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A systematic study on the influencing parameters and improvement of quantitative analysis of multi-component with single marker method using notoginseng as research subject

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ABSTRACT

A new quantitative analysis of multi-component with single marker (QAMS) method for 11 saponins (ginsenosides Rg_1, Rb_1, Rg_2, Rh_1, Rf, Re and Rd; notoginsenosides R_1, R_4, Fa and K) in notoginseng was established, when 6 of these saponins were individually used as internal referring substances to investigate the influences of chemical structure, concentrations of quantitative components, and purities of the standard substances on the accuracy of the QAMS method. The results showed that the concentration of the analyte in sample solution was the major influencing parameter, whereas the other parameters had minimal influence on the accuracy of the QAMS method. A new method for calculating the relative correction factors by linear regression was established (linear regression method), which demonstrated to decrease standard method differences of the QAMS method from 1.20%±0.02% - 23.29%±3.23% to 0.10%±0.09% - 8.84%±2.85% in comparison with the previous method. And the differences between external standard method and the QAMS method using relative correction factors calculated by linear regression method were below 5% in the quantitative determination of Rg_1, Re, R_1, Rd and Fa in 24 notoginseng samples and Rb_1 in 21 notoginseng samples. And the differences were mostly below 10% in the quantitative determination of Rf, Rg_2, R_4 and N-K (the differences of these 4 constituents bigger because their contents lower) in all the 24 notoginseng samples. The results indicated that the contents assayed by the new QAMS method could be considered as accurate as those assayed by external standard method. In addition, a method for determining applicable concentration ranges of the quantitative components assayed by QAMS method was established for the first time, which could ensure its high accuracy and could be applied to QAMS methods of other TCMs. The present study demonstrated the practicability of the application of the QAMS method for the quantitative analysis of multi-component and the quality control of TCMs and TCM prescriptions.

1. Introduction

In recent years, the quantitative analysis of multiple major components in traditional Chinese medicines (TCMs) has been considered an effective approach for quality control purposes. However, this approach has been limited due to the need for high-purity (> 98%) commercial standard substances. To solve this problem, a method that uses single standard substances for the quantitative analysis of multi-component (termed quantitative analysis of multi-component with single marker, or QAMS) in TCMs was established and has been adopted toward the quality evaluation of Coptidis Rhizoma in the Chinese Pharmacopoeia 2010 edition. Additionally, several studies have reported the application of the QAMS method for the quantitative analysis of multi-component in TCMs and Chinese patent medicines [1–7] including rhubarb, Sanhuang Tablet, Angelicae Dahuricae Radix, Paeoniae Radix Rubra, Lonicerae Japonicae Flos etc.

Abbreviations: QAMS, quantitative analysis of multi-component by single marker; RCF, relative correction factor; LRG method, linear regression method for calculating RCFs; AVG method, average method for calculating RCFs; SMD, standard method difference (the difference between contents assayed by external standard method and QAMS method)

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The QAMS method is based on the principle of the linear relationship between a detector response and the levels of components within certain concentration ranges. The relative correction factor (RCF) of the components co-existing in a TCM is calculated using the standard substance of each analyte. For the QAMS method of a TCM, the component, the standard substance of which is inexpensive and can easily be obtained, is first chosen as the internal referring substance. The concentration of the internal referring substance in the TCM is determined using external standard method (namely standard curve method), whereas the concentrations (c) of the other analytes in the TCM are calculated according to Eq. (1) with both their RCFs (f_r) and peak areas (S).

$$c_x = \frac{S_x}{k_0 \times f_r} \tag{1}$$

where x and 0 represent the analyte and the internal referring substance, respectively, and k_0 is the slope of the standard curve of internal referring substance.

