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Simultaneous determination of seven major triterpenoids in *Pyrola decorata* H. Andres by LC-MS method

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A liquid chromatography-mass spectrometry method was developed and validated for the quantitative determination of seven triterpenoids, 3-β-*O*-α-L-arabinopyranosylsiaresinolic acid-28-*O*-β-D-glucopyranosyl ester, ziyuglycoside I, pomolic acid, maslinic acid, colosic acid, oleanolic acid and ursolic acid in *Pyrola decorata* H. Andres. Chromatographic separation was achieved on a Hypersil C_{18} column using isocratic elution followed by a linear gradient elution of methanol and water as mobile phase. The analytes were ionized by atmospheric pressure chemical ionization source and determined on selected ion monitoring mode. All analytes showed good linearity ($r^2 \geq 0.9984$) within the test ranges and the recovery rates were 94.5% – 103.3%. Satisfactory precision and reproducibility were obtained with relative standard deviation less than 5%. The method was simple, accurate and performed well in application to the determination of twenty commercial samples of *P. decorata* collected from different regions of China. It could be used for the quality control of both plant materials and preparations of *P. decorata*.

1. Introduction

The herb of *Pyrola decorata* H. Andres is an important traditional Chinese medicine (TCM). It is recognized as "Lu xian cao" in Chinese and has been officially listed in the Chinese pharmacopoeia (Pharmacopoeia of PR China 2010). *P. decorata* extract has been confirmed by various studies to possess a wide range of pharmacological activities, such as anti-inflammatory and analgesic effects against rheumatoid arthritis, myocardial protective and vasodilating effects, antibacterial activity, as well as inhibitory effects of platelet aggregation and histamine release (Duan et al. 1992; Kosuge et al. 1985; Wang 1989; Yang et al. 1989; Kagawa et al. 1992; Aoyagi et al. 1998).

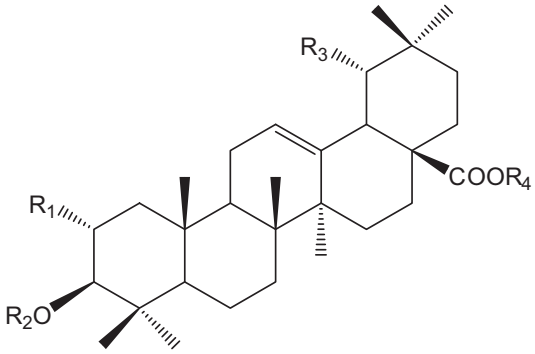
P. decorata contains a number of constituents including ursane and oleanane type triterpenoids, flavonoids, hydroquinones, naphthoquinones, phenolic glycosides, and so on. It has been reported that ursolic acid (UA), the major triterpenoid in *Pyrola* species, is one of the active constituents contributing to the anti-inflammatory and analgesic effects of Pyrolaceae (Kosuge et al. 1985). Moreover, UA, oleanolic acid (OA) and their derivates are considered as the active principles of many medicinal plants with hepato-protective, anti-tumor and anti-HIV properties (Kinjo et al. 1999; Tian et al. 2006; Ma et al. 2005; Ryu et al. 1994; Ma et al. 1998). As part of our study on cytotoxic constituents in *P. decorata*, we isolated several triterpenoid compounds from the ethyl acetate soluble fraction of the crude 95% ethanol extract of *P. decorata* by bioassay guided fractionation and purification method (Chen et al. 2006). Since triterpenoids are the major constituents responsible for the pharmacological activities of *P. decorata*, using them as the "marker compounds" for the chemical evaluation or quality standardization of this medicinal herb is reasonable. However, no studies on the determination of triterpenoid compounds in either *P. decorata* plant material or related preparations are available so far. An analytical method for the quantitative determination of the major triterpenoids in *P. decorata* is therefore required for the quality control and effective clinic use of this medicinal herb.

