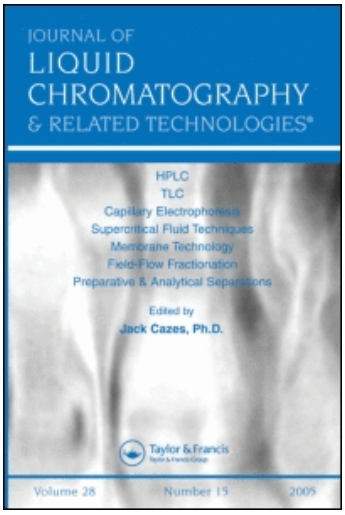

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Bin Li[a]; Lian-Wen Qi[a]; Xiao-Dong Wen[a]; Jun Cao[a]; Ping Li[a]; Jin-Xiang Zeng[a]; Hua Yang[a]; Jing Zhao[a]; Rong Hu[a]
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Rapid and Sensitive Analysis of Tannins and Monoterpene Glycosides in *Radix Paeoniae Alba* Products by HPLC-MS

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Abstract: A quantitative HPLC-MS method was developed for rapid and sensitive analysis of 5 tannins (gallotannins and proanthocyanidin types) and 5 monoterpene glycosides (pinane type) in 70% ethanol extract of Radix Paeoniae Alba (RPA). The total analysis time was less than 10 minutes without a loss in resolution using a short column packed with 1.8 μm porous particles, six times faster than the performance of conventional columns of 5.0 μm porous particles. This assay was fully validated with respect to precision, repeatability, and accuracy. All calibration curves showed good linear regression ($r^2 > 0.9961$), and recoveries of all analytes fell in the ranges of 92.0–104.1%. The developed method was successfully applied to analysis of the 10 marker compounds in 6 processed RPA products and 27 commercial samples from different pharmacies in China. The quantitative results demonstrated that samples processed in different ways and from different provinces showed varied qualities based on the contents of active components. Robustness and applicability of the method offered a rapid, sensitive, and validated method for routine analysis and quality control of this herbal medicine. Meanwhile, the LC/MS method developed here provided a more economical alternative for laboratories looking into improving separation and increasing throughput, without the need to upgrade the existing HPLC system to a special ultra fast HPLC system.

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Keywords: HPLC-MS, Quality control, Radix Paeoniae Alba, Rapid and sensitive analysis, Tannins and monoterpene glycosides, Ultra fast HPLC

INTRODUCTION

In 2004, the US FDA and the European Parliament claimed the approval of herbal mixtures with unknown ingredients, if convincing evidence is provided for their safety and efficacy.[1] Nowadays, combinatorial multicomponent therapies of medicines in one preparation to treat different conditions have received approval from the FDA, owing to the prevalence of chronic diseases and limitations of chemical entities.[2] These raised the interest of many pharmaceutical companies of herbal medicines all over the world. Correspondingly, safety and efficacy, as well as quality control, have become important concerns about complex herbal medicines.

Radix Paeoniae Alba (RPA), the root of *Paeonia lactiflora* Pall. (Ranunculaceae family), has been commonly used in traditional medicine to tonify the blood, alleviate pain, and to treat inflammation and cancer.[3] Chemical investigations and pharmacological studies have demonstrated that the major bioactive compounds in RPA include monoterpene glycosides and tannins.[4] Tannins in RPA, commonly not considered by researchers, are reported for their abilities to scavenge radicals and to associate with proteins, metals, amines, and polysaccharides through hydrophobic, ionic, and hydrogen bonding interactions.[5] It is widely accepted that the efficacy of herbal medicines has contributed to the holistic actions of multicomponents on multitargets. The analytical methodologies employed for herbal medicine analysis and their quality control should be able to perform assays as rapidly and efficiently as possible, measure low levels, and provide definite evidence to confirm both the identity and the quantity of marker constituents.