The utilization of QAMS method for the quality control of TCMs involves three steps, which are establishment, validation, and application of the method (Fig. 1). QAMS method should be established on the premise of both the high accuracy and wide applicability of the RCFs, as RCFs are the critical parameters that influence the accuracy of QAMS method. Approximately, RCF could be calculated by the ratio of slopes of the analyte's and the internal referring substance's standard curves (i.e. $f_r \approx k_n/k_0$), when their intercepts are small enough to be ignored (i.e. standard curve could be approximated as $S = k \times c$). However, this method has high requirement on standard curves, not to mention that the intercept of standard curve could not be ignored in most circumstances due to systematic and/or random errors. Therefore, usually, in most QAMS related studies, the final RCF of an analyte is calculated using the average of several RCFs from the internal referring substance and the analyte detected under multiple concentration levels [6], which would be referred to as average method (AVG method) in the following passages. However, this approach with AVG method suffers from fluctuations in the RCFs at different concentration levels, especially when the concentration level was low. Several parameters may cause the fluctuations in the RCFs, such as the experimental instrument, structural characteristics, the selection of the detection wavelength, the purities of the standard substances, and the retention indexes of the analytes [8]. Therefore, a comprehensive methodological investigation of QAMS method, including external parameters (e.g., environment, chromatographic instruments, columns and operators), and internal parameters (e.g., quantitative components, internal referring substances and chromatographic method) is necessary, in order to ensure the high repetitiveness and reliability of QAMS method. In addition, the standard curves of internal referring substances should be corrected before using the QAMS method.

Although many studies have reported the application of QAMS method for quantitative analysis of TCMs, very few focused on the accuracy or influencing parameters of QAMS method. According to Gao's review [8], several parameters may affect the accuracy of QAMS method. However, it didn't provide experimental information to illustrate which parameter was the major one, or how would those parameters affect QAMS method. And no other reports could be found focused on the accuracy of QAMS method. In Hou's study [6] the influence of several parameters on the convention factors (i.e. RCF) in QAMS method were investigated, including chromatographic conditions, standard solution concentration, chromatographic instrument and columns, however, the influence of these parameters on the accuracy of QAMS method was not discussed. Due to the growing application of QAMS method in the quality control of TCMs, further studies are needed to investigate the major influencing parameters of QAMS method and their influence intensities on the accuracy of QAMS method. Then corresponding methods could be established to control the influencing parameters. And if there were influence of some parameters which could not be ignored, an applicable range should be set to ensure the high accuracy of QAMS method.

Therefore, in the present study, the major influencing parameters (including the contents and structure types of quantitative markers and internal referring substances, and the purities of standard substances) and applicable ranges of QAMS method are studied with notoginseng and several saponins as investigation subjects and quantitative markers, respectively.

Notoginseng, which is derived from the dried root or rhizome of *Panax notoginseng* F.H. (Burk.) Chen, is used for either bleeding or promoting blood circulation, the major active components of which are saponins. Thus far, 37 panaxatriol(Ptriol)-type saponins and 56 panaxadiol(Pdiol)-type saponins have been isolated from notoginseng. In our previous study, a reliable fingerprint method

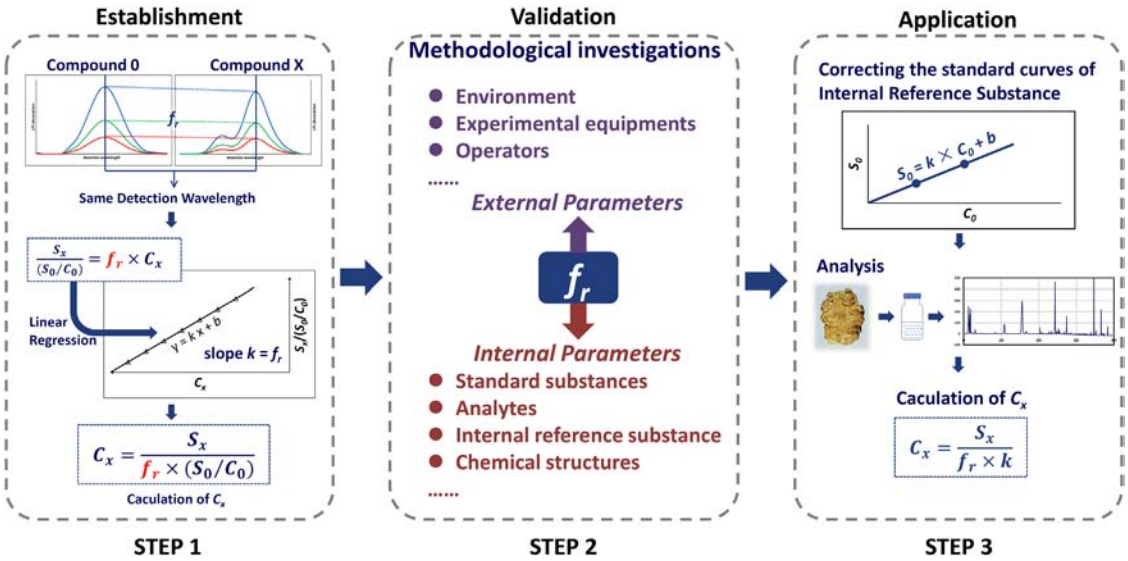


Fig. 1. Establishment, validation, and application of the QAMS method for the quality control of TCMs.

