Structural Characterization of an Artefact and Simultaneous Quantification of Two Monoterpenes and Their Artefacts of Isolation in White-Peony Root

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Sodium benzoylpaeoniflorinsulfonate (**3**), a new artefact, was isolated from sulfur-fumigated white-peony root and characterized by mass and NMR spectroscopy. A HPLC method was developed for the simultaneous determination of sodium paeoniflorinsulfonate (**1**), paeoniflorin (**2**), sodium benzoylpaeoniflorinsulfonate (**3**), and benzoylpaeoniflorin (**4**). The method developed was successfully applied to quantify the four compounds in 14 white-peony-root samples. The quantity of four constituents in sulfur-fumigated white-peony root may be regarded as an index for the quality assessment of this Chinese medicine.

Introduction. – White-peony root, a processed product from the root of *Paeonia lactiflora* Pall, is a kind of traditional Chinese medicine (TCM), which is used for alleviating pain, tonifying blood, and regulating menstruation [1]. In China, white-peony root, which stipulated by law can be used as raw material for functional food [2], is used extensively not only in Chinese medicinal formulations but also in functional-food industry.

The traditional processing methods, under the guidance of the theory of the traditional Chinese medicine science, have been proved to be important to enhance the curative effect or reduce the toxicity before the herbs can be used in the prescription of traditional medicines. The traditional processing method for white-peony root consists of decorticating, boiling, drying, and slicing the root of *Paeonia lactiflora* Pall [1]. In some production sites, however, sulfur fumigation is used between the procedures of boiling and drying in the preparation of white-peony root in order to preserve its white color and inhibit bacterial growth [3].

Several recent studies focused on the influence of sulfur fumigation on the phytochemical profiles of white-peony root. Two artefacts formed from paeoniflorin, namely sodium paeoniflorinsulfonate [4] and paeoniflorinsulfonate [3], were isolated from sulfur-processed white-peony root. Several analytical methods have been established for the qualification and/or quantification of these artefacts from sulfur-fumigated white-peony root, for example, HPLC/UV [5] and HPLC/MS [6]. These findings revealed that sulfiting-agents processing or sulfur fumigation can cause a dramatic decrease in the levels of paeoniflorin with the concomitant formation of the artefacts in sulfur-fumigated white-peony root, and that sulfonate derivatives can be

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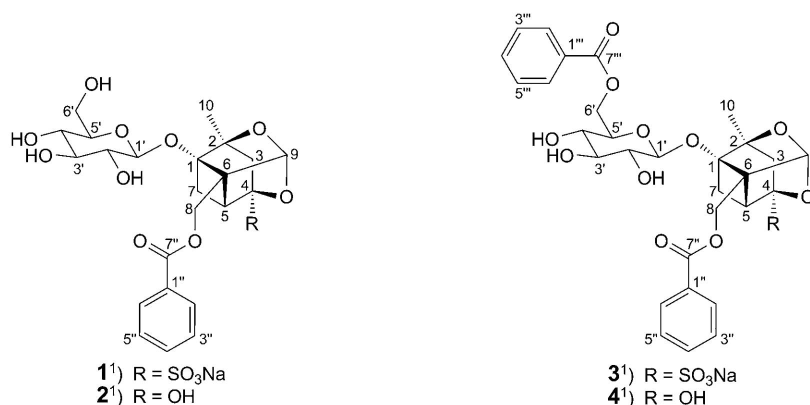
formed from monoterpene compounds possessing a hemiketal unit in the process of sulfur fumigation.

A new artefact, sodium benzoylpaeoniflorinsulfonate (**3**), which is formed from benzoylpaeoniflorin (**4**), has been isolated for the first time from fumigated white-peony root. It was reported that paeoniflorin (**2**) and benzoylpaeoniflorin (**4**) are the major monoterpene compounds possessing a hemiketal unit in white-peony root [7]. However, in some sulfur-fumigated white-peony root, paeoniflorinsulfonate became the most prominent constituent [5]. In the Pharmacopoeia of People's Republic of China, only paeoniflorin (**2**) was considered as the single chemical marker to control the quality of white-peony root. Apparently, only quantitative determination of paeoniflorin (**2**) is not suitable for the quality control of sulfur-fumigated white-peony root due to a dramatic decrease in the content of **2** in this kind of white-peony root.