To date, a number of analytical methods have been developed for analyzing marker compounds in complex RPA matrices, including high performance liquid chromatography (HPLC),[6–11] capillary zone electrophoresis,[12] and HPLC mass spectrometry (MS).[13] These methods, however, dealt with just one or several constituents, which are not coincidental with the characteristics of herbal medicines containing multicomponents producing effects on multitarget. Tannins are often ignored when evaluating the quality of RPA and its related products. Moreover, the operation time of some studies lasted up to 100 minutes in a single analysis. Recently, an improvement in chromatographic performance has been achieved by the introduction of an ultra fast HPLC system. Ultra fast HPLC, using a short column packed with 1.7–1.8 μm porous particles, has enhanced retention time, reproducibility, chromatographic

resolution, improved sensitivity, and increased operation speed, which can be proven in some published papers.[14] Li et al. developed an ultra fast HPLC method coupled with time-of-flight MS analysis for chemical evaluation of RPA and Radix Paeoniae Rubra.[15] The analytical time was greatly reduced. However, quantitative determination was absent, which is indispensable for the quality control of herbal medicines. Furthermore, the requirement of the expensive ultra fast HPLC system has kept the instrument more of a research tool than a routine tool for analysis of herbal medicines.

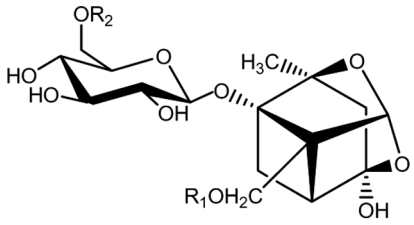
This paper marks the first report on a conventional HPLC system using a 1.8 μm particles column coupled with electrospray ionization (ESI) MS for rapid separation and sensitive determination of 10 tannins and monoterpene glycosides in RPA. Using the developed method, a total of 6 different processed products and 27 commercial crude drugs from different pharmacies have been investigated in this study. The LC/MS method developed here also provided a more economical alternative for laboratories looking into improving separation and increasing throughput without the need to upgrade the existing HPLC system to a special ultra fast HPLC system.

EXPERIMENTAL

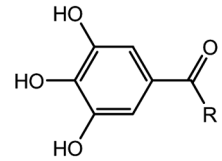
Samples, Chemicals, and Reagents

Six different processed products, prepared by Key Laboratory of Modern Chinese Medicines of China Pharmaceutical University, were obtained from RPA sample YC1 collected from Zhejiang Province. Twenty-seven batches of commercial crude drugs were purchased from different pharmacies in China and authenticated by one of the authors, Prof. Ping Li. The voucher specimens and samples were deposited in the department of Pharmacognosy, China Pharmaceutical University, Nanjing, China.

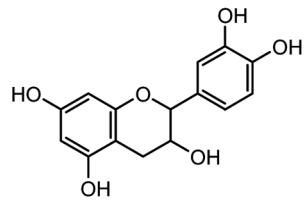
Ten reference compounds (P1 – P10), namely desbenzoylpaeoniflorin (P1), (+)-catechin (P2), albiflorin (P3), paeoniflorin (P4), galloylpaeoniflorin (P5), benzoylpaeoniflorin (P6), gallic acid (P7), methyl gallate (P8), ethyl gallate (P9), and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (P10) were isolated previously from the dried roots of *Paeonia lactiflora* Pall. by repeated Silica Gel, Sephadex LH-20 and RP-C_{18} Silica Gel column chromatography in our laboratory. Their structures were identified by spectroscopic methods (UV, IR, MS, ^{1}H NMR and ^{13}C NMR). The geniposide obtained from the National Institute for the Control of Pharmaceutical and Biological Product was identified as the internal standard (IS). The purities of these reference compounds were determined to be more than 95% by normalization of the peak areas detected by



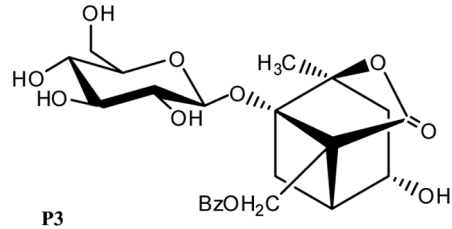
Compd.	R_1	R_2
P1	H	H
P4	Bz	H
P5	Bz	Galloyl
P6	Bz	Bz