Several previous papers have described the determination of triterpenoids in other natural medicines by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection, gas chromatography or capillary electrophoretic method (Singh et al. 2002; Zhao et al. 1998; Guo et al. 2005). These methods are subject to problems of lower selectivity, complex sample derivatization procedure and lack of applicability in routine analysis. Liquid chromatograph combined with mass spectrometry (LC-MS) method has been gradually used for the analysis of various compounds in TDMs by virtue of good specificity and high sensitivity. In this study, an original LC-MS method was developed for the simultaneous determination of seven major triterpenoids in *P. decorata*. The target triterpenoids are 3-β-*O*-α-L-arabinopyranosylsiaresinolic acid-28-*O*-β-D-glucopyranosyl ester (ASGE), ziyuglycoside I (3-β-*O*-α-L-Arabinopyranosylpomolic acid-28-*O*-β-D-glucopyranosyl ester, APGE), pomolic acid (PA), maslinic acid (MA), colosic acid (CA), OA and UA. Their structures are listed in Fig. 1. A validation study proved that the method was simple, accurate, and reliable. The proposed method was successfully applied to the determination of twenty commercial samples of *P. decorata* collected from different regions of China.

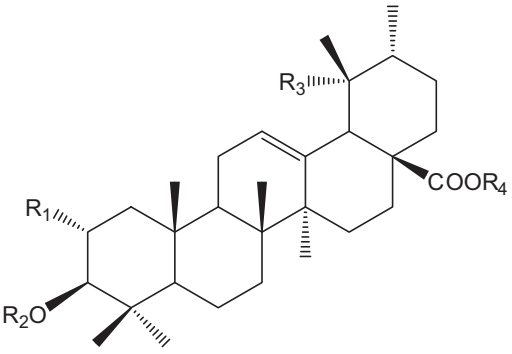
2. Investigations, results and discussion

2.1. Optimization of extraction solvent

As shown in Fig. 1, the analytes involve both triterpene sapogenins and triterpenoid saponins. These two types of com-



	R_1	R_2	R_3	R_4
ASGE	H	Ara	OH	Glc
MA	OH	H	H	H
OA	H	H	H	H



	R_1	R_2	R_3	R_4
APGE	H	Ara	OH	Glc
PA	H	H	OH	H
CA	OH	H	H	H
UA	H	H	H	H

Fig. 1: Structures of the seven triterpenoids

pounds differ greatly in physico-chemical properties such as polarity and solubility. On the one hand, the sapogenins, namely PA, MA, CA, OA and UA, are hydrophobic and prone to be dissolved in non-polar solvents such as chloroform and diethyl ether. On the other hand, the saponins, namely APGE and ASGE, are comparatively hydrophilic and readily soluble in aqueous ethanol or aqueous methanol for the existences of sugar chains in the molecules. For this reason, it was necessary to select an extraction solvent suitable for both types of compounds. Ethanol, ethyl acetate and diethyl ether are the recommended extraction solvents for the determination of OA and UA in the Chinese pharmacopoeia. In previous studies, triterpenoid saponins were often extracted with 70%−80% ethanol (Chen et al. 2005; Wei et al. 2000; Ren et al. 2005). In this study, diethyl ether, ethyl acetate, and 70%, 90%, and 100% ethanol aqueous solutions (v/v) were used to screen the optimal solvent for the extraction of the triterpenoids in a *P. decorata* Herb sample from Hangzhou city (sample No. 8) was used for optimization. Extraction procedure was the same as mentioned above except for the variety of extraction solvent.

The results shown in Table 1 indicated that the extraction efficiencies of individual analytes were quite variable depending on the solvent. For APGE and ASGE, 70% ethanol was the most suitable solvent. However, the contents for all other compounds increased up to 90% ethanol concentration and the contents of ASGE and APGE only decreased by about 10% from 70% to 90% ethanol. The contents of the saponins were significantly lower in diethyl ether and ethyl acetate contrary to those of the sapogenins. At last, 90% ethanol was selected as the extraction solvent with consideration of the maximum total extraction content and good extraction efficiencies for the compounds of minor contents.

2.2. Optimization of MS detection

HPLC with UV detection was attempted for determination during the early stage of method development. Owing to the absence of chromophores in the analytes, the detection was done at the non-specific wavelength of 210 nm. But at this wavelength, baseline resolution of the analytes and pigments or other constituents in the extract could not be achieved even by optimizing the separation conditions. UV detection was also failed to detect the minor contents of ASGE, APGE, PA, and MA for the existence of high background noise.