In this article, we describe the structural elucidation of the artefact **3**. In addition, the four constituents paeoniflorin (**2**), benzoylpaeoniflorin (**4**), and the derived artefacts from them, sodium paeoniflorinsulfonate (**1**) and sodium benzoylpaeoniflorinsulfonate (**3**), were simultaneously determined by HPLC in 14 white-peony-root samples. Their contents could be used to evaluate and compare the quality of white-peony root. Factors that influenced the contents of the constituents are also discussed.



Results and Discussion. – *Structural Elucidation.* In the ^{1}H- and ^{13}C-NMR spectra of compound **3**, the signals associated with two benzoyl and one glucosyl groups were readily recognized. By comparison with the NMR data of paeoniflorin, albiflorin, and benzoylpaeoniflorin, the monoterpene system was confirmed in compound **3**. The ^{1}H- and ^{13}C-NMR data of compound **3** (*Table 1*) were assigned by analysis of the HMQC spectrum. In the HMBC spectrum of **3** (*Fig. 1*), the 2 H–C(3) were correlated with C(1), C(2), C(4), C(5), and C(7), H–C(5) with C(1), C(3), C(4), C(6), and C(8), H–C(8) with C(1), C(5), C(6), and C(9), H–C(9) with C(2), C(4), C(5), C(6), and C(8), and H–C(10) with C(1), C(2), C(3), and C(4), confirming that compound **3** and benzoylpaeoniflorin (**4**) contained the same monoterpene skeleton. The attachment of two BzO groups and of a β-glucose moiety at C(6′), C(8), and C(1), respectively, were

[1]) Trivial atom numbering; for systematic names, see *Exper. Part.*

Table 1. *^{1}H- and ^{13}C-NMR Data of* **4** *and* **3**[1]). In $(D_6)DMSO$; δ in ppm, J in Hz.

	4		**3**	
	δ(H)[a])	δ(C)[b])	δ(H)[a])	δ(C)[b])
C(1)		88.1		88.2
C(2)		85.2		86.6
CH_2(3)	1.59 (*s*), 1.72 (*d*, J = 12.0)	43.9	1.75 (*d*, J = 13.0), 2.09 (*d*, J = 13.0)	39.5
C(4)		105.0		93.4
H−C(5)	2.41 (overlap)	42.6	2.94 (*d*, J = 6.8)	41.8
C(6)		70.9		70.8
CH_2(7)	1.56 (*d*, J = 6.0), 2.41 (overlap)	22.0	1.72 (*d*, J = 10.6), 2.57 (overlap)	23.0
CH_2(8)	4.63 (*d*, J = 15.6)	60.8	4.67 (*s*)	60.7
H−C(9)	5.32 (*s*)	100.4	5.44 (*s*)	103.4
Me(10)	1.15 (*s*)	19.4	1.16 (*s*)	19.7
H−C(1′)	4.48 (*d*, J = 7.8)	99.0	4.52 (*d*, J = 7.7)	98.9
H−C(2′)	3.06 (*dd*, J = 13.2, 8.4)	73.8	3.05 (*m*)	73.8
H−C(3′)	3.15–3.24 (*m*)	77.0	3.16 (overlap)	77.0
H−C(4′)	3.15–3.24 (*m*)	70.2	3.16 (overlap)	70.7
H−C(5′)	3.50 (*t*, J = 8.4)	74.0	3.48 (*t*, J = 7.3)	74.0
CH_2(6′)	4.36 (*dd*, J = 11.7, 7.2), 4.62 (*dd*, J = 7.2)	64.7	4.15 (*dd*, J = 9.5, 7.3), 4.67 (*dd*, J = 11.5, 2.0)	64.8
C(1″)		130.1		130.1
H−C(2″)	7.98 (*d*, J = 7.8)	129.5	7.98 (*d*, J = 7.8)	129.4
H−C(3″)	7.54 (*t*, J = 7.8)	129.2	7.56 (*t*, J = 7.8)	129.3
H−C(4″)	7.66 (*t*, J = 7.8)	129.7	7.67 (*t*, J = 7.8)	133.8
H−C(5″)	7.54 (*t*, J = 7.8)	129.2	7.56 (*t*, J = 7.8)	129.3
H−C(6″)	7.98 (*d*, J = 7.8)	129.5	7.98 (*d*, J = 7.8)	129.4
C(7″)		166.2		166.2
C(1‴)		130.0		130.0
H−C(2‴)	7.98 (*d*, J = 7.8)	129.7	7.96 (*d*, J = 7.8)	129.7
H−C(3‴)	7.54 (*t*, J = 7.8)	129.1	7.52 (*t*, J = 7.8)	129.1
H−C(4‴)	7.66 (*t*, J = 7.8)	133.8	7.65 (*t*, J = 7.8)	133.7
H−C(5‴)	7.54 (*t*, J = 7.8)	129.1	7.52 (*t*, J = 7.8)	129.1
H−C(6‴)	7.98 (*d*, J = 7.8)	129.7	7.96 (*d*, J = 7.8)	129.7
C(7‴)		165.9		166.0
OH−C(2′)	5.19 (*d*, J = 4.8)		5.11 (*d*, J = 4.8)	
OH−C(3′)	5.11 (*d*, J = 4.8)		5.06 (*s*)	
OH−C(4′)	5.31 (*d*, J = 4.8)		5.25 (br. *d*, J = 4.8)	
OH−C(4)	6.93 (*s*)			

