, 40, 60, 80, and 100 μg/mL. Standard was analyzed by HPLC using the method described above to produce a standard curve ranging from 0.75 to 100 μg/mL for each compound. Mean areas ($n$=3) generated from the standard solutions were plotted the concentrations to establish calibration equation.

**Sample preparation of perilla leaves** The leaves of 30 perilla varieties (each 0.1 g) were extracted with 20 mL of 50% EtOH at 25°C for 60 min, and then filtered with Whatman No. 2 filter paper. Prior to analysis, all samples were filtered through a 0.45-μm membrane filter (Millipore).

**Statistical analysis** All determinations were based on at least 3 independent replicate samples for each perilla leaves. Results were analyzed by using Sigma Plot 2001.

## Results and Discussion

**Identification of isolated RA and CA** The structures of isolated compounds (Fig. 1A), RA and CA were confirmed by spectroscopic analysis and comparison with values previously reported (16).

**Profile of RA and CA** The HPLC chromatogram of RA and CA was shown in Fig. 2A. The retention times were as follows: RA ($t_R$=17.2 min) and CA ($t_R$=7.6 min). To quantitatively analyze RA and CA, calibration curves were constructed in the range 0.75-100 μg/mL. The concentrations of RA and CA were determined on the basis of the peak areas in the chromatogram as follows: RA, $y=22.427x-65.113$, $R^2=0.998$; CA, $y=43.519x-371.880$, $R^2=0.999$. When the sample solution was analyzed in the evaluated method, the peaks were identified by comparison of the retention times with those corresponding to authentic sample purified from perilla leaves.

**Optimization of extraction conditions for RA and CA** Effects of the extraction time, temperature, and solvent were investigated in order to obtain the maximal yield of RA and CA. The perilla leaves (cv. Bora and Namchen) were extracted using the initial protocol (0.1 g, 20 mL), different solvent systems (EtOH, MeOH, and MeCN, each

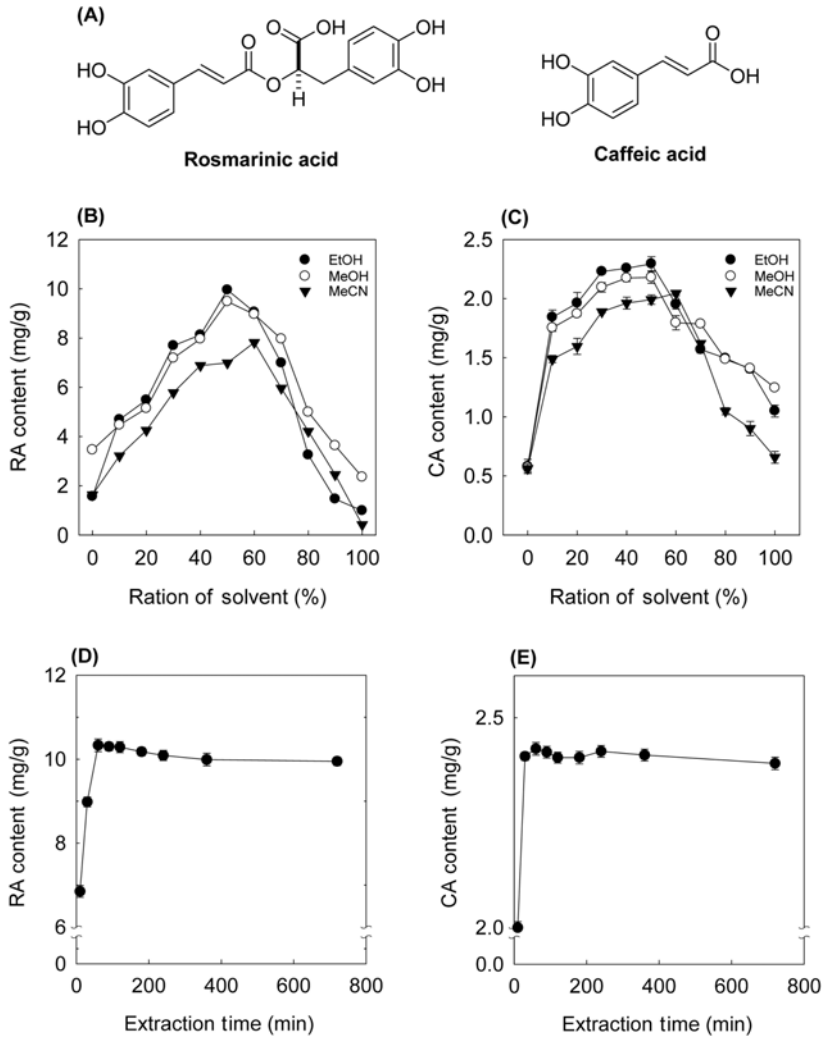


**Fig. 1. Chemical structures and comparison of rosmarinic acid (RA) and caffeic acid (CA) contents using different extraction solvent and extraction time from perilla leaves (cv. Bora).** (A) Chemical structures of RA and CA; (B, C) contents of RA and CA using different extraction solvent at 25°C for 12 hr from perilla leaves (cv. Bora); (D, E) contents of RA and CA using 50% EtOH at 25°C for different extraction time from perilla leaves (cv. Bora).

0-100%), different extraction time (10, 30, 60, 90, 120, 180, 240, 360, and 720 min), and temperature (25 and 50°C), respectively. At first, we tested the cv. Bora using solvent polarity for 12 hr at 25°C. Figure 1B and 1C showed a plot of the extraction efficiency of RA and CA contents under the different extraction solvent systems. Note that as the concentrations of solvent (EtOH, MeOH, and MeCN) increases up to the 50%, the extraction rates of the RA and CA contents significantly increased, and then dramatically decreased at higher concentrations (>50%) of solvent.

The 50% EtOH, 50% MeOH, and 60% MeCN were the solvents that produced higher contents of RA and CA, with only small differences among them. Especially, the best extraction solvents for RA (9.95±0.57 mg/g) and CA (2.30±0.13 mg/g) contents were 50% EtOH from the cv. Bora, and same results were obtained for the cv. Namchen. In order to evaluate the effects of the temperature on the extraction efficiency of the RA and CA contents, the cv. Bora and Namchen extractions were performed at 25 and 50°C. Interestingly, the extractions of phenolic acids have a great impact on the temperature (31), as the results of this study, the effect of the temperature was not detected to improve the extraction of RA and CA (data not shown). Therefore, the extraction temperature of these compounds was carried out at 25°C. In order to investigate the influences of the extraction time, we studied the extraction depending on 50% EtOH for 10, 30, 60, 90, 120, 180, 240, 360, and 720 min by increasing extraction time at 25°C. For the 50% EtOH the extraction yield increased up to 60 min (cv. Bora: RA=10.33±0.14 and CA=2.43±0.11 mg/g, cv. Namchen: RA=4.81±0.22 and CA=2.40±0.20 mg/g) and then became fairly constant (Fig. 1D and 1E), so this time was chosen as the optimal extraction time. As a result, the best extract conditions were obtained by employing 50% EtOH for 60 min at 25°C.

**Identification of RA and CA from varieties extracts using HPLC and LC-ESI/MS** Identification of individual phenolic acids were performed by comparison of HPLC retention time ($t_R$), photodiode array UV-Vis spectroscopic, and LC-ESI/MS spectrometric data,

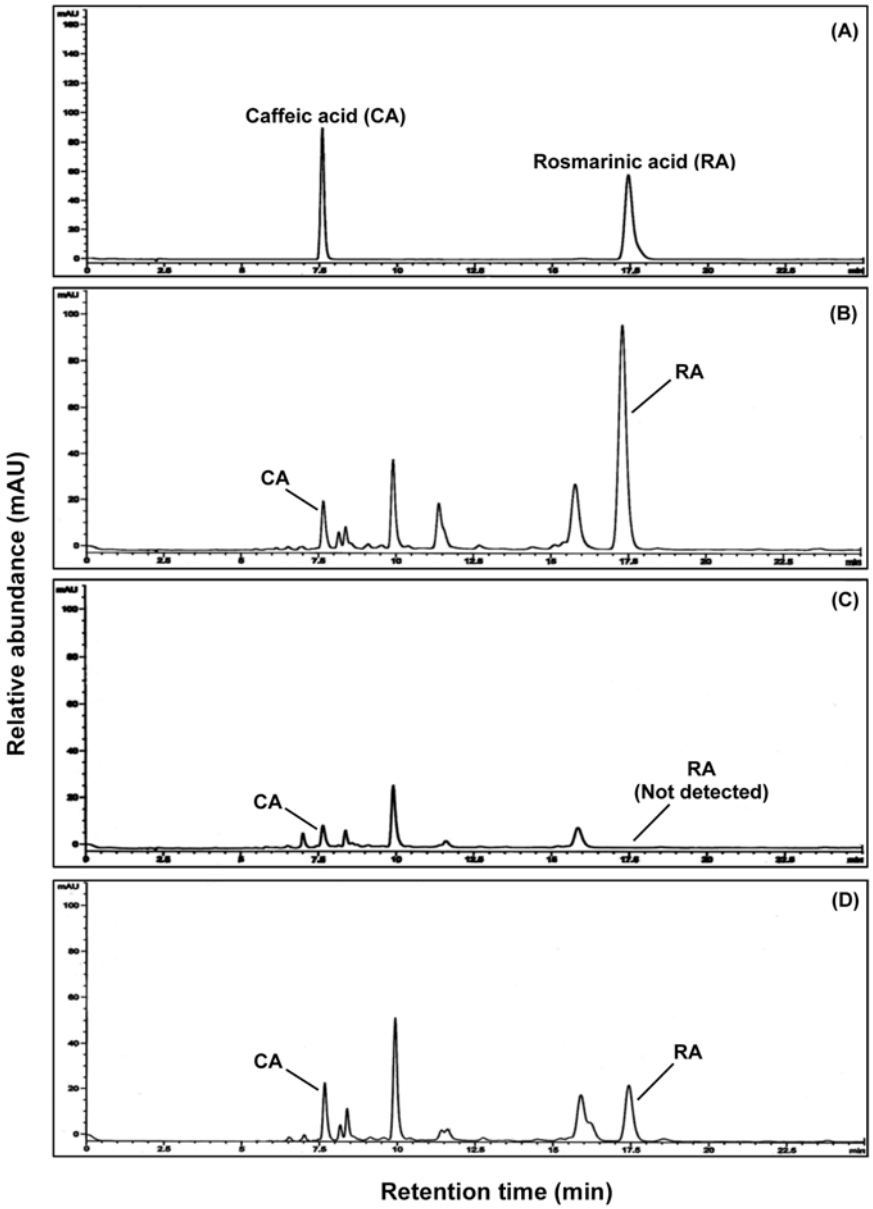


**Fig. 2. Comparison of HPLC chromatograms concern to RA and CA from standards and the leaves of perilla variety 1, 19, and 27.** (A) Standards, (B) perilla variety 1, (C) perilla variety 19, (D) perilla variety 27.

respectively. The HPLC analysis showed that a major peaks and minor peaks were present in the perilla leaves extracts. Among them, the most major peak ($t_R$=17.2 min) was identified as RA and the peak ($t_R$=7.6 min) was identified as CA by comparison of the retention times with those of the isolated RA and CA. This HPLC method allowed analysis of RA and CA contents in perilla leaves extracts within 20 min.

To further characterize individual components of the extracts, LC-ESI/MS spectra were obtained. The HPLC analytical conditions for the LC-ESI/MS were the same as those used for the HPLC-PDA analysis, except for that the of the eluant. The negative ion mode was selected for the quantitative analysis of the RA and CA due to the simplicity and stability of mass spectra as well as the lower background noise (32). Therefore, ESI/MS spectrometry analysis of 2 phenolic acids produced intact molecular ions $[M-H]^-$ at *m/z* 358.9 and 179.0, corresponding to RA and CA (data not shown).

**Comparison of RA and CA contents in Korean varieties** RA and CA contents in 32 perilla leaves varieties (6 subfamilies) grown in Miryang was listed in Table 1 and their contents showed significant differences. The highest RA content was 8.53±0.57 mg/g in perilla variety 1 (*P. frutescens* Brit. var. *acuta* Kudo I), whereas the lowest was not detected (variety 3, 9, 14, 19, and 23-25). Also, as shown in Table 1, the highest CA content was 2.33±0.11 mg/g in perilla variety 30 (*P. frutescens* Brit. var. *viridis* Makino), whereas the lowest was 1.65±0.11 mg/g in perilla variety 19. Significant differences in RA contents (0.00-8.53 mg/g) were observed, but CA contents (1.65-2.33 mg/g) were much lower than those of RA in all varieties. In particular, high concentrations of RA were

**Table 1. Mean values and standard deviation of rosmarinic acid (RA) and caffeic acid (CA) contents from perilla leaves**

| Number | Perilla taxa | RA (mg/g)[1)] | CA (mg/g) |
|---|---|---|---|
| 1 | *P. frutescens* Brit. var. *acuta* Kudo I | 8.53±0.57 | 2.12±0.22 |
| 2 | *P .frutescens* Brit. var. *acuta* Kudo II | 2.06±0.17 | 2.04±0.11 |
| 3 | *P. frutescens* Brit. var. *acuta* Kudo III | ND | 1.74±0.08 |
| 4 | *P. frutescens* Brit. var. *acuta* Kudo IV | 1.20±0.07 | 1.89±0.15 |
| 5 | *P. frutescens* Brit. var. *acuta* Kudo V | 4.59±0.16 | 1.94±0.22 |
| 6 | *P. frutescens* Brit. var. *acuta* Kudo VI | 4.39±0.35 | 2.09±0.21 |
| 7 | *P. frutescens* Brit. var. *crispa* Hand-Mazz | 3.96±0.31 | 1.90±0.14 |
| 8 | *P. frutescens* Brit. var. *crispa* Hand-Mazz. *f.atropurpurea* I | 0.41±0.08 | 1.84±0.34 |
| 9 | *P. frutescens* Brit. var. *crispa* Hand-Mazz. *f.atropurpurea* II | 0.34±0.04 | 1.69±0.08 |
| 10 | *P. frutescens* Brit. var. *crispa* Hand-Mazz. *f.atropurpurea* III | 0.35±0.15 | 1.70±0.18 |
| 11 | *P. frutescens* Brit. var. *crispa* Hand-Mazz. *f.atropurpurea* IV | 4.20±0.23 | 1.98±0.19 |
| 12 | *P. frutescnes* Brit. var. *japonica* Hara. for. *discolor* Makino I | 3.39±0.31 | 2.16±0.24 |
| 13 | *P. frutescnes* Brit. var. *japonica* Hara. for. *discolor* Makino II | 0.94±0.05 | 2.02±0.07 |
| 14 | *P. frutescnes* Brit. var. *japonica* Hara. for. *discolor* Makino III | ND | 1.69±0.12 |
| 15 | *P. frutescnes* Brit. var. *japonica* Hara. for. *discolor* Makino IV | 1.48±0.30 | 2.13±0.26 |
| 16 | *P. frutescens* Brit. var. *japonica* Hara I | 1.16±0.10 | 2.06±0.08 |
| 17 | *P. frutescens* Brit. var. *japonica* Hara II | 0.38±0.07 | 1.79±0.17 |
| 18 | *P. frutescens* Brit. var. *japonica* Hara III | 7.21±0.51 | 2.01±0.40 |
| 19 | *P. frutescens* Brit. var. *japonica* Hara IV | ND | 1.65±0.11 |
| 20 | *P. frutescens* Brit. var. *japonica* Hara V | 4.29±0.32 | 2.10±0.09 |
| 21 | *P. frutescens* Brit. var. *japonica* Hara VI | 3.35±0.13 | 2.20±0.19 |
| 22 | *P. frutescens* Brit. var. *japonica* Hara VII | 2.78±0.07 | 1.98±0.06 |
| 23 | *P. frutescens* Brit. var. *japonica* Hara VIII | 0.48±0.09 | 1.70±0.14 |
| 24 | *P. frutescens* Brit. var. *japonica* Hara IX | ND | 1.66±0.18 |
| 25 | *P. frutescens* Brit. var. *japonica* Hara X | 0.40±0.09 | 1.88±0.11 |
| 26 | *P. frutescens* Brit. var. *japonica* Hara XI | 2.18±0.17 | 1.92±0.21 |
| 27 | *P. frutescens* Brit. var. *japonica* Hara XII | 2.99±0.24 | 2.26±0.18 |
| 28 | *P. frutescens* Brit. var. *japonica* Hara XIII | 4.88±0.22 | 2.08±0.16 |
| 29 | *P. frutescens* Brit. var. *japonica* Hara XIV | 1.84±0.11 | 2.10±0.09 |
| 30 | *P. frutescens* Brit. var. *viridis* Makino | 2.20±0.19 | 2.33±0.11 |
| 31 | Bora | 10.33±0.14 | 2.43±0.11 |
| 32 | Namchen | 4.81±0.22 | 2.40±0.20 |

[1)]Yields of RA and CA are reported dry weight; average value±SD in triplicate; ND, not detected.

found in *P. frutescens* Brit. var. *acuta* Kudo subfamily (variety 1, 5, and 6), whereas low levels were recorded for *P. frutescens* Brit. var. *japonica* Hara subfamily with the exception of varieties 18, 20, and 28 (RA content: 7.21± 0.51, 4.29±0.32, and 4.88±0.22 mg/g, respectively). On the other hand, CA concentrations ranged between 1.65± 0.11 and 2.33±0.11 mg/g in 6 subfamilies and showed little differences among subfamilies. In particular, the HPLC chromatograms of 3 perilla leaves variety (1, 19, and 27) had significant differences between RA and CA concentrations were shown in Fig. 2B-2D.

In the present work, 2 phenolic acids including RA and CA were isolated from the leaves of *P. frutescens* cv. Bora. On the basis of isolated phenolic acids, the best condition for the RA and CA of perilla leaves was to digest the dry sample in 50% EtOH for 60 min at 25°C. Moreover, we were screened for RA and CA contents from leaves of 32 Korean *P. frutescens*. Among 32 varieties, perilla variety 1 had the highest RA content (8.53±0.57 mg/g) and perilla variety 30 showed the highest CA (2.33 ±0.11 mg/g). It was observed that RA content showed a markedly higher than that of CA from Korean varieties. These methods for extraction and analyses of RA and CA may contribute to rational approach for a high quality species.

## Acknowledgments

This study was supported by a Post Doctoral Course Program of Yeongnam Agricultural Research Institute, National Institute of Crop Science, and Rural Development Administration, Republic of Korea.

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