LC-MS method with SIM acquisition provides good selectivity, which simplifies the sample pretreatment procedure. In the present study, the analytes could be quantified with no co-elution interference using this method. APCI source was chosen as the ionization source because reduced analysis time and better peak shape were obtained in the APCI mode compared with the ESI mode whose flow rate was usually limited to less than 0.3 mL/min. For all analytes, the ionizations were more pronounced and the signal intensities were higher using negative ion mode than positive mode. Therefore, the APCI source was operated in the negative ion mode.

2.3. Optimization of LC conditions

Three groups of structural isomers are existing in the analytes. Namely, ASGE and APGE have a nominal molecular weight of 766; PA, MA, and CA have a nominal molecular weight of 472, whereas OA and UA have a nominal molecular weight of 456. The isomers ionized to identical ions and thus needed to be separated chromatographically. However, it was a bit difficult to separate these isomers with very similar chemical structures. Several different analytical columns, mainly C_{18} columns from various manufacturers were tested for better performance and assurance of consistent quality. As a result, the Hypersil C_{18} column gave the best resolution for OA and UA which desired increased resolution specially. This column was also capable of providing good reproducibility and consistent retention of all analytes throughout the study.

Methanol, which showed better selectivity to all analytes than acetonitrile, was employed as the organic mobile phase. Since the chromatographic characteristics of triterpene sapogenins and triterpenoid saponins differ greatly from each other, constant mobile phase could not lead an effective separation of all analytes within a relatively short analysis time. In this study, it was decided to use two sections of elution. The isocratic elution was firstly used in order to separate ASGE and APGE. The following linear gradient elution ensured not only satisfactory separation of the left five analytes but also a reasonable analysis time of 70 min, including re-equilibration. Typical chromatograms of the mixed standard and *P. decorata* extract are shown in Fig. 2. The chromatogram presented no baseline drift as occurred commonly in UV detection. Typical retention times of ASGE, APGE, PA, MA, CA, OA, and UA were approximately 15.1, 16.8, 24.8, 30.2, 31.7, 41.5 and 48.9 min, respectively.

Table 1: Effect of solvent on the extraction of the seven major triterpenoids from *P. decorata*

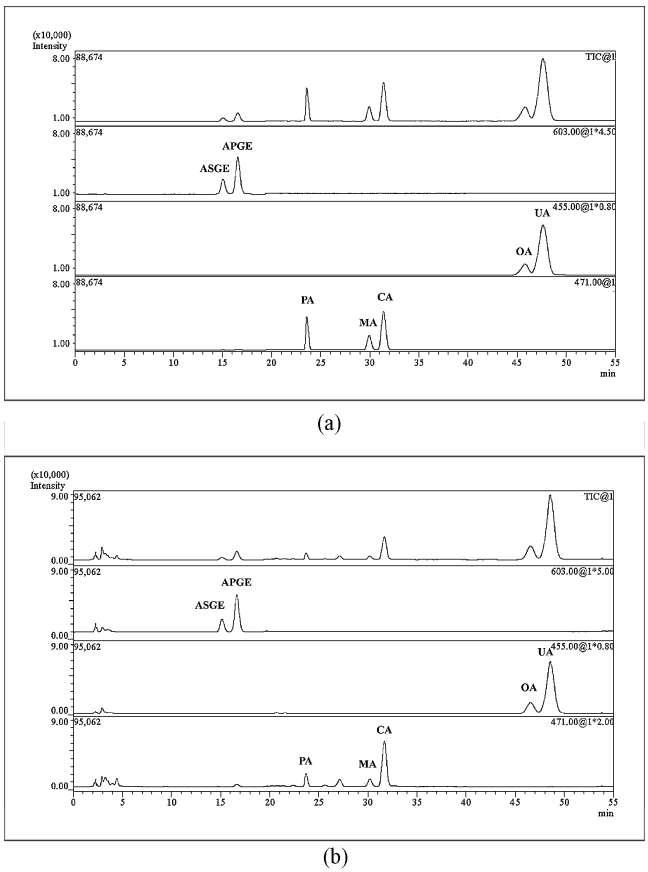
Solvent	Content (mg/g)							
	Compound							
	ASGE	APGE	PA	MA	CA	OA	UA	Total
Diethyl ether	0.037	0.047	0.052	0.043	0.402	0.942	3.373	4.896
Ethyl acetate	0.048	0.052	0.075	0.070	0.426	1.137	4.086	5.893
70% Ethanol	0.101	0.216	0.081	0.077	0.418	0.712	2.755	4.360
90% Ethanol	0.090	0.201	0.093	0.106	0.631	1.074	4.130	6.325
100% Ethanol	0.066	0.159	0.089	0.094	0.602	1.105	4.199	6.313