[a]) At 600 MHz. [b]) At 150 MHz.

evident from the HMBC spectrum which showed correlations of H−C(6′) to C(7‴), H−C(8) to C(7″), and H−C(1′) to C(1). These findings suggested that compound **3** possesses a C-atom connectivity identical to that of benzoylpaeoniflorin (**4**). However, surprisingly, a close comparison of the ^{1}H- and ^{13}C-NMR spectra of **3** and those of **4** (*Table 1*) revealed that there were some obvious differences between them. These differences concern C(4), which moved upfield by 11.6 ppm (from δ 105.0 in **4** to δ 93.4 in **3**), C(3) and C(9), which moved upfield by 4.4 ppm and downfield by 3 ppm,

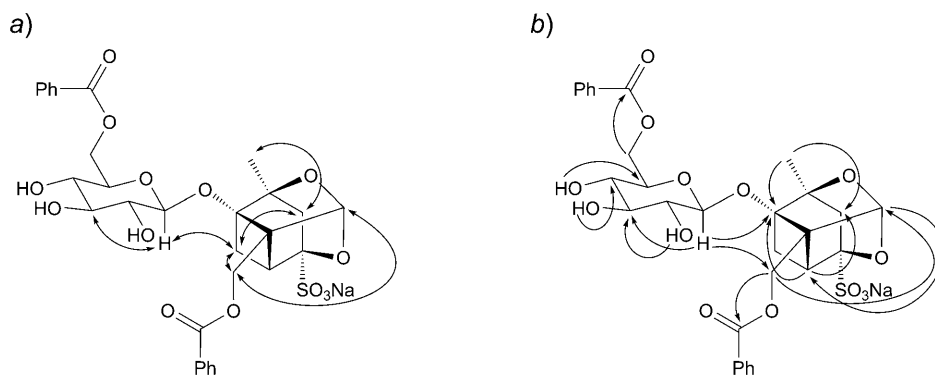


Fig. 1. a) *Key NOESY correlations* (H ↔ H) *and* b) *HMBCs* (H → C) *for* **3**

respectively, H–C(5), which moved downfield by *ca.* 0.5 ppm. In addition, the OH–C(4) signal (δ 6.93), which was observed in the ^{1}H-NMR spectrum of **4**, was not detectable anymore in **3**. This indicated that an unknown group which replaced OH–C(4) linked to C(4) of **3**, but the group did not consist of C- and H-atoms because no additional C- and H-atoms were present in the NMR spectra of **3**. Previous researchers [4] reported that an artefact, sodium paeoniflorinsulfonate (**1**), was isolated from sulfited *Paeonia lactiflora*, and a notable C(4) upfield shift (10.5 ppm) and H–C(5) downfield shift (0.95 ppm) in the NMR data of this compound was observed by comparison with that of paeoniflorin (**2**). All their facts led to the conclusion that the tertiary OH group (present at C(4) in benzoylpaeoniflorin (**4**)) was converted into a sodium sulfonate moiety in compound **3**.

The structure of **3** was further confirmed by ESI-MS, which showed a molecular ion at *m*/*z* 693 ($[M+\mathrm{Na}]^+$) in the positive-ion mode and a molecular ion at *m*/*z* 647 ($[M-\mathrm{Na}]^-$) in the negative-ion mode. This corresponds to a mass increase of 86 for compound **3** relative to benzoylpaeoniflorin (**4**), suggesting the molecular formula $C_{30}H_{31}NaO_{14}S$.