Table 1
The structure types, contents (%, w/w) in notoginseng (*P.N.*) samples, standard substance purities, standard curves, linear ranges, relative correction factors referring to Rg_1, concentration (mg mL^{-1}) calculation formulas, and applicable concentration range (ACR, mg mL^{-1}) of the 11 quantitative components.

Saponins' structure type	Contents in *P.N.* samples	Standard substance purities	Standard curves	Linear ranges (mg mL^{-1})	RCF		Concentration calculation formulas of QAMS				ACR of *LRG*-QAMS
					$f_{r\text{-}LRG}$	$f_{r\text{-}AVG}$	*LRG*-QAMS	RSD ($n=6$)	*AVG*-QAMS	RSD ($n=6$)	
Panaxatriol type											
Rg_1[a]	0.5–6%	99.8%	$y=4311.7x+2.2$	0.010–3.00			$C_{Rg1}=S_{Rg1}/4308.5$	1.08%	$C_{Rg1}=S_{Rg1}/4725.4$	6.14%	
Re	0.2–2%	99.3%	$y=3754.7x-5.0$	0.015–1.00	0.87	0.85	$C_{Re}=S_{Re}/3776.5$	1.86%	$C_{Re}=S_{Re}/3929.0$	6.66%	0.013–1.00
R_1[a]	0.2–2%	95.8%	$y=3599.8x+1.0$	0.015–1.00	0.83	0.83	$C_{R1}=S_{R1}/3626.7$	2.03%	$C_{R1}=S_{R1}/3941.7$	6.18%	0.003–1.00
Rh_1	< 0.2%	94.8%	$y=5135.9x+26.7$	0.038–0.20	1.19	1.34	$C_{Rh1}=S_{Rh1}/5157.7$	1.82%	$C_{Rh1}=S_{Rh1}/6246.6$	6.93%	0.052–0.20
Rf	< 0.2%	96.6%	$y=4848.2x+2.8$	0.0040–0.20	1.12	1.09	$C_{Rf}=S_{Rf}/4915.8$	1.50%	$C_{Rf}=S_{Rf}/5085.6$	6.38%	0.005–0.20
Rg_2[a]	< 0.2%	99.7%	$y=4575.4x-2.3$	0.006–0.20	1.06	0.93	$C_{Rg2}=S_{Rg2}/4677.9$	1.93%	$C_{Rg2}=S_{Rg2}/4220.3$	4.44%	0.007–0.034
Panaxadiol type											
Rb_1[a]	0.5–6%	98.2%	$y=3183.2x-38.0$	0.0030–3.00	0.74	0.71	$C_{Rb1}=S_{Rb1}/3208.6$	2.08%	$C_{Rb1}=S_{Rb1}/3343.0$	7.01%	0.159–3.00
Rd[a]	0.2–2%	92.3%	$y=3530.0x+17.9$	0.005–1.00	0.82	0.77	$C_{Rd}=S_{Rd}/3569.3$	1.95%	$C_{Rd}=S_{Rd}/3532.1$	5.82%	0.035–1.00
R_4[a]	< 0.2%	100%	$y=2982.0x-3.8$	0.006–0.20	0.69	0.62	$C_{R4}=S_{R4}/3015.9$	1.68%	$C_{R4}=S_{R4}/2818.8$	6.18%	0.013–0.20
N-K	< 0.2%	99.1%	$y=3566.5x+3.3$	0.006–0.20	0.82	0.82	$C_{N\text{-}K}=S_{N\text{-}K}/3616.1$	1.58%	$C_{N\text{-}K}=S_{N\text{-}K}/3797.0$	6.58%	0.017–0.20
Fa	< 0.2%	98.1%	$y=2839.1x+0.3$	0.006–0.20	0.66	0.63	$C_{Fa}=S_{Fa}/2882.1$	1.60%	$C_{Fa}=S_{Fa}/2911.1$	6.37%	0.006–0.20

