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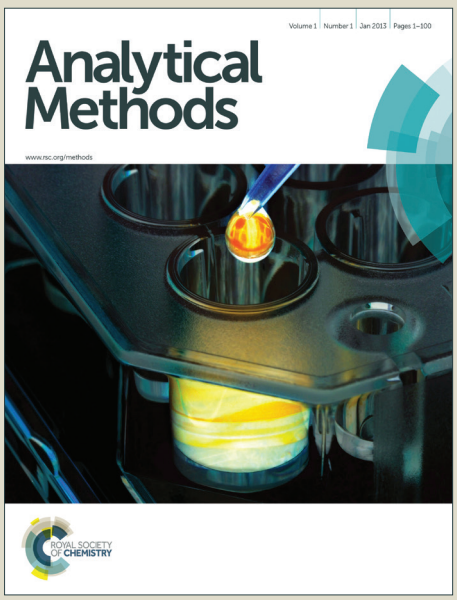

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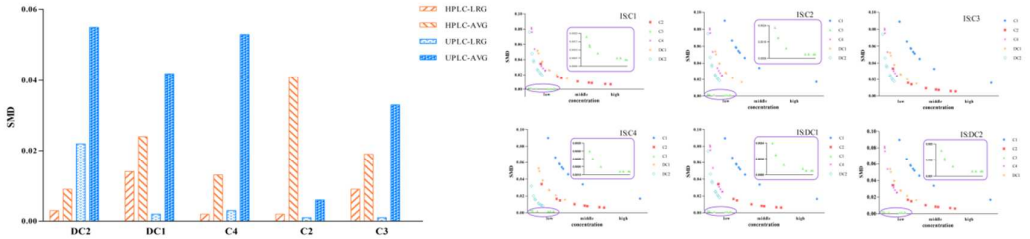
Graphical Abstract:



Linear regression method (LRG) had higher accuracy than average method (AVG) and the concentration of quantitative component was the major influencing parameter of the accuracy of QAMS method.

Systematic study on QAMS method for simultaneously determination of triterpenoid saponins in *Ilex Pubescens* by HPLC and UPLC

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Abstract

Quantitative analysis of multi-components with single marker (QAMS) method was firstly established for simultaneous determination of six triterpenoid saponins (ilexgenin A (C_1), ilexsaponin A1 (C_2), ilexsaponin B1 (C_3), ilexsaponin B2 (C_4), ilexsaponin B3 (DC_1), ilexoside O (DC_2)) in *Ilex Pubescens* by Ultra Performance Liquid Chromatography (UPLC) and High Performance Liquid Chromatography (HPLC), respectively. Using C1 as the internal reference, the relative correction factors (RCF) of the other five triterpenoid saponins were calculated and statistically evaluated. The durability of the method was verified with five different LC instruments and five different C_{18} columns under various chromatographic conditions. External standard method after methodology verification was chosen to check the accuracy and feasibility of QAMS method. The results showed that RCFs of all compounds with good reproducibility (RSD<5.0%) were obtained whether on different chromatography instruments or under various chromatographic conditions. The relative retention value method could be adopted for accurately position the chromatographic peak of the six constituents, with their values of RSD ranging between 0.5%~1.6%. Meanwhile, no significant differences were found in the quantitative results of the six saponins in 9 batches of medicines calculated by the QAMS method and external standard method, whether on HPLC system or UPLC system. The QAMS method established in our research for simultaneous determination of the six saponins is accurate and feasible to evaluate the quality of *Ilex pubescens*, especially when the standard substance is inconvenient to obtain.

Keywords: QAMS, *Ilex Pubescens*, UPLC, HPLC, triterpenoid saponins

1. Introduction

The root of *Ilex pubescens* Hook. et Arn (native name, Mao-dong-qing), is well-known Chinese herbal medicine commonly used in South China for treatment of cardiovascular disease and hypercholestaemia [1] as main ingredient in many formulae such as Mao-Dong-Qing injection, Jian-Xin-Kang tablet, Yan-Jian-Ning decoction, and the saponins were investigated to be the main active component. So far, more than 30 individual saponins in *Ilex pubescens* have been isolated and identified [2-4], which showed activities against hypertension, platelet aggregation, thrombosis, inflammatory atherosclerosis, cardiac ischemia, and so on. These inherent saponins work 'synergistically' and could be considered as 'marker compounds' for the chemical evaluation or standardization of *Ilex pubescens*.

There have been some reports on the quantification of saponins in Ilex pubescens using HPLC-UV [5-7], HPLC-ELSD [8-10] and HPLC-ESI/MS [11-12]. However, Most reports only selected one or two saponins as performance index for quantitative analysis due to reference substances except ilexgenin A were unavailable in market. Among these researches, even if a small number of published articles quantified the unusual saponins self-made in the laboratory in Ilex pubescens, the assay using external standard calculation method could not be widely applied due to lacks of reference substances still. With the development of modernization process of TCMs, simultaneous determination of multi-components for quality control of TCMs becomes more and more popular and acknowledged. So establishment of a convenient and easily available multi-component quantification method for Ilex pubescens is necessary and urgent. A quantitative analysis of multi-components by single marker (QAMS) was firstly proposed by Wang et al in China for the quality control of Akebiae Caulis [13]. The QAMS method is based on the principle that the quantity or concentration of the compounds was proportional to the response of detector within certain concentration ranges. The relative correction factor (RCF) and relative retention time (RT_R) of the components co-existing in TCMs is calculated by taking a typical active ingredient with

characteristic of sufficient abundance in chromatograms and easy availability as the internal reference substance (IS) according to formula (1).

$$RCF_x = \frac{f_x}{f_i} = \frac{A_x / c_x}{A_i / c_i} \quad (1) \qquad c_x = \frac{c_i}{RCF_x} \times \frac{A_x}{A_i} \quad (2) \qquad c_x \times RCF_x = \frac{A_x}{(A_i / c_i)} \quad (3)$$

where A_x and C_x represent peak area and concentration of the analyte, respectively. A_i and C_i are peak area and concentration of IS accordingly.

Formula (2) transformed from formula (1) indicates that once RCF_x for each target compounds is determined, simultaneous analyzing multi-components in TCMs is becoming feasible for QAMS method can overcome the bottleneck problem of lacks of reference substances.

By now QAMS method has been paid more attention by pharmacopoeia of Europe, India and American, and it has been adopted for quantitative analysis of multi-component from more than 20 kinds of herbals by American herbal pharmacopoeia [14]. HPLC and UPLC method has been widely introduced as a useful multi-component approach for quality control of TCM [15]. With the deeper understanding of QAMS method, Wang et al found that RCFs suffered from relatively large fluctuations at low concentration level, and thus established a novel RCF calculating method named LRG (linear regression) by using the linear relationship between c_x and $(A_x \times c_i)/A_i$ to calculate RCFs by linear regression (formula 3). The standard method difference (SMD) calculated according to LRG method was found to be lower than that calculated using the old one (average method, AVG method).

In our study, a simple and more easily popularized method namely QAMS for simultaneous quantification of six triterpenoid saponins in *Ilex Pubescens* by using HPLC and UPLC, respectively, was developed. By using C1 as internal reference substance, RCFs of other five components were calculated and systematically evaluated by investigating the influencing parameters including chromatographic conditions, standard solution concentration, chromatographic instrument and columns. Furthermore, a

novel RCFs calculation method namely LRG (linear regression) was also applied for data processing and evaluated. The present research would contribute to gain better understanding the potential use of QAMS method in other TCMs.

2. Materials and methods

2.1 Chemicals and reagents

The reference substances listed in Fig. 1 were isolated from Radix Ilex pubescens, and their structures were fully characterized by chemical and spectroscopic method in our laboratory. Purity analysis proved to be above 98% by using an area normalization method. Acetonitrile (HPLC grade) was obtained from Merck Co. and distilled water used was purchased from Huarun yibao drinks (China) Co. All other chemicals used were of analytical grade.

2.2 Plant materials

Nine Commercial herb samples of *Radix Ilex pubescens* were purchased from drug stores or markets in Guangdong, Guangxi, Jiangxi, Yunnan, Zhejiang and Henan province in China and authenticated by professor Jin-song Zhou from Guangzhou University of Traditional Chinese Medicine.

2.3 Instrument and Chromatographic conditions

Analyses were performed on (1) Agilent 1260, (2) Shimadzu LC-20A and（3）Waters Alliance liquid chromatographic system. Column used including Waters Symmetry C_{18} HPLC column (4.6 mm×250 mm, 5 μm), Phenomenex Synergi Hydro-RP C_{18} column (4.6 mm×250 mm, 5 μm) and Acchrom Unitary C_{18} HPLC column (4.6 mm×250 mm, 5 μm). Mobile phase was composed of 0.05% aqueous phosphoric acid (A) and acetonitrile (B) using a gradient elution of 25% B at 0-10 min, 25-30% B at 10-12 min, 30-35% B at 12-20 min and 35-65% B at 20-45 min. The flow rate was set at 1.0 $mL \cdot min^{-1}$ with temperature maintained at 25℃/30℃/35℃， and the injection volume was 10 μL. The detection wavelength was set at 210nm.

UPLC determination was carried out using (1) Waters Acquity UPLC system and (2) Thermo scientific UPLC system. YMC-Triart C_{18} UPLC column (2.0 mm×100 mm, 1.9 μm) and Waters Acquity HSS T3 UPLC column (2.1 mm×100 mm, 1.8 μm) was applied. The column temperature was maintained at 25℃/30℃/35℃, and the injection volume was 1 μL. Separation was achieved using the following gradient elution program of 0-2 min, 25% B, 2-4 min, 25-38% B, 4-6.5 min, 38% B, 6.5-10 min, 38-65% B, 10-11 min, 65-85% B, 11-13 min, 85% B. The flow rate of each column was set at 0.25 $mL \cdot min^{-1}$. The detection wavelength was set at 210nm.

2.4 Preparation of sample solutions

Approximately 1.0g of finely ground sample powder was extracted with 10ml methanol (A.R.) for 30min at room temperature for twice repeatedly. The extraction solution was combined after filtration, and diluted with methanol to a certain concentration equivalent to 0.04 $g \cdot mL^{-1}$ of the raw *Ilex Pubescens* material. The extract was filtered through a membrane filter (0.22μm pore size) prior to injection.

2.5 Preparation of standard solutions

The appropriate amounts of C1, C2, C3, C4, DC1 and DC2 were accurately weighed and dissolved in methanol separately to make the stock solutions (0.20 $mg \cdot mL^{-1}$ for C3, C4, DC1 and DC2, 0.30 $mg \cdot mL^{-1}$ for C2 and 1.00 $mg \cdot mL^{-1}$ for C1). Subsequently, different amounts of each stock solution were mixed and diluted to appropriate concentration ranges as working solution for construction of calibration curves and detection of RCFs. All stock solution and working solution were stored at 4℃.

3. Results and discussion

3.1 Method Validation

Calibration curves

A total of 20μL and 1μL of different concentration of mixed standard working solution were injected in HPLC and UPLC system, respectively, for the construction of calibration curves. The standard curves for

all six saponins showed good linearity with correlation coefficient higher than 0.9990 within the test ranges (Table 1).

Table 1.

Precision, Repeatability, Stability and Recovery test

The precision was examined from five consecutive injections of mixed standard working solution at low, middle and high concentration levels; **The repeatability** was tested by injecting six independently prepared samples, which were prepared according to the method outlined in the section of "2.4"; **Stability** was tested with sample solution that was stored at room temperature and analyzed at 3, 6, 9, 12 and 24h; **Recovery tests** were carried out to investigate the accuracy of the method by spiking mixed standard solution at middle concentration level to samples (six portions) with known content from the same batch. The radio of detected and added amounts was used to calculate the recovery. The results (Fig 1 and Table 2) suggested that the established UPLC and HPLC method meet the requirement for quantitative analysis.

Fig. 1

Table 2.

3.2 Durability of RCFs and RT_R of QAMS method

Effect of different instruments and column: Phenomenex synergi C_{18}, Waters symmetry C_{18} and Unitary C_{18} column (4.6 × 250 mm, 5 μm) were used to study the effect of different HPLC instrument and columm , and YMC-Triart C_{18} (2.0 mm×100 mm, 1.9 μm) and Waters Acquity HSS T_3 (2.1 mm×100 mm, 1.8 μm) UPLC column were applied for UPLC system. The results showed (Table 3 and Table 4) that RCFs and RT_R to internal standard had good reproducibility with RSD < 4.3%

Table 3

Table 4

Effect of different column temperatures: The SHIMADZU LC-20A system with phenomenex synergi C_{18} column and Waters Acquity UPLC system with waters acquity HSS T3 UPLC column were applied to investigate the effect of different column temperatures on RCFs and RT_R. The RSDs (summarized in Table 5 and Table 6) for all target compounds were less than 3.8%.

Table 5

Table 6

Effect of different concentration: Effect of concentration of target compound was examined from five consecutive injections of mixed standard working solution at low, middle and high concentration levels. The results (Table 7) indicated that concentration level had no influence on the RCFs with RDS <3.0%.

Table 7

3.3 Establishment of QAMS method for the quantitative analysis of six triterpenoid saponins in *Ilex Pubescens.*

Compared with external standard method, QAMS method was established to determine 9 batches of *Ilex Pubescens* using HPLC and UPLC method respectively (Table1 8 and 9). C1 was chosen as the internal referring substance. Meanwhile, different instruments, relative retention value (RT_R), concentration of target compound, columns as well as various column temperatures were investigated to evaluate the durability of RCFs. Standard method difference (SMD), calculated according to formula (4) was applied to evaluated LRG method and AVG method.

$$SMD = \frac{(C_{ES} - C_{QAMS})}{C_{ES}} \times 100\% \qquad (4)$$

c_{ES} and c_{QAMS} represent the concentrations of an analyte assayed by external standard method and QAMS method, respectively.

The results obtained by LRG and AVG method showed good similarity with content ratio of C2, C3, C4, DC1 and DC2 to that calculated by external standard method being of 94.7%~103.6%, 95.7%~102.4%, 96.6%~102.5%, 93.3%~103.7% and 92.2%~105.0% for HPLC analysis, and 99.6%~100.1%, 99.8%~100.1%, 99.5%~99.9%, 99.6%~100.0% and 94.8%~98.7% for UPLC analysis, respectively. The SMDs of LRG method were smaller than that of AVG method significantly both on the HPLC and UPLC determination (Fig. 2). LRG method was demonstrated to be higher accuracy and more suitable for the calculation of RCFs than AVG method in QAMS method.

Table 8 and 9

Fig. 2

The contents of 6 saponins from different areas exhibit an obvious difference in our study. However, the content of total saponins from 9 batches *Ilex Pubescens* was calculated to be similar (fig. 3), mostly in the range of 10~15 mg/g. Additionally, the content of C1 and C2 accounted for larger proportion (about 61% of the total saponins). So, C1 and C2 can be used as index components in the quality assessment of *Ilex Pubescens*.

Fig. 3

3.4 Investigation of internal standard and quantitative component concentration on accuracy of the LRG-QAMS

The concentration of internal standard reference and quantitative components (<0.05mg/ml, low; >0.10mg/ml, high) were investigated individually to evaluate their influence to the SMDs. As shown in Fig. 4 and Fig. 5, the concentration of internal standard reference had minimal influence on the SMD values. However, the SMDs got a clear downward trend when the concentration of the quantitative components increased, which verified the conclusion that came out from Wangs' research [15]. A high linear correlation between SMD and *1/C* was obtained (Table 10), which indicated that SMDs can be

controlled within 10.0% by setting exactly range of the concentration of quantitative components to confirm the reliability of QAMS method.

Fig. 4 and 5

Table 10

4. Conclusion

In the present study, a QAMS method was established and applied for the simultaneous determination of 6 triterpenoid saponins in 9 batches of *Ilex pubescens* by HPLC and UPLC. The contents of each target compounds were stable and similar in these two methods. Linear regression method was used to compare with the average method in order to evaluate the accuracy of the QAMS. The validation of the methodology and verification of durability and system suitability investigation performed in our research indicated that QAMS method possessed high accuracy and feasibility. The concentration of quantitative component was found to be the major influencing parameter of the accuracy of QAMS method. The method developed in this study will be useful for providing an efficient and feasible quality assessment of *Ilex pubescens* as well as other TCMs, which solves the problems associated with the absence of some rare standard substances.

5. Acknowledgments

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List of tables:

Table 1. Liner relationship about HPLC and UPLC

component	HPLC		UPLC	
	Standard curve	Liner range(mg/ml)	Standard curve	Liner range(mg/ml)
C1	y=5161765.26x-35641.61 (R^2=0.9982)	0.10-3.00	y = 2140.3x - 503.7 R^2 = 0.9998	0.04-1.20
C2	y=2964065.32x-5368.94 (R^2=0.9986)	0.03-0.90	y = 1267.2x - 242.29 R^2 = 0.9997	0.02-0.60
C3	y = 2447261.57x - 46.88 (R^2=0.9987)	0.03-0.90	y = 1083.6x + 39.43 R^2 = 0.9996	0.01-0.30
C4	y=2322875.23x-3993.13 (R^2=0.9988)	0.03-0.90	y = 977.48x - 113.09 R^2 = 0.9993	0.01-0.30
DC1	y=1572508.51x-3477.94 (R^2=0.9981)	0.03-0.90	y = 664.45x - 74.072 R^2 = 0.9996	0.02-0.60
DC2	y=1689464.90x-1805.33 (R^2=0.9986)	0.01-0.30	y = 733.42x - 402.85 R^2 = 0.9996	0.01-0.30

Table 2. Method validation test of HPLC and UPLC

component	HPLC					UPLC				
	Precision	Repeatability	Stability	Recovery		Precision	Repeatability	Stability	Recovery	
	RSD/%	RSD/%	RSD/%	x / %	RSD/%	(RSD/%)	（RSD/%）	(RSD/%)	x / %	RSD/%
DC2	0.6～1.7	0.2	2.6	94.1	1.8	0.3～3.2	1.5	1.2	94.2	2.5
DC1	0.5～1.6	2.3	1.1	93.3	4.0	0.6～2.7	3.5	0.4	97.9	3.9
C4	0.6～1.5	3.0	2.1	104.4	4.2	1.6～3.8	0.6	0.5	104.3	1.7
C2	1.2～1.8	4.9	1.9	103.4	4.4	0.1～0.6	0.5	0.4	101.2	4.3
C3	1.1～3.6	4.8	1.4	91.2	2.8	0.1～2.1	0.3	0.5	94.6	0.9
C1	1.1～3.0	0.9	2.5	108.7	4.3	0.1～0.5	0.1	0.7	105.4	2.1

Table 3. Effect of different HPLC instruments and columns upon RCF and RT_R

Instrument	column	RCF					RT_R				
		$f_{DC2/C1}$	$f_{DC1/C1}$	$f_{C4/C1}$	$f_{C2/C1}$	$f_{C3/C1}$	$RT_{R\ DC2/C1}$	$RT_{R\ DC1/C1}$	$RT_{R\ C4/C1}$	$RT_{R\ C2/C1}$	$RT_{R\ C3/C1}$
Waters e2695-2998	phenomenex synergi	0.327	0.305	0.450	0.574	0.474	0.380	0.415	0.621	0.651	0.678
	Waters symmetry C18	0.327	0.309	0.453	0.566	0.504	0.388	0.423	0.642	0.663	0.696
	Unitary C18	0.330	0.315	0.445	0.554	0.481	0.399	0.438	0.627	0.666	0.684
Agilent1260	phenomenex synergi	0.332	0.313	0.460	0.564	0.529	0.399	0.436	0.629	0.662	0.683
	Waters symmetry C18	0.346	0.312	0.466	0.586	0.519	0.406	0.442	0.648	0.672	0.700
	Unitary C18	0.323	0.320	0.456	0.563	0.515	0.399	0.438	0.627	0.666	0.684
LC-20A	phenomenex synergi	0.345	0.318	0.458	0.599	0.512	0.411	0.446	0.643	0.670	0.696
	Waters symmetry C18	0.349	0.335	0.457	0.587	0.518	0.415	0.451	0.663	0.681	0.714
	Unitary C18	0.347	0.333	0.451	0.575	0.531	0.411	0.446	0.643	0.670	0.696
	mean	0.336	0.318	0.455	0.574	0.509	0.401	0.437	0.638	0.667	0.692
	RSD	3.1%	3.9%	1.0%	2.8%	4.3%	2.9%	2.6%	2.0%	1.2%	1.6%

Table 4. Effect of different UPLC instruments and columns upon RCF and RT_R

Instrument	column	RCF					RT_R				
		$f_{DC2/C1}$	$f_{DC1/C1}$	$f_{C4/C1}$	$f_{C2/C1}$	$f_{C3/C1}$	$RT_{R\ DC2/C1}$	$RT_{R\ DC1/C1}$	$RT_{R\ C4/C1}$	$RT_{R\ C2/C1}$	$RT_{R\ C3/C1}$
Waters Acquity UPLC	waters acquity HSS T3	0.344	0.321	0.457	0.593	0.508	0.443	0.468	0.673	0.731	0.758
	YMC-Triart C18	0.345	0.322	0.474	0.579	0.513	0.454	0.479	0.707	0.742	0.777
Thermo scientific UPLC	waters acquity HSS T3	0.355	0.327	0.473	0.605	0.525	0.436	0.463	0.660	0.722	0.750
	YMC-Triart C18	0.381	0.348	0.500	0.607	0.554	0.446	0.471	0.689	0.729	0.768
mean		0.356	0.329	0.476	0.596	0.525	0.445	0.470	0.682	0.731	0.763
RSD		4.9%	3.8%	3.7%	2.2%	3.9%	1.7%	1.4%	3.0%	1.1%	1.5%

Table 5. Effect of column temperature upon RCF and RT_R (HPLC)

column temperature	RCF					RT_R				
	$f_{DC2/C1}$	$f_{DC1/C1}$	$f_{C4/C1}$	$f_{C2/C1}$	$f_{C3/C1}$	$RT_{R\ DC2/C1}$	$RT_{R\ DC1/C1}$	$RT_{R\ C4/C1}$	$RT_{R\ C2/C1}$	$RT_{R\ C3/C1}$
25℃	0.327	0.305	0.450	0.574	0.474	0.380	0.415	0.621	0.651	0.678
30℃	0.327	0.315	0.456	0.572	0.506	0.386	0.423	0.640	0.662	0.688
35℃	0.325	0.314	0.457	0.566	0.507	0.391	0.430	0.640	0.672	0.698
mean	0.326	0.311	0.454	0.571	0.496	0.386	0.423	0.634	0.662	0.688
RSD	0.5%	1.8%	0.8%	0.7%	3.8%	1.5%	1.7%	1.7%	1.6%	1.4%

Table 6. Effect of column temperature upon RCF and RT_R (UPLC)

column temperature	RCF					RT_R				
	$f_{DC2/C1}$	$f_{DC1/C1}$	$f_{C4/C1}$	$f_{C2/C1}$	$f_{C3/C1}$	$RT_{R\ DC2/C1}$	$RT_{R\ DC1/C1}$	$RT_{R\ C4/C1}$	$RT_{R\ C2/C1}$	$RT_{R\ C3/C1}$
25℃	0.335	0.307	0.453	0.588	0.508	0.443	0.468	0.673	0.731	0.758
30℃	0.336	0.309	0.458	0.590	0.506	0.445	0.472	0.681	0.740	0.764
35℃	0.339	0.315	0.450	0.567	0.513	0.452	0.479	0.698	0.753	0.773
mean	0.337	0.311	0.453	0.582	0.509	0.447	0.473	0.684	0.741	0.765
RSD	0.7%	1.4%	0.9%	2.2%	0.7%	1.1%	1.1%	1.9%	1.5%	1.0%

Table 7. Effect of concentration upon RCF

Concentration level	HPLC					UPLC				
	$f_{DC2/C1}$	$f_{DC1/C1}$	$f_{C4/C1}$	$f_{C2/C1}$	$f_{C3/C1}$	$f_{DC2/C1}$	$f_{DC1/C1}$	$f_{C4/C1}$	$f_{C2/C1}$	$f_{C3/C1}$
low	0.323	0.305	0.448	0.574	0.481	0.342	0.311	0.438	0.595	0.509
middle	0.322	0.304	0.454	0.578	0.480	0.338	0.311	0.455	0.593	0.515
high	0.326	0.304	0.454	0.577	0.480	0.344	0.313	0.463	0.594	0.510
mean	0.324	0.304	0.452	0.576	0.480	0.341	0.312	0.452	0.594	0.511
RSD	0.6%	0.2%	0.8%	0.4%	0.1%	0.9%	0.4%	2.8%	0.2%	0.6%

Table 8. Comparison of external standard method, LRG-QAMS method and AVG-QAMS method for *Ilex Pubescens* determination by HPLC

locations	External standard method (mg/g)						LRG-QAMS method (mg/g)										AVG-QAMS method (mg/g)									
	DC2	DC1	C4	C2	C3	C1	DC2		DC1		C4		C2		C3		DC2		DC1		C4		C2		C3	
							Content	LRG/ES	Content	LRG/ES	Content	LRG/ES	Content	LRG/ES	Content	LRG/ES	Content	AVG/ES	Content	AVG/ES	Content	AVG/ES	Content	AVG/ES	Content	AVG/ES
Guangdong1	0.59	1.17	0.58	3.08	0.49	3.51	0.59	99.0%	1.13	96.9%	0.56	97.4%	3.06	99.3%	0.48	98.8%	0.58	97.9%	1.12	96.0%	0.56	96.3%	3.20	103.7%	0.48	97.9%
Guangxi	0.73	2.06	1.38	3.08	2.12	3.17	0.73	100.4%	2.05	99.5%	1.41	102.5%	3.08	99.9%	2.11	99.4%	0.72	99.2%	2.03	98.6%	1.40	101.3%	3.22	104.2%	2.09	98.5%
Guangdong2	1.10	2.71	1.21	4.45	2.48	3.39	1.11	101.3%	2.71	99.8%	1.23	101.6%	4.45	100.0%	2.45	99.1%	1.10	100.1%	2.68	98.9%	1.22	100.5%	4.65	104.3%	2.43	98.1%
Yunnan	0.72	1.49	1.29	2.68	0.83	5.34	0.70	98.1%	1.44	96.2%	1.29	99.9%	2.61	97.4%	0.81	97.1%	0.70	96.9%	1.42	95.3%	1.27	98.8%	2.72	101.6%	0.80	96.2%
Guangdong3	0.33	1.06	1.51	1.52	2.14	10.00	0.30	92.2%	0.99	93.3%	1.49	98.9%	1.44	94.7%	2.05	95.7%	0.30	91.1%	0.98	92.4%	1.48	97.8%	1.50	98.8%	2.03	94.8%
Henan	0.84	2.73	1.02	4.74	2.29	2.42	0.86	102.6%	2.78	101.8%	1.05	103.0%	4.83	101.9%	2.31	100.9%	0.85	101.3%	2.75	100.8%	1.04	101.9%	5.04	106.4%	2.29	99.9%
Jiangxi	1.27	1.40	0.55	6.93	0.51	3.82	1.29	101.0%	1.36	97.3%	0.53	96.6%	6.90	99.6%	0.50	98.2%	1.27	99.8%	1.35	96.3%	0.52	95.6%	7.20	103.9%	0.50	97.3%
Guangdong4	1.39	1.27	0.54	6.76	0.38	2.79	1.43	103.1%	1.25	98.6%	0.53	98.1%	6.87	101.5%	0.38	100.1%	1.42	101.8%	1.24	97.7%	0.52	97.0%	7.16	105.9%	0.37	99.1%
Zhejiang	1.21	3.57	0.54	5.64	1.94	2.10	1.27	105.0%	3.70	103.7%	0.54	100.2%	5.85	103.6%	1.99	102.4%	1.26	103.8%	3.66	102.7%	0.53	99.1%	6.10	108.1%	1.97	101.4%

Table 9. Comparison of external standard method, LRG-QAMS method and AVG-QAMS method for *Ilex Pubescens* determination by UPLC

locations	External standard method (mg/g)						LRG-QAMS method (mg/g)										AVG-QAMS method (mg/g)							
	DC2	DC1	C4	C2	C3	C1	DC2		DC1		C4		C2		C3		DC2		DC1		C4		C2		C3	
							Content	LRG/ES	Content	LRG/ES	Content	LRG/ES	Content	LRG/ES	Content	LRG/ES	Content	AVG/ES	Content	AVG/ES	Content	AVG/ES	Content	AVG/ES	Content	AVG/ES
Guangdong1	0.58	1.17	0.48	3.28	0.53	3.47	0.57	97.4%	1.16	99.8%	0.48	99.5%	3.27	99.9%	0.53	100.0%	0.55	94.2%	1.12	95.8%	0.45	94.5%	3.25	99.4%	0.51	96.8%
Guangxi	0.75	2.29	1.19	3.31	2.12	3.09	0.73	98.0%	2.29	99.9%	1.19	99.9%	3.31	99.9%	2.11	99.9%	0.71	94.7%	2.19	95.9%	1.13	94.9%	3.29	99.4%	2.05	96.7%
Guangdong2	1.22	2.71	1.02	4.30	2.40	3.03	1.20	98.7%	2.71	99.9%	1.02	99.8%	4.30	99.9%	2.40	99.9%	1.16	95.4%	2.60	95.9%	0.97	94.9%	4.28	99.4%	2.32	96.7%
Yunnan	0.60	1.66	1.08	2.83	0.92	4.95	0.59	97.5%	1.65	99.8%	1.07	99.8%	2.83	99.8%	0.92	99.9%	0.57	94.2%	1.59	95.8%	1.02	94.8%	2.81	99.3%	0.89	96.6%
Guangdong3	0.28	1.02	1.26	1.67	2.00	9.92	0.26	94.8%	1.02	99.6%	1.25	99.8%	1.66	99.6%	2.00	99.8%	0.26	91.6%	0.98	95.6%	1.19	94.8%	1.65	99.1%	1.93	96.5%
Henan	0.73	2.75	0.88	4.42	2.06	2.00	0.72	98.1%	2.75	100.0%	0.88	99.9%	4.42	100.0%	2.06	100.0%	0.69	94.8%	2.64	96.0%	0.83	94.9%	4.40	99.5%	1.99	96.7%
Jiangxi	1.17	1.32	0.46	6.71	0.54	3.70	1.15	98.6%	1.32	99.8%	0.45	99.5%	6.71	99.9%	0.54	100.0%	1.11	95.3%	1.26	95.8%	0.43	94.5%	6.68	99.4%	0.52	96.7%
Guangdong4	1.23	1.32	0.45	6.27	0.42	2.59	1.21	98.7%	1.32	99.8%	0.45	99.5%	6.26	100.0%	0.42	100.1%	1.17	95.4%	1.26	95.8%	0.42	94.6%	6.23	99.5%	0.40	96.9%
Zhejiang	1.08	3.44	0.46	5.23	1.85	1.95	1.07	98.6%	3.44	100.0%	0.45	99.6%	5.23	100.1%	1.85	100.0%	1.03	95.3%	3.30	96.0%	0.43	94.6%	5.21	99.5%	1.79	96.8%

Table 10. Linear relationships between SMDs and the reciprocals of the concentrations of six analytes and applicable concentration ranges

analyte	Liner regression equation	Minimum value of concentration (mg/ml)
C2	$y = 0.0018x+0.0004$ $R^2=1$	1.81×10^{-2}
C3	$y = 0.000019x+0.000239$ $R^2=1$	1.90×10^{-4}
C4	$y = 0.0017x+0.000035$ $R^2=1$	1.70×10^{-2}
DC1	$y = 0.0022x-0.0012$ $R^2=1$	2.17×10^{-2}
DC2	$y = 0.0011x+0.0009$ $R^2=1$	1.11×10^{-2}

Titles for the figures:

Fig. 1 Chemical structures of the 6 saponins A and the chromatographs of the 6 components quantified in this study B as well as 6 *Ilex Pubescens* samples C

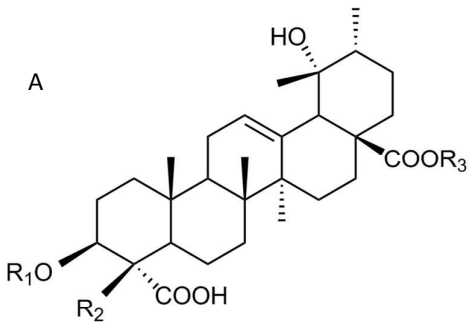
Fig. 2 Standard method difference (SMD) between LRG and AVG method of HPLC and UPLC

Fig. 3 content of total saponins in 9 batches *Ilex Pubescens* samples detected by HPLC and UPLC

Fig. 4 The standard method differences (SMDs) of C1 (C2, C3, C4, DC1, DC2) (A ~ F) in 9 batches Ilex Pubescens samples assayed by the LRG-QAMS method using other 5 components at different concentration level (<0.05mg/ml, low; >0.10mg/ml, high) as internal standard reference respectively

Fig. 5 The standard method differences (SMDs) of 6 saponins in 9 batches Ilex Pubescens samples assayed by the LRG-QAMS method calculated by the concentration of each component at different concentration level (<0.05mg/ml, low; >0.10mg/ml, high) using C1, C2, C3, C4, DC1 and DC2 (E~J) as internal standard reference respectively

Fig. 1



	R_1	R_2	R_3
1. Ilexoside O	-Xyl-Glc-Rha	CH_3	-Glc
2. Ilexsaponin B3	-Xyl-Glc	CH_3	-Glc
3. Ilexsaponin B2	-Xyl-Glc-Rha	CH_3	H
4. Ilexsaponin A1	H	COOH	-Glc
5. Ilexsaponin B1	-Xyl-Glc	CH_3	H
6. Ilexgenin A	H	COOH	H

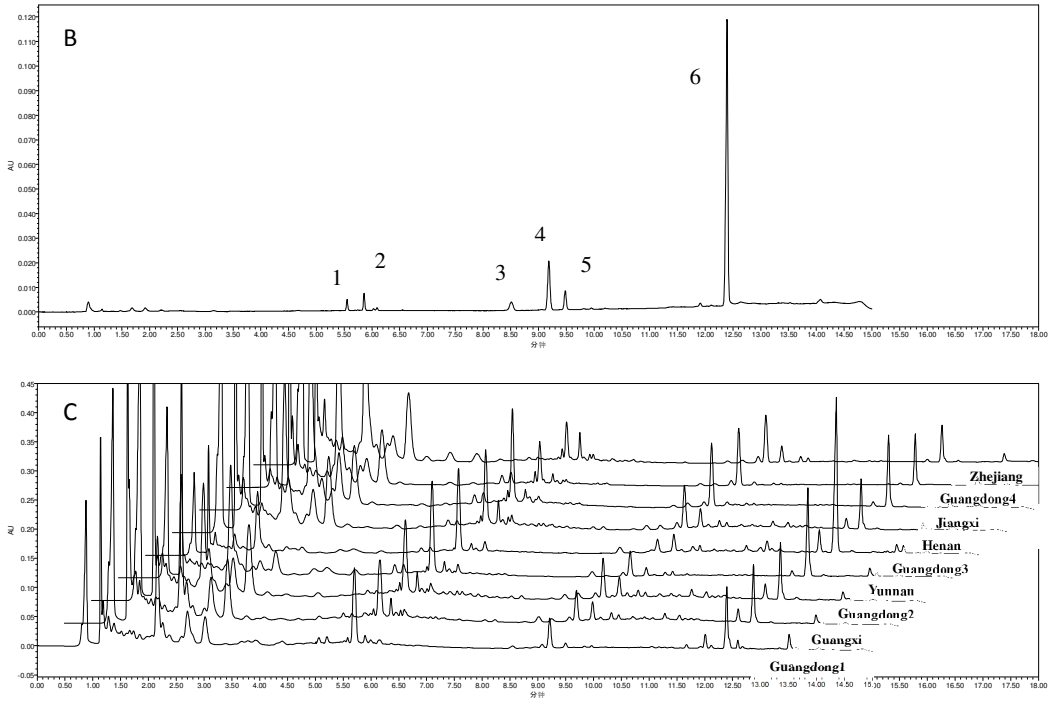
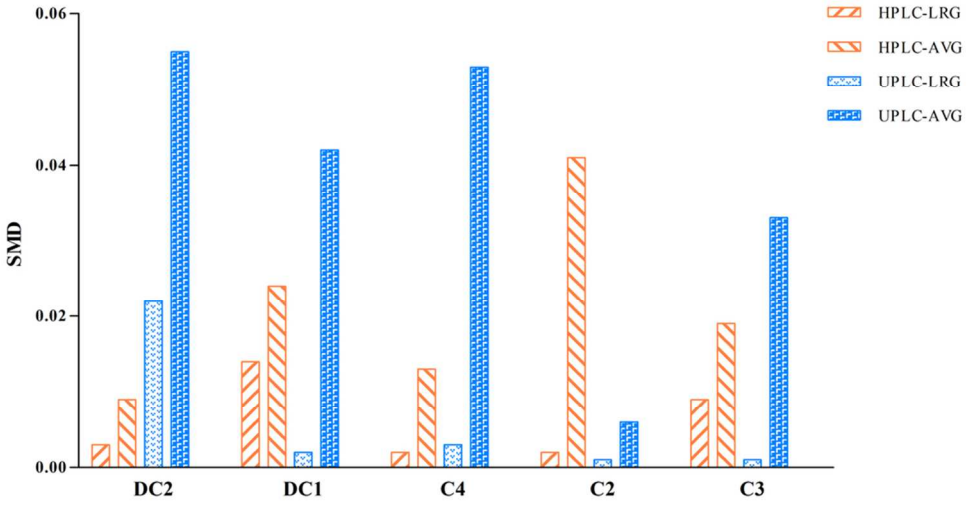


Fig. 1.Chemical structures of the 6 saponins A and the chromatographs of the 6 components quantified in this study B as well as 6 *Ilex Pubescens* samples C



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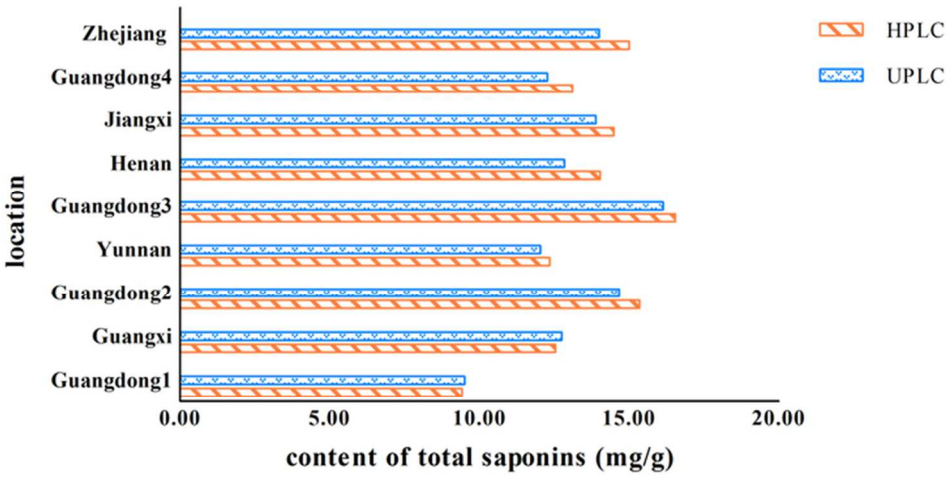

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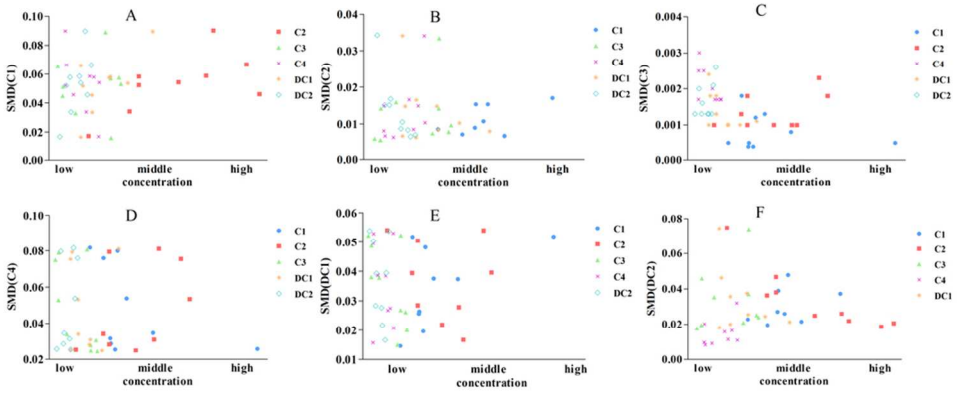

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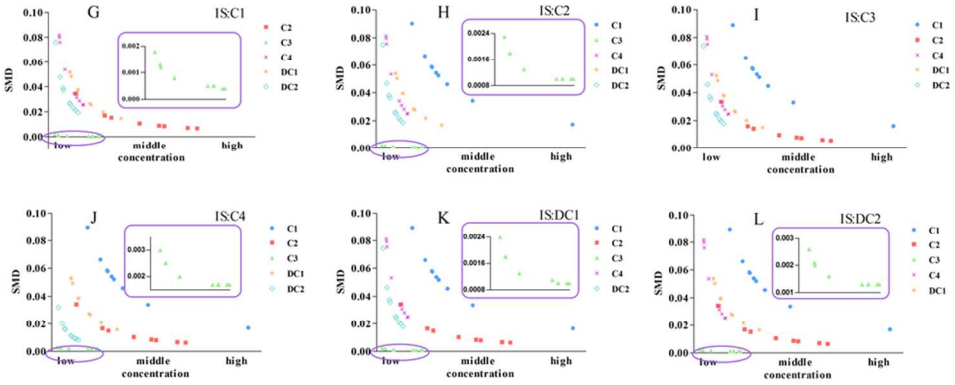

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