Method Validation. As clearly shown in *Table 2*, all of the marker substances showed good linearity (correlation coefficient $r > 0.9998$) in the test ranges, with *LOD*s (limits of detection) and *LOQ*s (limits of quantification) for the four compounds ranging from 45 to 125 ng and from 150 to 417 ng, respectively. *Table 3* shows the results of the precision test for the four compounds, and indicates that the overall intra-day and inter-day RSDs (relative standard deviations) were 1.23–2.48 and 1.55–2.76%,

Table 2. *Regression Data and Limits of Detection* (*LOD*) *and Quantification* (*LOQ*) *for the Four Constituents* **1**–**4**

	Linear range	*r*	Range [µg]	*LOD* [ng]	*LOQ* [ng]
1	$y = 3894.3x + 4146.9$	0.9999	26.25–420.0	79.0	262
2	$y = 4902.3x - 850.20$	0.9998	12.50–200.0	125	417
3	$y = 5770.7x - 330.80$	0.9999	3.000–48.00	45.0	150
4	$y = 6861.5x + 3352.8$	0.9999	7.250–116.0	54.0	181

Table 3. *Precision and Repeatability of the HPLC Analysis of the Four Constituents* **1**–**4**

	Precision		Repeatability ($n=5$)	
	intra-day RSD [%] ($n=6$)	inter-day RSD [%] ($n=3$)	mean [mg/g]	RSD [%]
1	1.81	2.05	12.4	1.12
2	2.19	2.76	2.32	1.93
3	1.23	1.55	0.33	2.15
4	2.48	2.64	0.21	1.86

respectively, and that the overall repeatability variations were less than 2.15%. Additionally, as shown in *Table 4*, the overall recoveries were determined to be between 94.77 and 100.6% for all compounds. The recovery results indicated that the established method was accurate enough for the determination of the four components. Therefore, the method is precise, accurate, and sensitive enough for the simultaneous quantitative evaluation of the four compounds **1**–**4** in white-peony-root samples.

Table 4. *Recovery Study for the Four Constituents* **1**–**4** ($n=3$)

	Content in sample [mg]	Spiked amount [mg]	Found amount [mg]	Recovery [%]	RSD [%]
1	0.6285	0.6778	1.3107	100.6	1.87
2	0.1338	0.1420	0.2731	98.13	3.65
3	0.0161	0.0153	0.0306	94.77	4.36
4	0.0122	0.0185	0.0299	95.67	3.13

Sample Analysis. Fourteen samples were analyzed by the established HPLC method, and the contents of the four compounds **1**–**4** analyzed are listed in *Table 5*. From the data, it was found that sodium paeoniflorinsulfonate (**1**) and sodium benzoylpaeoniflorin sulfonate (**3**) did not exist in *Samples 10*–*14* but in *Samples 1*–*9*. *Sample 10* (*Fig. 2*,*b*) contains paeoniflorin (**2**) as the major component and has a notable peak of benzoylpaeoniflorin (**4**). However, in the chromatogram of the samples *4* and *7* (*Fig. 2*,*c* and *d*), two obvious new peaks (**1** and **3**), were observed, with a concomitant sharp decrease of the peaks of **2** and **4**. These results indicated that the *Samples 1*–*9* were from sulfur-fumigated white-peony root, and the artefacts were the typical constituents of fumigated white-peony root. The data also showed that the total contents of these compounds varied markedly between *Samples 1*–*9*. Moreover, the content of compound **1** (mean 12.7 mg/g) in the *Samples 1*–*9* was higher than that of compound **2** (mean 2.07 mg/g). As sulfonates can easily form from hemiketals [4], it was thought that this may explain the issue. Similar results were observed for the contents of compound **3** (mean 0.28 mg/g) and **4** (mean 0.19 mg/g).

Unlike paeoniflorin (**2**), there have been no reports so far relevant to biological activities and toxicities of these artefacts. Thus, cautious use of sulfur-fumigated white-peony root is advisable. The presented HPLC method is suitable for controlling the quality of sulfur-fumigated white-peony root and could also be used to differentiate sulfur-fumigated white-peony root from nonfumigated white-peony root. As part of our work, studies to determine the pharmacological activity of these artefacts are in progress.