[a] The component was chosen as the internal referring substance; The standard curves established in our previous study [10] were used; The concentration (mg mL^{-1}) calculation formulas are the average formulas when 6 components were used as internal referring substances individually ($n=6$); RCF–relative correction factor calculated with either the new method–LRG method ($f_{r\text{-}LRG}$) or the previous method–AVG method ($f_{r\text{-}AVG}$) referring to Rg_1, more data could be found in the Supplementary material; *LRG*-QAMS–QAMS method based on LRG method; *AVG*-QAMS–QAMS method based on AVG method; *C*–the concentration (mg mL^{-1}) of an analyte; *S*–the chromatographic peak area (mAU s) of an analyte.

of notoginseng was established, and the parameters that could affect the fingerprint were investigated [9]. Then, a method for the simultaneous determination of 11 saponins in notoginseng was also established [10], as well as a method for quantitative analysis of 5 saponins (ginsenosides Rg_1, Rb_1, Re, Rd, and notoginsenoside R_1) using QAMS method for the quality evaluation of notoginseng and its slices [11]. However, the contents of certain trace components (e.g., ginsenosides Rg_2, Rh_1, notoginsenosides R_4, Fa) in notoginseng were too low to obtain high-purity standard substances, making it difficult to quantitatively determine those components. Therefore, the establishment of QAMS method for the quantitative analysis of these trace components along with the major components is also especially necessary.

2. Materials and methods

2.1. Chemicals and reagents

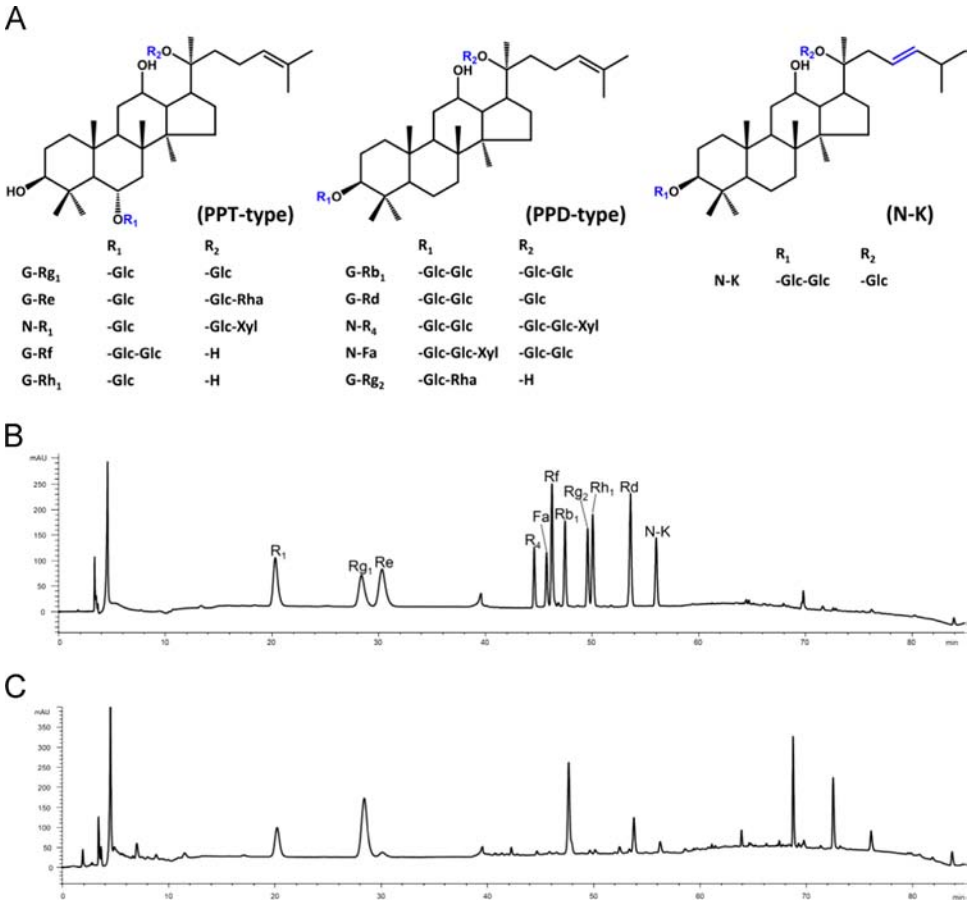
Ginsenosides Rg_1, Re, Rb_1, Rd, Rg_2, Rf, and notoginsenoside R_1 were purchased from the department of Organic Chemistry, College of Health Science, Jilin University. