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Application of novel HPLC method to the analysis of regional and seasonal variation of the active compounds in *Paeonia lactiflora*

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Abstract

Alcohol extracts of the dried root of paeony (*Paeonia lactiflora*), an Oriental medicinal herb, have been reported to contain cardiovascular protective monoterpene glycosides such as paeoniflorin and albiflorin. To analyze these compounds, a novel HPLC method with good precision was developed by a gradient elution making use of Inertsil ODS-3 column and acetonitrile–H_2O as the eluent. At the optimum analytical condition, albiflorin and paeoniflorin were resolved well with the retention times of 14.30 and 17.74 min, respectively. By applying this method, the contents of paeoniflorin and albiflorin in 12 regional samples of paeony root were compared. The contents of these compounds in the seasonal samples from different parts of 4 year-grown paeony were also analyzed, with the highest values in the root sample of June, which was expected to be applicable to a cultivation management for producing paeony root with good functional activity.
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Keywords: Application; Gradient HPLC; Variation; Active compounds; *Paeonia lactiflora*

1. Introduction

Paeonia lactiflora is an Oriental medicinal herb containing monoterpene glycoside compounds such as paeoniflorin, albiflorin and benzoyloxypaeoniflorin, and its dried root as a predominantly used form is called as Paeoniae Radix (Goto et al., 1996; Ikeda et al., 1996). Solvent extracts of Paeoniae Radix have been used for cleansing heat, cooling blood and invigorating blood circulation and so forth in Chinese herb medicine (Wu & Sheu, 1996). Recently, many studies have been done to assign specific functional activities such as inhibitory effect on the change of extracellular calcium concentration and vasodilator effect for the purified compounds and isolated fractions from *P. lactiflora* (Goto et al., 1996; Sugaya et al., 1991). It has been reported that the contents of the active components in *P. lactiflora* are quite different according to the environmental factors like habitat and harvest time (Shimizu, Hashimoto, Ishikawa, Kurosaki, & Morita, 1979). Hence, it has been strongly required that a precise analytical method be developed for cultivation management for *P. lactiflora*, evaluation of the functional activities for extracts and purified fractions from it, and development of functional foods containing active components.

Until now, the active components in *P. lactiflora* have been routinely determined by HPLC, TLC and capillary electrophoresis as follows. Making use of

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Lichrospher RP-18 column and water–acetonitrile–methanol–acetic acid (80:15:5:1, v/v) as an eluent Wen, Huang, and Liu (1992) have reported on an isocratic separation of paeoniflorin in herbal extracts such as Huang Chi Chien Chung Tong with methyl paraben as the internal standard. Some HPLC methods using isocratic elution protocols have also been developed with reversed-phase Zorbax CN, μBondapak C_{18} and Symmetry C_{18} column to determine the contents of paeoniflorin, the main quality index of *P. lactiflora* as the major component, and albiflorin in many herbal extracts, callus cultures, pharmaceutical preparations and purified samples (Akada, Kawano, & Tanase, 1979, 1980; Asakawa, Hattori, Ueyama, Shinoda, & Miyake, 1979; Chen, Lu, & Jiang, 2004; Yamamoto, Machida, & Tomimori, 1982). Ikeda et al. (1996) analyzed the monoterpene constituents in Paeoniae Radix from various collection areas with HPLC employing Wakosil II 5 C_{18}HG column at a linear gradient elution of 0.01 M potassium phosphate buffer (pH 2.6): acetonitrile and found paeoniflorin and oxypaeoniflorin as the main components amounting to the contents of 0.12–9.61% and 0.06–10.80%, respectively. Zhang, Shen, and Cheng (2004) determined the major constituents in a traditional Chinese medicinal preparation by using Hypercil C_{18} column at a linear gradient elution of acetic acid–water (0.1:100, v/v) and acetic acid–acetonitrile (0.1:100, v/v). They could separate paeoniflorin and albiflorin around the retention time of 45 min and reported the recovery of paeoniflorin at 94.3%. Okamoto and Noguchi (1986) have reported on a TLC scanning analysis by using a 3-cyanopropyltrichlorosilane-coated TLC plate and a mobile phase of methanol–water (10:90, v/v), with the detection range and limit of detection (LOD) of 0.5–10 and 0.5 μg for paeoniflorin. Wu and Sheu (1996) developed a method combining capillary zone electrophoresis and micellar electrokinetic chromatography to separate eight paeony components and reported on the LOD values for them in the range of 2.6–23.7 μg/ml.

The correct measurement for the active compounds like paeoniflorin and albiflorin in different forms of *P. lactiflora* like Paeoniae Radix is very important as the data from it can be used for grading the preparations from *P. lactiflora*, dose optimization for them to develop functional foods and cultivation management for producing *P. lactiflora* with good functional activity. The analytical methods used for this goal should be precise and repeatable in sample analysis and be done within a reasonable analytical time. As a consequence, this study was conducted to develop a novel HPLC method which shows a good resolution in sample analysis with the resultant good precision and repeatability, followed by an application of the above method to the analysis of regional and seasonal variation of the active compounds in *P. lactiflora*.

2. Materials and methods

2.1. Herb samples

The regional samples of Paeoniae Radix marketed in Korea and the seasonal samples from May to October which were prepared from different parts of 4 year-grown *P. lactiflora* at Uisung, Kyungsangbuk-do, Korea, were crushed with a dry mixer (Samsung Electronic Co., Korea) after drying in a convection oven at 40 °C, and the resulting powder was used for HPLC analysis.

2.2. Reagents

Standard samples of paeoniflorin (mw 480.47) and albiflorin (mw 480.46), which have the purity greater than 99%, were purchased from Wako Pure Chemical (Japan). HPLC-grade acetonitrile, methanol and water were obtained from Burdick and Jackson (USA). Ethanol, methanol and other solvents were of guaranteed reagent grade from various suppliers.

2.3. HPLC apparatus

A gradient HPLC system consisting of intelligent pumps (model PV-980, Jasco, Japan), a variable wavelength ultraviolet detector (model UV-975, Jasco) having a wavelength setting of 230 nm, an intelligent column thermostat (model CO-966, Jasco) maintained at 35 °C, and an autosampler (model AS-950-10, Jasco) was used throughout this study. The column used was Inertsil ODS-3 C_{18} (250 × 4.6 mm i.d., GL Sciences, Japan) having a particle size of 5 μm. A guard column (GL Sciences) containing the same C_{18} packing as above was positioned in front of the analytical column to protect it from contamination. Data acquisition was performed with Borwin chromatography software (revision 1.2150, Jasco).

2.4. Sample preparation for HPLC analysis

Two grams of a powdered sample were extracted in an Erlenmeyer flask with 70 ml of 70% ethanol in static mode with a reciprocal water bath incubator overnight at 45 °C. During extraction, the contents of the flask were shaken gently for 1 min every 30 min during the initial 2 h. The contents in the flask were filtered through Whatman #1 filter paper (Whatman Co., UK). The residue in the flask was washed with small portions of 70% ethanol and was filtered again. The final volume of the combined filtrate was adjusted to 100 ml. It was transferred into a round-bottomed flask and was concentrated under reduced pressure with a rotary evaporator (Büchi, Switzerland). The concentrate was then dissolved with HPLC-grade methanol to the final volume

of 5 ml. One milliliter of the resulting solution was filtered through 0.45 μm PVDF syringe filter (Gelman Co., USA) and was used as the sample for HPLC analysis.

2.5. Chromatographic procedure

Gradient elution concerning acetonitrile–water system was carried out at the flow rate of 1.0 ml/min and the injection volume was 10 μl. Prior to analysis, the analytical column was equilibrated with the initial eluent composition for 1 h at room temperature and then for 30 min at the operating temperature of 35 °C. Two solvents used for gradient elution were acetonitrile and water containing 0.1% trifluoroacetic acid. Gradient elution was performed at different acetonitrile concentrations before and after gradient formation, and gradient time in min. After sample injection, for example, an isocratic elution of 15:75 (v/v) acetonitrile–water was done during the first 15 min. Then, the first gradient elution from 15:75 (v/v) acetonitrile–water to 40:60 (v/v) acetonitrile–water was carried out from 15 to 30 min. Elution was maintained at this eluent composition until the 50 min mark, followed by the second gradient elution back to 15:75 (v/v) acetonitrile–water until the 55 min mark. In this method, a reequilibration time amounting to 10 min was required before starting the next measurement.

3. Results and discussion

3.1. Optimization of gradient elution

In our previous experiments, the standard samples of paeoniflorin and albiflorin were easily separated by isocratic elution employing acetonitrile–water system having the acetonitrile ratios of 15–50%. In the case of isocratic elution, however, the resolution of paeoniflorin and albiflorin in a sample was not so satisfactory, possibly due to impurities present in the analytical sample, as previously shown in the elution profiles of the water and alcohol extracts prepared from Paeoniae Radix (Akada, Kawano, & Tanase, 1980; Asakawa et al., 1979). Hence, our effort was focused on the development of a new HPLC method with good resolution and precision which is applicable to the analysis of the active compounds in *P. lactiflora*. For this purpose, an optimization of gradient elution was carried out by changing acetonitrile concentrations before and after gradient formation, and gradient time.

Table 1 shows the comparison with retention times of the standard samples of paeoniflorin and albiflorin at different gradient types of acetonitrile–water having the acetonitrile concentrations of 15–20% and 40–50% before and after the first gradient elution. As shown in Table 1, the gradient times required for the first gradient elution significantly affected the retention times of the active compounds, resulting in the retention times of albiflorin between 4.29 and 15.02 min and those of paeoniflorin between 8.89 and 18.75 min. Based on the degree of peak resolution required for sample analysis, the initial and final acetonitrile concentration of 15% and 40%, and the first gradient elution from 15 to 30 min were selected as the optimized condition for the HPLC analysis of the ethanol extracts of the paeony preparations including Paeoniae Radix. At this optimized condition, the peak resolution of the active compounds in them was found very good compared with the cases of isocratic elution (Akada et al., 1979, 1980; Asakawa et al., 1979; Wen et al., 1992). The HPLC method established here also seemed to be an improvement in the gradient separation of the active compounds in *P. lactiflora* in terms of analysis time because paeoniflorin and albiflorin have been separated by a gradient HPLC around 43–46 min from a previous report (Zhang et al., 2004).

Table 1
Retention times of the standard samples of albiflorin and paeoniflorin at different gradient types of acetonitrile–water

Acetonitrile ratio/time (min)	Retention time (min)	
	Albiflorin	Paeoniflorin
15% (10) → 40% (15)	15.09	17.18
20% (10) → 40% (15)	7.96	8.89
20% (15) → 40% (20)	4.29	8.91
20% (15) → 50% (20)	7.83	8.89
15% (15) → 40% (20)	15.02	18.75
15% (15) → 45% (20)	14.88	18.50
15% (15) → 50% (20)	14.48	18.00
15% (15) → 40% (25)	13.65	17.02
15% (15) → 50% (25)	14.27	17.71
15% (15) → 40% (30)[a]	14.30	17.74

[a] Optimized condition.

The gradient HPLC chromatograms for the standard samples of paeoniflorin and albiflorin, and the ethanol extracts of two different Paeoniae Radix were compared at the above optimized gradient condition and at a fast eluting gradient condition [20% acetonitrile (15 min) → 50% acetonitrile (20 min)]. As shown in Fig. 1, the elution of paeoniflorin and albiflorin was done in the same retention times between the standard samples and the ethanol extracts in both gradient conditions. The peak resolution in the sample chromatogram at the optimized gradient condition was very good with no baseline drift caused by impurities in the sample and the retention times of albiflorin and paeoniflorin were found at 14.30 and 17.74 min, respectively. Compared with a previous method which used solid-phase extraction with a commercial cartridge and did an isocratic elution with

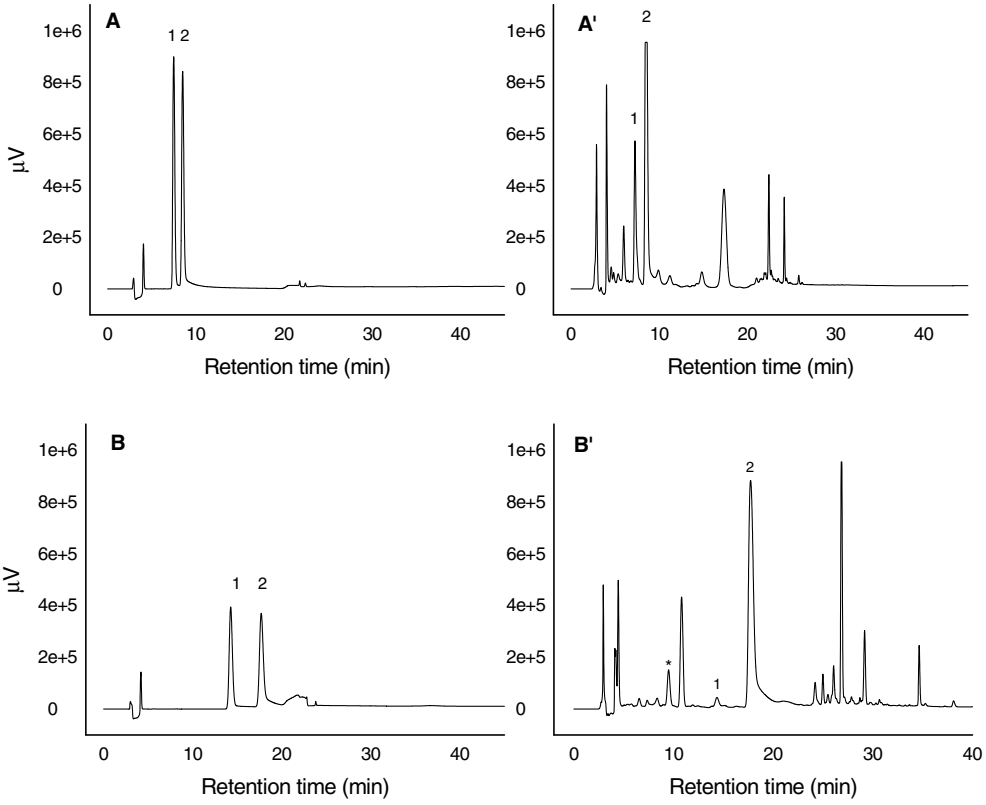

Fig. 1. Gradient HPLC chromatograms of the standard samples of albiflorin and paeoniforin (A and B) and the ethanol extracts of two different Paeoniae Radix (A′ and B′). A and A′ show the HPLC chromatograms at a fast eluting gradient condition [20% acetonitrile (15 min) → 50% acetonitrile (20 min)], whereas, B and B′ depict those at the optimized gradient condition [15% acetonitrile (15 min) → 40% acetonitrile (30 min)]. Arabic numerals 1 and 2, respectively, represent the peaks of albiflorin and paeoniflorin, whereas, the peak with asterisk in panel B′ indicates benzoyloxypaeoniflorin.

0.03% formic acid–acetonitrile (83:17, v/v), the gradient method of this study showed better resolution between paeoniflorin and albiflorin with a little delay in retention time by a few minutes (Sheng, Li, Zhang, & Guo, 2004). Also, the solid-phase extraction method which they used for the pretreatment of serum samples seemed to be inappropriate for the powdered samples of Paeoniae Radix of this study considering its procedure in their report. In addition to the peaks of paeoniflorin and albiflorin, two peaks around the retention times of 10 min and six peaks having the retention times of 24–35 min were also separated. It seemed that they are other monoterpene glycoside constituents found in the alcohol and water extract of Paeoniae Radix (Ikeda et al., 1996; Ikuta, Kamiya, Satake, & Saiki, 1995; Wu & Sheu, 1996). As an output of our purification and identification study making use of an ODS-2 C_{18} preparative column (250 × 20 mm i.d., GL Sciences) and ^{1}H NMR, the peak indicated by an asterisk in panel B′ eluting just before 10 min was identified as benzoyloxypaeoniflorin which has the highest platelet aggregation inhibitory activity among the isolated compounds from a butanol extract of Paeoniae Radix (data not shown).

3.2. Calibration curves

Fig. 2 shows the calibration curves for paeoniflorin and albiflorin prepared by an external standard method. As shown in Fig. 2, the calibration curves were linear and reproducible, as evidenced by the correlation coefficients (r) of 0.9962 and 0.9957 for paeoniflorin and albiflorin, respectively. Paeoniflorin was more responsive than albiflorin at a fixed concentration and the upper limit of linear responses was normally obtained at 2.0 mg/ml. The lower limit of linear responses, which normally represents sensitivity or LOD, was attained at 0.1 mg/ml in both compounds.

The coefficient of variability (standard deviation/mean × 100, %) values for paeoniflorin and albiflorin in Paeoniae Radix after 5 repetitive measurements were 4.5% and 3.7%, respectively, which shows a good repeatability of the present HPLC assay (Kim et al., 1993; Kim, Oh, Nam, Min, & Suh, 1983). The recoveries for paeoniflorin and albiflorin in this analysis were 97.7 ± 2.4 and 96.9 ± 0.1% (n = 2), respectively. The recoveries for the active compounds in Paeoniae Radix obtained from the present analysis were similar or better

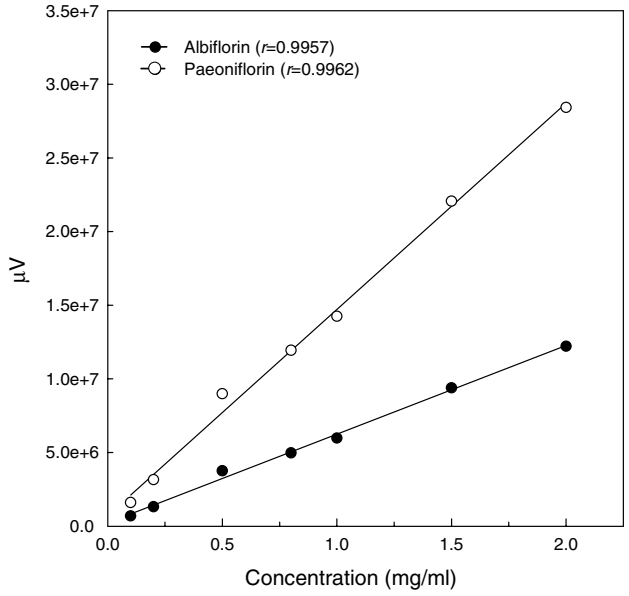

Fig. 2. Calibration curves for the active compounds in *P. lactiflora*.

than those of previous works (Chen et al., 2004; Zhang et al., 2004).

3.3. Regional variation of the active compounds in Paeoniae Radix

Based on the resolution and repeatability obtained, the gradient HPLC method of this study was assumed as a novel method which accurately measures the contents of paeoniflorin and albiflorin in *P. lactiflora* and functional food products containing these compounds. As shown in the HPLC chromatogram of Fig. 1, this method also seemed to have a strong potential able to be used for the analysis of other monoterpene glycosides reportedly known to be present in the solvent extracts of *P. lactiflora* such as lactiflorin, oxypaeoniflorin and benzoyloxypaeoniflorin (Ikeda et al., 1996; Ikuta et al., 1995).

The regional variation of the active compounds in 12 samples (10 from domestic areas and the remaining 2 from a foreign country) of Paeoniae Radix marketed in Korea is shown as an example (Table 2). The contents of paeoniflorin were in the range of 517.3–2780.2 mg/100 g dried sample, whereas those of albiflorin were from 43.9 to 724.8 mg/100 g dried sample. In general, paeoniflorin was present at higher levels in most domestic samples compared with two foreign samples. Whereas, there was no meaningful trend in the content of albiflorin between two groups of Paeoniae Radix. It has been reported that cultural characteristics, climate, soil condition, modification processes such as heat treatment during the preparation of Paeoniae Radix, impurities present at the commercial samples of Paeoniae Radix and extraction solvents affect the contents of the

Table 2
Regional variation of the active compounds in Paeoniae Radix

Cultivation region	Albiflorin (mg/100 g dried sample)	Paeoniflorin (mg/100 g dried sample)
1[a]	43.9	1689.9
2[a]	547.6	1970.3
3[a]	190.9	1676.6
4[a]	513.2	517.3
5[a]	45.6	1345.9
6[a]	724.8	2233.7
7[a]	432.0	1685.3
8[a]	296.6	2020.1
9[a]	521.3	2631.8
10[a]	581.7	2780.2
11[b]	543.4	1366.3
12[b]	423.3	941.0

[a] Domestic origin.
[b] Foreign origin.

active compounds in Paeoniae Radix, resulting in the conspicuous regional variation (Akada et al., 1979; Ikeda et al., 1996; Okamoto & Noguchi, 1986; Shimizu et al., 1979). The regional variation of paeoniflorin found in the samples of this study also seemed to reflect the governing factors described above.

3.4. Seasonal variation of the active compounds in P. lactiflora

The seasonal variation of paeoniflorin and albiflorin in different parts of 4 year-grown *P. lactiflora* was traced (Fig. 3). The active compounds were present at the highest level in root compared with leaf and branch. Although the contents of paeoniflorin in root were maintained at a similar level during the harvest time, those of albiflorin were diminished conspicuously after June. In leaf, paeoniflorin was also present at a higher level around 1000 mg/100 g dried sample during the late spring and summer, afterwards decreased drastically. Whereas, the contents of albiflorin in leaf were in a low level from May to October. In branch, the contents of paeoniflorin gradually decreased from the level of 500 mg/100 g dried sample during the whole harvest time and those of albiflorin increased to the maximum value of 113.7 mg/100 g dried sample in July, followed by a gradual decrease until October. From these facts, it was found that the contents of the active compounds in different parts of *P. lactiflora* grown in Korea showed characteristic patterns of change as reported previously for the same herb cultivated in Japan, which indicated that the conversion of the active compounds in each part of *P. lactiflora* and the translocation of them to the other parts actively take place (Shimizu et al., 1979). As the root of *P. lactiflora* is processed into Paeoniae Radix for commercial purpose, the contents of the active compounds in root are very important for a cultivation management of *P. lactiflora*. Based on it, the optimum

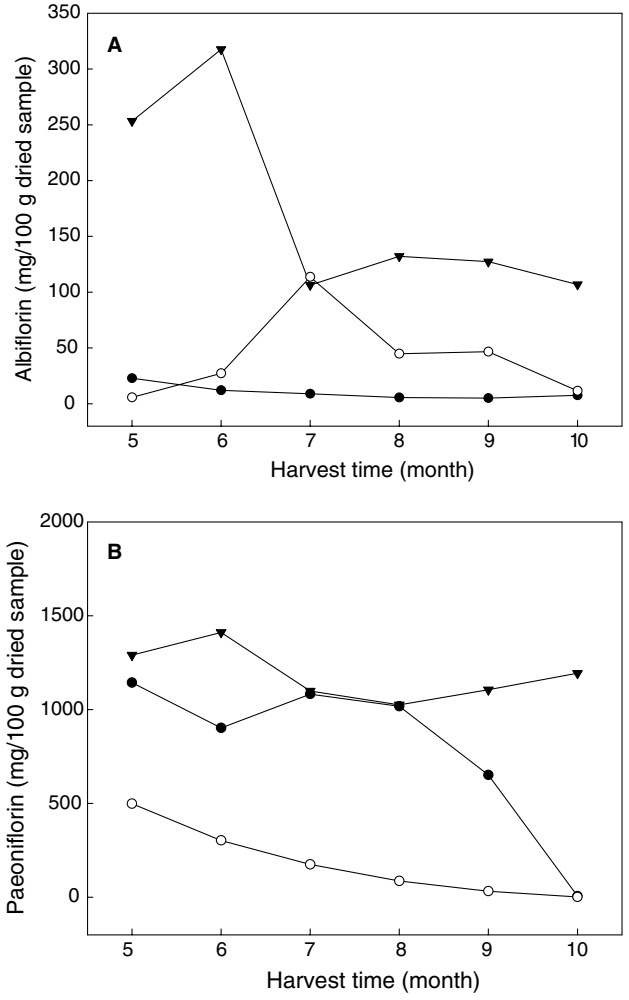

Fig. 3. Seasonal variation of albiflorin (panel A) and paeoniflorin (panel B) in 4 year-grown *P. lactiflora* in leaf (●), branch (○) and root (▼).

harvest time of the 4 year-grown *P. lactiflora* of this study was determined as June.

4. Conclusions

Two active compounds (paeoniflorin and albiflorin) in *P. lactiflora* were separated by a gradient HPLC method making use of a reversed-phase (ODS C_{18}) column and the chromatographic resolution of the eluted peaks was evaluated according to the gradient types of acetonitrile–water, resulting in the establishment of a good separation concerning resolution, analysis time and precision. Considering its fine repeatability and recovery of analysis, this method might be a routine analytical tool for quantitative analysis of the active compounds in paeony root and food products containing them. It might also find its applicability to the determination of regional variation of the active compounds and to a cultivation management for *P. lactiflora* with good functional activity.

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