Table 5. *Abundance of the Four Constituents* **1**–**4** *in White-Peony-Root Samples*

Sample	Abundance [mg/g]			
	1	**2**	**3**	**4**
1	9.71	3.39	0.16	0.28
2	10.7	0.71	0.28	0.16
3	9.02	0.46	0.25	0.09
4	12.8	2.24	0.31	0.22
5	13.5	2.92	0.35	0.11
6	13.3	5.42	0.17	0.35
7	16.0	0.25	0.43	0.08
8	16.1	2.72	0.21	0.25
9	12.9	0.48	0.40	0.14
Mean	12.7	2.07	0.28	0.19
10	–[a])	27.1	–[a])	1.22
11	–[a])	31.2	–[a])	1.35
12	–[a])	28.6	–[a])	1.19
13	–[a])	33.5	–[a])	1.28
14	–[a])	31.7	–[a])	1.31

[a]) Not detected.

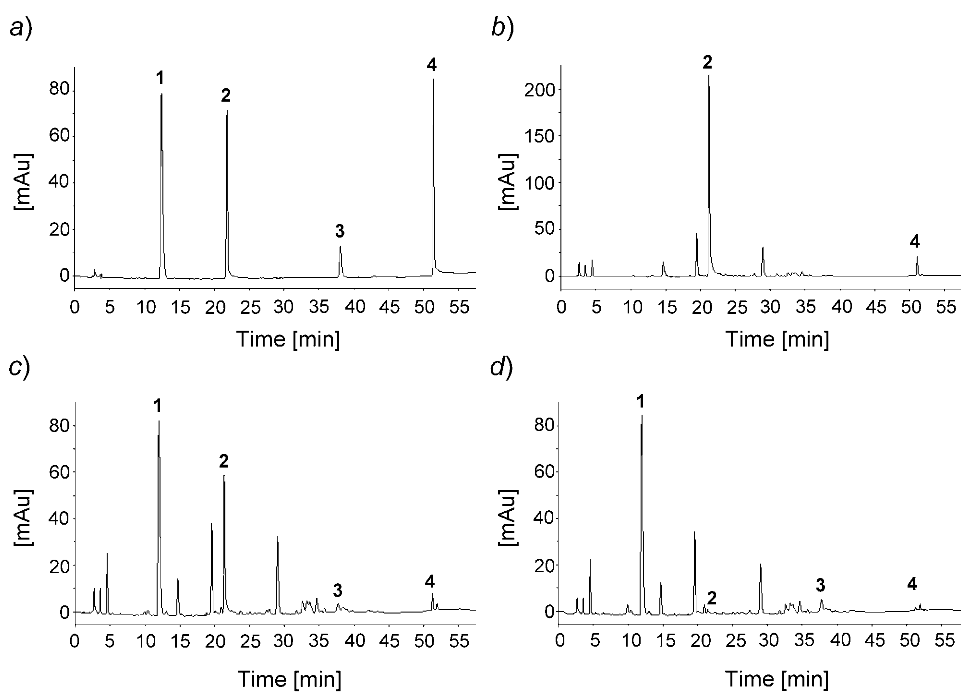


Fig. 2. *Typical HPLCs of* a) *standard mixture,* b) *white-peony root from Zhongjiang* (*Sample 10*), c) Sample 4, *and* d) Sample 7

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Experimental Part

General. Sodium paeoniflorinsulfonate (**1**), paeoniflorin (**2**), sodium benzoylpaeoniflorinsulfonate (**3**), and benzoylpaeoniflorin (**4**) were isolated from the white-peony roots, and their structures were confirmed by the comparison of their respective NMR spectra with the published data [4][8]. HPLC Analysis suggested that their purity were > 98%. MeCN was of HPLC grade from *Fisher Laboratories Ltd.* (USA). Anal.-grade H_3PO_4 and EtOH were obtained from *Beijing Chemical Corporation* (Beijing, P. R. China), CC = Column chromatography. ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz), and 2D Spectra: *Bruker-AV-II-600-MHz* spectrometer; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS: *Finnigan-LCQ*$_{DECA}$ ion-trap mass spectrometer (San Jose, CA, USA), capable of analyzing ions up to m/z 2000.

Materials. White-peony-root *Samples 1–9* were purchased from different drug stores and *Hehuaci Traditional Chinese Medicine Market* in Chengdu. *Samples 10–14* were purchased from their habitats plantation in Zhongjiang and Pengzhou, Sichuan, China. These samples were authenticated by *H. Z.* (Sichuan University, Chengdu, China).