Table 2: Results of linear regression and LOQ

Compound	Concentration range (μg/mL)	Regression equation	r^2	LOQ (μg/mL)
ASGE	0.125−2.50	$y = 108127x + 1340.8$	0.9991	0.040
APGE	0.25−5.00	$y = 150814x - 5154.5$	0.9992	0.040
PA	0.50−10.00	$y = 175510x - 6634$	0.9989	0.025
MA	0.50−10.00	$y = 149374x - 1781.3$	0.9988	0.025
CA	1.25−25.00	$y = 157848x - 3461.7$	0.9995	0.025
OA	1.25−25.00	$y = 100198x + 6477.5$	0.9984	0.040
UA	5.00−100.00	$y = 110479x + 8530.1$	0.9988	0.040

2.4. Method validation results

As shown in Table 2, all analytes exhibited good linearity with correlation coefficient $r^2 \geq 0.9984$ in the concentration ranges. LOQs for ASGE, APGE, PA, MA, CA, OA and UA were 0.045, 0.045, 0.030, 0.030, 0.030, 0.050, and 0.050 μg/mL, respectively. The wide linear range and high sensitivity of the method allowed its application to various samples. The results of precision and reproducibility are shown in Table 3, indicating that precision and reproducibility for all analytes were very tight (1.21%−4.53% RSD).As shown in Table 4, the recoveries for all analytes were satisfactory at all of the three concentrations levels with RSD less than 5%.



(a)

(b)

Fig. 2: LC-MS chromatograms of mixed standards (1) and *P. decorate* sample No. 8 (2)

2.5. Application to P. decorata samples

As the results presented in Table 5, the LC-MS method described here was successfully applied to the determination of triterpenoids in twenty commercial samples of *P. decorata* collected from different regions of China. All the seven triterpenoids were detected and UA appeared to be the most abundant triterpenoid in all of the twenty samples. However, the quantity ratio of these triterpenoids and the content of each triterpenoid varied significantly among different samples. This might be caused by many reasons such as plant origins, collecting season, drying process and storage conditions. Since the composition and contents of bioactive compounds influenced the pharmacological activities of TCM directly, the quality of *P. decorata* could be effectively evaluated by means of quantitative determination of the seven major triterpenoids using the proposed LC-MS method.

2.6. Conclusions

In spite of the fact that triterpenoids contribute significantly to the bioactivity of *P. decorata*, no study was reported so far for the quantitative determination of triterpenoid compounds in this herbal medicine. Here, we report for the first time an LC–MS method for the simultaneous determination of seven major triterpenoids in the 90% ethanol extract of *P. decorata*. The method performed well in the validation study and was successfully applied to the evaluation of twenty *P. decorata* samples. Since it is a simple, accurate, and reliable method, we expect that it might be used in the future for the standardization and quality control of the medicinal herb *P. decorata* and products containing it.

Table 3: Precision and reproducibility of the seven major triterpenoids in *P. decorata* (n = 5)

Compound	Precision		Reproducibility	
	Mean ± SD (μg/mL)	RSD (%)	Mean ± SD (mg/g)	RSD (%)
ASGE	1.01 ± 0.03	2.80	0.093 ± 0.003	3.43
APGE	1.99 ± 0.04	2.02	0.200 ± 0.009	4.53
PA	3.98 ± 0.09	2.37	0.092 ± 0.003	3.33
MA	4.02 ± 0.09	2.15	0.108 ± 0.004	3.52
CA	10.10 ± 0.12	1.21	0.630 ± 0.014	2.27
OA	10.14 ± 0.29	2.89	1.075 ± 0.045	4.18
UA	40.22 ± 0.97	2.43	4.116 ± 0.076	1.85

3. Experimental

3.1. Materials and reagents

Dried herb samples of *P. decorata* were purchased from drug stores or markets in different regions of China and identified by Professor Qishi Sun (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). HPLC-grade methanol was obtained from Caledon Laboratories Ltd. (Georgetown, Canada). Distilled water, prepared from demineralized water, was used throughout the study. All the other reagents were of analytical grade.