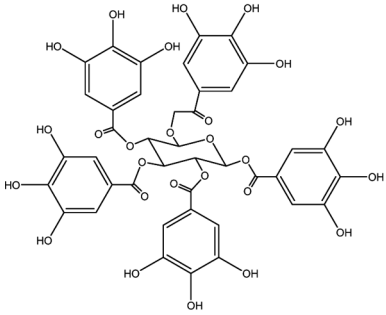
Compd.	R
P7	OH
P8	OCH_3
P9	OCH_2CH_3



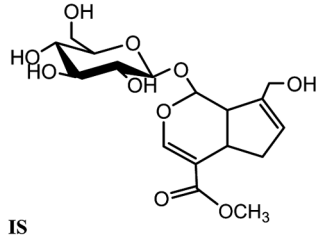
P2



P3



P10



IS

Figure 1. Chemical structures of tested constituents.

HPLC-ESI MS, and proved to be very stable in menthol solution at 4°C in a refrigerator. Figure 1 shows the chemical structures of these compounds.

HPLC grade ACN was purchased from Merck (Darmstadt, Germany); HPLC grade formic acid with a purity of 96% was obtained from Tedia (Fairfield, OH, USA); distilled water was further purified by Milli-Q system (Millipore, Bedford, MA, USA); other chemicals were analytical grade. All solvents and samples were filtered through 0.22 μm membrane filters before injecting into the HPLC-MS.

Apparatus and Chromatographic Conditions

Analysis was performed on an Agilent 1100 series LC system (Agilent, Germany) equipped with a binary pump, a micro degasser, an auto plate sampler, and a thermostatically controlled column compartment. An Agilent Zorbax SB-C_{18} column (4.6×50 mm, 1.8 μm) was employed. The column temperature was maintained at 30°C. The mobile phase consisting of 0.3% formic acid (A) and acetonitrile (B) was delivered at a flow rate of 1.2 mL/min. The flow rate of mobile phase was split at the column outlet to allow 20% eluent to flow into the mass spectrometer. The gradient elution was programmed as follows: 0–2.0 min, 10% B; 2.0–5.0 min, 10–12% B; 5.0–6.0 min, 12–30% B; 6.0–8.0 min, 30–31% B; 8.0–13.0 min, 31–35% B; and 13.0–15.0 min, 35–100% B. A five minute reequilibration time was used after each analysis so that the system was back to the initial mobile phase composition.

Analytes were detected using an Agilent single quadrupole mass spectrometer equipped with ESI source. The MS analysis worked using the selected ion monitoring (SIM) mode in negative mode. The mass spectrometric conditions were as follows: drying gas (N_2) flow rate, 10 L/min; drying gas temperature, 320°C; nebulizer gas (N_2) pressure, 35 psi; capillary voltage, 4000 V; Quad temperature, 100°C; fragment, 100 V; full scan range, 120–1000 m/z. The standard compounds, as well as extracted samples, were analyzed with SIM mode by monitoring the deprotonated molecular ions $[M-H]^-$ and the formate adduct ion $[M+HCOO]^-$. For simultaneous quantitative analysis, selected ions scan for each compound was restricted to a specific retention time window (Chanel 1: 0–2.3 min, m/z 289.1 and 421.1; 2.3–7.0 min, m/z 433.1 and 525.1; 7.0–10.0 min, m/z 629.0 and 631.0; Chanel 2: 0–2.3 min, m/z 169.2 and 183.2; 2.3–4.0 min, m/z 433.1; 4.0–6.0 min, m/z 197.2; 6.0–10.0 min, m/z 938.8). All the acquisition and analysis of data were controlled by Chemstation software (Agilent Technologies, USA).

Sample Preparation

All samples were dried at 40°C until constant weight. Each dried sample was ground to a fine powder (40 mesh) using a pulverizer. A 0.2 g portion of powder was accurately weighed and extracted three times under reflux with 70% ethanol for 30 min each (70% ethanol volume: 20, 15, and 15 mL). The extracts were combined and filtered and transferred into a 50 mL volumetric flask, which was made up to its volume with 70% ethanol. A 0.1 mL portion of the filtrate, added with the IS solution (the concentration of the IS was kept at 10 μg/mL as the standard solution), was then diluted with methanol to 1 mL. The solution obtained

was filtrated through a 0.22 μm membrane prior to HPLC-ESI MS analysis.

Calibration Curves, Limits of Detection (LOD) and Quantification (LOQ)

Methanol stock solution containing 10 reference standards was prepared and diluted to appropriate concentrations for the establishment of calibration curves. The concentration of the IS was 10 μg/mL for all analyses. The limits of detection (LOD) and limits of quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Precision and Accuracy

The intra-day variation was determined by analyzing the same mixed standard solution 5 times within one day. While for the inter-day variability test, the solution was examined for 3 consecutive days. Recovery was used to evaluate the accuracy of the method. Ten analytical compounds with known amounts were added to approximate 0.1 g of the RPA from YC1, and then the mixture was extracted and analyzed as described in sample preparation section. The total amount of each analyte was calculated from the corresponding calibration curve.

RESULTS AND DISCUSSION

Optimization of HPLC and MS Conditions

The optimization of the chromatographic conditions was focused on enhancing the detection of the analytes as well as minimizing the analysis time. Several mobile phase systems were tested, including acetonitrile–water, methanol–water with or without formic acid, and various gradient elution processes of these solvent systems. At last, a solvent system consisting of aqueous formic acid (0.3%, v/v) and acetonitrile was adopted as the mobile phase. Higher flow rate (2 mL/min) resulted in narrower peaks and shorter analytical time; however, the backpressure increased up to 300 bar and the resolution of adjacent peaks was affected, which is unsuitable for conventional HPLC analysis. The flow rate of 1.2 mL/min, finally adopted in this method produced a shorter analytical time, less than 10 min and moderate column pressure at about 190 bar for the RPA sample (Figure 2). The optimum chromatographic conditions provided better chromatographic

peaks shapes, greater baseline stability, and higher ionization efficiency than those of other systems.

Under the gradient condition as described previously, the peaks P2 and P8, P4 and P9, did not achieve baseline separation from each other. In order to obtain better sensitivity and resolution, acquisition was further performed using SIM by two channels simultaneously. Compared with positive ion analyses, better analytical selectivity and sensitivity for compounds were obtained by acquiring spectra in negative ion analyses using acidic mobile phase. Overall analysis time was reduced to 10 min, comparably shorter than that in other published methods.[6,7,13]

Identification of the 10 Target Analytes in RPA Samples

The m/z of the target analytes was extracted from the total ion chromatogram (TIC) and the mass of the compounds was obtained. During negative ion ESI MS, the deprotonated molecular ions $[M-H]^-$ were the base peaks in the spectra of 6 compounds (P2, P5 and P7–10). Instead, main ions derived from compound P1, P3–4 and P6 were the formate adduct ion $[M+HCOO]^-$ resulting from use of formic acid as mobile phase modifier. These results were similar to those of a previous study.[16,17] Table 1 summarizes the selected ions of the 10 marker components in RPA. In general, the peak area of the most abundant ion was calculated for quantitative analysis in SIM mode for better sensitivity.

Table 1. Selected negative ion mass measurements for the marker constituents in RPA by HPLC-ESI MS

No.	t_R (min)	Formula	Selected ion	Experimental (m/z)
SIM 1				
P1	0.704	$C_{16}H_{24}O_{10}$	$[M+HCOO]^-$	421.1
P2	1.986	$C_{15}H_{14}O_6$	$[M-H]^-$	289.1
IS	2.700	$C_{17}H_{24}O_{10}$	$[M+HCOO]^-$	433.1
P3	3.797	$C_{23}H_{28}O_{11}$	$[M+HCOO]^-$	525.1
P4	4.671	$C_{23}H_{28}O_{11}$	$[M+HCOO]^-$	525.1
P5	7.582	$C_{30}H_{31}O_{15}$	$[M-H]^-$	631.0
P6	9.611	$C_{30}H_{32}O_{12}$	$[M+HCOO]^-$	629.1
SIM 2				
P7	0.913	$C_7H_6O_5$	$[M-H]^-$	169.2
P8	2.121	$C_8H_8O_5$	$[M-H]^-$	183.2
IS	2.701	$C_{17}H_{24}O_{10}$	$[M+HCOO]^-$	433.1
P9	4.762	$C_9H_{10}O_5$	$[M-H]^-$	197.2
P10	7.796	$C_{41}H_{32}O_{26}$	$[M-H]^-$	938.8