Extraction and Isolation. The powdered white-peony root (2.0 kg) was extracted three times with 80% EtOH at 40°. After evaporation of EtOH, the residue was suspended in H_2O and successively extracted with $CHCl_3$ and AcOEt. The H_2O fraction (25.0 g) was then subjected to CC (silica gel, gradient $CHCl_3$/MeOH 10:1 → 3:1): *Fr. 1* (4.3 g), *Fr. 2* (3.9 g), and *Fr. 3* (3.0 g). *Fr. 3* (3.0 g) was subjected to medium-pressure CC (*Büchi*, *Labortechnik AG*, Switzerland; *RP-C*$_{18}$ column, EtOH/H_2O 10:90 → 25:75): *Fr. 3A* (30 mg). *Fr. 3A* (30 mg) was purified by CC (*Sephadex LH-20*, MeOH): *sodium benzoylpaeoniflorinsulfonate* (=*(1a*R*,2*S*,3a*R*,5*S*,5a*R*,5b*S*)-5b-[(benzoyloxy)methyl]-5a-[(6-*O*-benzoyl-β-*D*-glucopyranosyl)oxy]hexahydro-5-methyl-2,5-methano-2*H*-3,4-dioxacyclobuta[*cd*]pentalene-2-sulfonic acid monosodium salt*; **3**; 22 mg). White amorphous powder. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (pos.): 693 ($[M+Na]^+$). ESI-MS (neg.): 647 ($[M-Na]^-$).

Sample Preparation. The dried powder of white-peony root (1.0 g) was accurately weighed and extracted with MeOH (40 ml) in an ultrasonic bath for 30 min. The extract was cooled to r.t., and MeOH was added to compensate for the loss of weight. Prior to use, all samples were filtered through a 0.45 μm micropore membrane, and 5 μl were injected into the HPLC instrument for analysis.

Chromatographic Conditions. Experiments were performed with a *Shimadzu-LC-10ATvp* system (*Shimadzu Corp.*, Kyoto, Japan) consisting of a *LC-10ATvp* binary pump, a *CTO-10Asvp* column oven, a *SCL-10Avp* system controller, and a *Class-vp* workstation, a *Dikma-Diamonsil*™*II-C*$_{18}$ column (5 μm, 250 mm × 4.6 mm i.d.) and a guard column (5 μm, 7.5 mm × 4.6 mm i.d.), and a mobile phase consisting of MeCN (A) and 0.3% aq. H_3PO_4 soln. (v/v, B) with gradient program of 10% A in 0–5 min, 10–18% A in 5–15 min, 18–27% A in 15–40 min, 27–45% A in 40–45 min, 45% A in 45–50 min, and 45–10% A in 50–55 min; flow rate 1.0 ml/min, column temp. 30°; detection at 230 nm.

Calibration Curves, Limits of Detection and Quantification. Standard stock solns. of sodium paeoniflorinsulfonate (**1**; 1.05 mg/ml), paeoniflorin (**2**; 0.50 mg/ml), sodium benzoylpaeoniflorinsulfonate (**3**; 0.12 mg/ml), and benzoylpaeoniflorin (**4**; 0.29 mg/ml) were prepared in MeOH. Working standard solns. containing each of the four compounds were prepared by diluting the stock solns. with MeOH to the proper volumes. Typical chromatograms are shown in *Fig. 2,a*. Each calibration curve contained six different concentrations and was performed in triplicate. Calibration curves were constructed by plotting peak areas *vs.* concentration of each analyte. The results are shown in *Table 2*.

Limits of detection (*LOD*) and quantification (*LOQ*) were defined by the value at a signal-to-noise ratio of 3 and 10, resp. The *LOD* and *LOQ* for each compound analyzed are also shown in *Table 2*.

Precision, Repeatability, and Accuracy. The intra-day and inter-day precision were determined by analyzing calibration samples during a single day and on three different days, resp. The intra-day variation was determined by analyzing the six replicates on the same day and the inter-day variation was

determined on three consecutive days. The relative standard deviation (RSD [%]) was taken as a measure of precision, and the results are shown in *Table 3*. In addition, to further evaluate the repeatability of the developed assay, the white-peony-root sample was treated and analyzed five times. The RSD was also taken as the measure of repeatability (*Table 3*).

Recovery experiments were performed to investigate the accuracy of the method. Known amounts of each solute were added to the sample detected, and the resulting spiked sample was subjected to the entire analytical sequence. Each solute was spiked at a close concentration with the sample, and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the nonspiked samples. Three replicates were performed for the test (*Table 4*).

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