The standards of the seven triterpenoids were isolated by the author from *P. decorata*. The structural characterizations were done by ^{1}H NMR, ^{13}C NMR and IR analyses and the spectra coincided well with the data reported for ASGE (Mimaki et al. 2001), APGE (Yosioka et al. 1971; Qin et al. 1986), PA (Li et al. 2003), MA (Taniguchi et al. 2002), CA (Taniguchi et al. 2002), OA (Liu et al. 1999) and UA (Liu et al. 1999). Purity analysis proved that their purities were all above 98%.

3.2. Instruments and analytical conditions

The analysis was performed on a LC-MS 2010A system (Shimadzu Co., Kyoto, Japan) consisting of an LC-10ADvp connected to a single quadrupole MS analyzer with an interface usable for either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) source. The LC separation was carried out on a Hypersil C_{18} column (250 × 4.6 mm, 5 μm, Yilite Co., Dalian, China) which was preceded by a guard C_{18} column (10 × 4.6 mm, 5 μm, Zhonghuida Co., Dalian, China). Isocratic elution followed by a linear gradient elution with methanol (A) and water (B) was used according to the following profile: 0−16 min, 60% A, 40% B; 16–55 min, 60–80% A, 40–20% B. Re-equilibration duration was 15 min between individual runs. The column oven was set at 30 °C and the flow rate was 0.8 mL/min. The injection volume was 5 μL.

Samples were ionized by APCI probe in negative ion mode under the following source conditions: nebulizing gas flow: 2.5 L/min; interface temperature: 400 °C; heat block temperature: 200 °C; CDL temperature: 250 °C; detector voltage: 1.6 kV; drying gas flow: 2.0 L/min. Quantitation was carried out in selected ion monitoring (SIM) mode with specific m/z at 603 for ASGE, APGE $[M-H-glc]^-$, 471 for PA, MA, CA $[M-H]^-$ and 455 for OA, UA $[M-H]^-$, respectively. Peak areas for all analytes were automatically integrated using Shimadzu LC-MS Solution Version 3.0 software.

Table 4: Recovery of the seven major triterpenoids in *P. decorata* (n = 3)

Compound	Added (g/mL)	Detected (g/mL)	Recovery (%)	RSD (%)
ASGE	0.50	0.50	100.67	4.02
	1.00	0.96	96.17	2.11
	1.25	1.22	97.60	3.36
APGE	1.00	0.97	96.83	3.16
	2.00	1.92	96.00	1.46
	2.50	2.53	101.33	2.58
PA	2.00	1.89	94.50	4.59
	4.00	4.07	101.75	3.08
	5.00	5.04	100.73	1.30
MA	2.00	1.93	96.33	3.38
	4.00	4.11	102.67	1.10
	5.00	4.99	99.80	3.16
CA	5.00	5.12	102.47	2.06
	10.00	9.90	99.03	4.50
	12.50	12.32	98.59	4.16
OA	5.00	4.94	98.87	1.75
	10.00	10.33	103.30	3.20
	12.50	12.65	101.23	2.66
UA	20.00	20.40	102.02	2.17
	40.00	40.46	101.14	1.36
	50.00	50.04	100.09	2.59

3.3. Extraction procedure

About 0.25 g of pulverized *P. decorata* (40 mesh) was extracted by refluxing with 10 mL of 90% ethanol for 1 h and then filtered. The extraction was repeated twice. After evaporating the extracted solution to dryness with reduced-pressure evaporation, the residue was dissolved in 25 mL of methanol using a volumetric flask. The obtained solution was filtered through a 0.45 μm millipore filter prior to injection.

3.4. Preparation of standard solutions

Individual primary stock solutions for the analytes were prepared by dissolving accurately weighed amounts of the standards in methanol. Mixed stock solution was prepared by combining an appropriate amount of each primary stock solution and diluting with methanol. The mixed stock solution was further diluted to make mixed working solutions with methanol. All solutions were stored at 4 °C and filtered with a 0.45 μm millipore filter before LC-MS analysis.

3.5. Method validation

Calibration curves were calculated by plotting the peak areas of mixed calibration solutions versus corresponding concentrations. Mixed calibration solutions were at six concentration levels over the range of 0.125−2.50 μg/mL for ASGE, 0.25−5.00 μg/mL for APGE, 0.50−10.00 μg/mL for PA, 0.50−10.00 μg/mL for MA, 1.25−25.00 μg/mL for CA, 1.25−25.00 μg/mL for OA and 5.00−100.00 μg/mL for UA. Linear regression analysis was performed by the external standard method. The value of limit of quantitation (LOQ) was calculated as the analyte concentration which gave rise to peak whose height was at least 10 times the baseline noise.

The precision of the LC-MS method was evaluated by five replicate injections of the mixed standard solution at the concentration of 1.00 μg/mL (ASGE), 2.00 μg/mL (APGE), 4.00 μg/mL (PA), 4.00 μg/mL (MA), 10.00 μg/mL (CA), 10.00 μg/mL (OA), and 40.00 μg/mL (UA). In order to test the reproducibility of the extraction procedure, one sample from Hangzhou city (sample No. 8) was extracted independently for five times. The precision and reproducibility were measured by relative standard deviation (RSD).

In the recovery test, it involved the addition of known amounts of the mixed standard solutions to 0.125 g of *P. decorata* sample from Hangzhou city (sample No. 8). The fortified samples were then extracted as described in the section "Extraction procedure" and determined with the LC-MS method. The added mixed standard solutions were prepared in the concentration range of calibration curve (high, middle and low) and triplicate experiments were done at each level. The ratio of detected and added amount was used to calculate the recovery.

Table 5: Contents of the seven major triterpenoids in twenty commercial samples of *P. decorata*

No.	Origin	Content (mg/g)						
		Compound						
		ASGE	APGE	PA	MA	CA	OA	UA
1	Shijiazhuang Hebei	0.044	0.046	0.127	0.164	0.530	0.814	1.126
2	Heyang Hunan	0.038	0.059	0.092	0.068	0.245	0.767	2.685
3	Yinchuan Gansu	0.034	0.088	0.139	0.083	0.412	0.631	2.017
4	Nanchang Jiangxi	0.072	0.089	0.082	0.122	0.288	0.497	3.108
5	Kunming Yunnan	0.021	0.075	0.071	0.069	0.179	0.253	0.832
6	Datong Shanxi	0.055	0.072	0.090	0.153	0.676	0.442	2.186
7	Wuhu Anhui	0.085	0.238	0.145	0.223	0.528	0.608	3.023
8	Hangzhou Zhejiang	0.096	0.201	0.093	0.104	0.631	1.087	4.117
9	Guiyang Guizhou	0.101	0.193	0.214	0.098	0.343	1.141	3.092
10	Shenyang Liaoning	0.056	0.121	0.133	0.392	0.996	1.035	4.424
11	Hefei Anhui	0.047	0.063	0.068	0.287	0.433	0.746	2.082
12	Haerbin Heilongjiang	0.040	0.164	0.103	0.160	0.195	0.382	1.578
13	Guangzhou Guangdong	0.025	0.066	0.142	0.091	0.739	0.903	3.493
14	Xian Shanxi	0.108	0.197	0.181	0.125	0.410	0.997	3.106
15	Shaoxing Zhejiang	0.062	0.226	0.098	0.107	0.587	1.353	4.577
16	Nanjing Jiangsu	0.060	0.101	0.105	0.134	1.106	0.853	3.418
17	Chengdu Sichuan	0.092	0.169	0.082	0.077	0.492	0.637	1.254
18	Changchun Jilin	0.042	0.061	0.079	0.102	0.866	0.779	2.097
19	Haikou Hainan	0.027	0.053	0.062	0.069	0.482	0.924	3.473
20	Zibo Shandong	0.071	0.085	0.115	0.103	0.449	0.966	2.840

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