Quantitative Analysis

Quantitation was carried out using the selected ion for each compound obtained in the scan mode. To improve quantitation precision and repeatability, an internal standard method was applied in this study. Searching for the internal standard that possesses similar structure and is not present in this herb would be reasonable for different classes of constituents. Geniposide was finally selected as the internal standard, and acceptable quantitative parameters were obtained.

To check the performance of this method, validated parameters such as LODs, LOQs, precision, and accuracy were studied, and the results are listed in Table 2. The calibration curves were established levels with good correlation coefficients (r^2) in a range from 0.9961 to 0.9997. The values of LODs and LOQs were in the pg range on column. For intra-day variations, the RSD of peak area ratios were less than 3.89%, while the inter-day variations of peak area ratios were less than 4.56%. These data demonstrated that this method provided acceptable precision for all analytes.

The accuracy of the developed method was established by recovery tests. Accurate standards were spiked with 100% of the amount found in the root extraction of the YC1, and then extracted and analyzed as

Table 2. Calibration curves, LODs and LOQs for 10 marker compounds and instrument accuracy

No.	Calibration curve	r^2	Test range (μg/mL)	LOD (pg)	LOQ (pg)	Precision	
						Intra-day RSD (%) (n = 5)	Inter-day RSD (%) (n = 3)
1	[a]$y = 0.0111x - 0.0005$	0.9980	0.48–15.36	4.85	13.58	1.35	1.77
2	$y = 0.0124x - 0.001$	0.9965	0.39–124.8	7.80	24.18	0.32	1.02
3	$y = 0.0182x - 0.003$	0.9972	0.38–88.32	7.68	22.27	0.97	1.15
4	$y = 0.0079x - 0.0005$	0.9997	3.53–86.93	5.68	15.90	0.73	3.88
5	$y = 0.0202x - 0.003$	0.9961	0.55–10.96	5.48	16.98	3.59	4.56
6	$y = 0.0031x + 0.00005$	0.9997	0.32–10.37	8.10	25.11	2.39	3.35
7	$y = 0.0350x + 0.001$	0.9986	0.94–45.12	8.64	24.19	0.73	1.33
8	$y = 0.776x - 0.0034$	0.9993	0.38–15.52	7.00	21.78	1.35	2.08
9	$y = 0.0189x - 0.02$	0.9984	0.40–13.01	6.40	21.12	0.62	1.61
10	$y = 0.0345x + 0.001$	0.9974	0.94–45.12	5.64	16.35	3.89	4.44

[a]y: concentration of analyte (μg/mL); x: peak area ratio (analyte peak area/IS peak area).

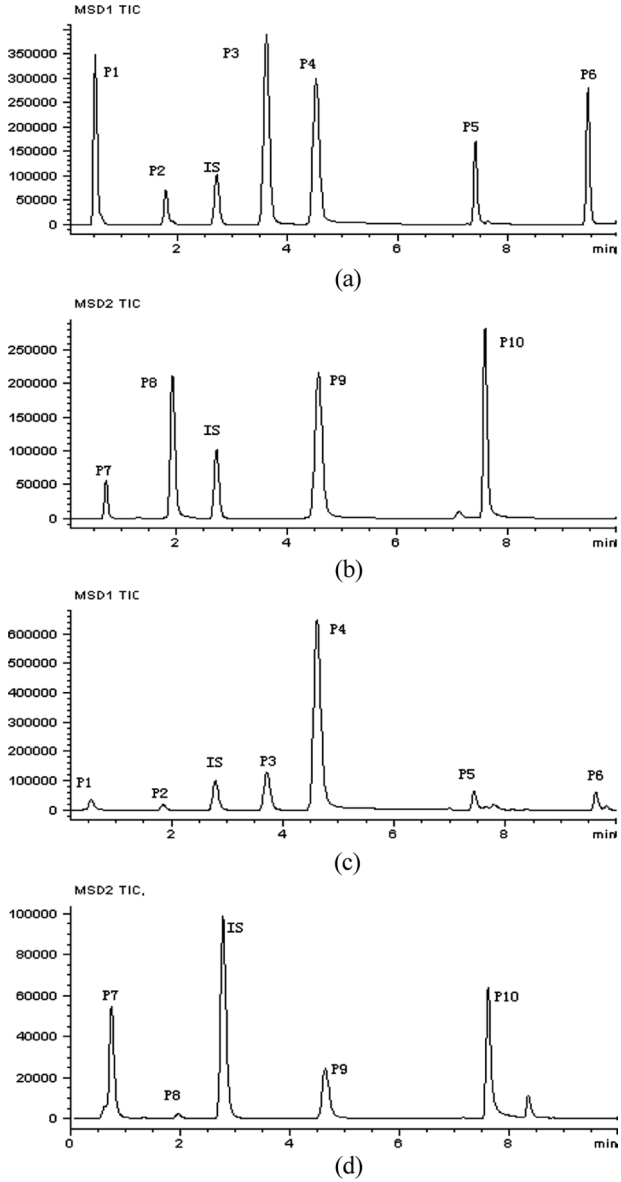


Figure 2. SIM chromatograms of the reference stock solution in Channel 1 (a) and Channel 2 (b), SIM chromatograms of 70% ethanol extract of Radix Paeoniae Alba sample YC1 collected from Zhejiang Province in Channel 1 (c) and Channel 2 (d).

Table 3. Mean contents of 10 bioactive components in RPA samples

No.	Collective site	Mean contents of commercial products (mg/g)									
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
YC1	Zhejiang Province	0.82	0.27	6.13	16.42	1.87	0.40	4.05	1.18	0.97	8.44
YC2	Hena Province	0.36	1.05	18.43	2.65	0.98	0.23	4.20	3.02	0.17	5.39
YC3	Nanchang, Jiangxi Province	0.26	0.60	10.13	2.96	0.47	0.25	2.95	3.86	0.34	2.76
YC4	Qinghai Province	0.32	0.80	16.28	6.49	1.39	0.33	6.90	0.10	0.41	5.98
YC5	Nanning, Guangxi Province	0.42	0.67	11.98	1.11	0.16	0.08	3.31	0.10	0.17	2.43
YC6	Helongjiang Province	0.14	0.10	16.07	2.81	0.98	0.22	0.98	ND	0.19	5.55
YC7	Zhejiang Province	0.34	1.11	15.14	7.51	1.13	0.35	3.96	0.24	0.74	5.65
YC8	Shangxi Province	0.34	0.66	11.01	1.04	0.46	0.17	5.52	ND	0.14	4.68
YC9	Beijing City	0.23	0.50	7.64	6.18	0.51	0.29	3.07	0.11	0.69	2.96
YC10	Jilin Province	0.25	0.46	12.19	2.60	0.64	0.20	4.31	0.11	0.18	4.43
YC11	Yinchuan, Ningxia Province	0.34	0.47	15.37	3.46	1.14	0.26	5.00	0.15	0.12	5.61
YC12	Fujian Province	0.19	0.68	13.28	3.28	0.57	0.26	3.30	0.79	0.27	3.28
YC13	Shengyang, Liaoning Province	0.29	0.51	9.16	5.44	0.61	0.26	3.72	0.18	0.00	3.77
YC14	Anhui Province	0.33	0.55	16.54	8.34	1.26	0.31	3.41	1.16	0.56	3.69
YC15	Shanghai City	0.44	0.66	10.63	8.81	1.40	0.39	5.53	0.70	0.24	6.30
YC16	Liuzhou, Guangxi Province	0.30	0.51	11.11	5.41	1.02	0.30	3.85	0.10	0.22	3.50
YC17	Shanxi Province	0.31	0.85	16.11	12.82	1.86	0.41	4.06	0.28	0.48	6.69
YC18	Yunnan Province	0.27	0.75	12.63	6.16	1.03	0.33	3.87	0.12	0.12	4.39
YC19	Sichuan Province	0.29	0.68	14.58	1.66	0.73	0.24	3.51	0.48	0.11	3.94
YC20	Tangshan, Hebei Province	0.39	0.64	10.64	3.30	0.55	0.10	4.04	0.10	0.10	0.86
YC21	Hainan Province	0.27	0.66	12.57	0.97	0.18	0.11	3.75	0.45	0.15	3.49

(*Continued*)

Table 3. Continued

No.	Collective site	Mean contents of commercial products (mg/g) P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
YC22	Bozhou, Anhui Province	2.27	2.99	13.95	20.74	2.68	2.12	4.28	1.31	0.58	4.77
YC23	Hunan Province	0.31	0.85	13.83	3.85	0.78	0.21	4.05	3.42	0.21	5.51
YC24	Tianjing Province	0.89	0.10	8.69	7.14	1.53	0.37	7.95	0.37	0.28	5.23
YC25	Guagndong Province	0.13	0.86	19.59	8.22	1.39	0.36	4.89	0.84	0.67	5.65
YC26	Guizhou Province	1.63	0.43	8.51	13.47	2.56	0.52	6.43	0.44	0.20	10.46
YC27	Hena Province	0.36	0.45	8.99	4.29	0.63	0.25	3.43	1.98	0.13	4.09
		Mean contents of processed products (mg/g)									
No.	Processing methods	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
	Mean contents of 10 bioactive components in RPA processed products with YC1										
YC1	–	0.82	0.27	6.13	16.42	1.87	0.40	4.05	1.18	0.97	8.44
PS1	light-frying	0.63	ND	4.81	13.69	0.23	0.35	4.22	2.15	0.12	7.29
PS2	processing with vinegar	0.80	0.12	6.41	16.50	0.91	0.39	4.53	2.50	0.32	8.33
PS3	processing with wine	0.13	0.18	6.03	2.92	0.79	0.42	0.94	0.13	0.26	8.25
PS4	cooking by stir-frying	0.94	0.10	7.46	15.19	0.78	0.37	5.20	2.10	0.10	8.44
PS5	carbonizing by stir-frying	0.60	0.18	6.47	6.86	0.99	0.15	4.38	3.34	0.35	1.78
PS6	stir-baked with soil	0.81	0.11	6.73	14.75	0.56	0.33	5.23	1.95	0.11	7.20

ND = not detected.

described in sample preparation section. Results indicated that the developed analytical method was accurate and reliable for determination of 10 compounds with the overall recovery in the range of 92.0–104.1%. Thus, the extraction procedure was shown to give excellent recovery of analytes.

Application to Real Samples

To demonstrate applicability of the proposed method, 6 processed products and 27 commercial samples from different batches were analyzed. Each sample was analyzed and the mean contents were summarized in Table 3. The results showed that the contents of main components varied to different extents in the commercial herbs and different processed products of RPA. The quantitative results demonstrate that all compounds in the 27 samples from different provinces showed obvious variations because of different origins, sources, cultural manner, harvest time, and manufacturing processes. It is also suggested that we should further study pharmacological effects of variously processed RPA in order to guide their clinical use. These results indicate that it is important to establish strict quality control standards to ensure the quality of the final preparations.

CONCLUSION

In this study, a HPLC-MS method was developed for quality evaluation of RPA, through simultaneous qualitative and quantitative analysis of tannins and monoterpene glycosides. The total analysis time on the short 1.8 μm particles column is about 10 min without a loss in resolution, which provides similar excellent performance with the special ultra fast HPLC system. Usage of HPLC-MS has become the most preferred choice for analysis of complex herbal medicines, and is likely to increase exponentially in the near future owing to its structural information, high selectivity, and sensitivity. Compared with other MS instruments, quadrupole MS has more potential for routine analysis and quality control of herbal medicines due to its relative low cost, availability, easy operation, and high precision for quantification. It can be foreseen that the combined use of HPLC and quadrupole MS, without the need to upgrade the existing HPLC system to special systems, will be an impressive alternative for analysis of various components in complex natural matrices as well as quality control purposes.

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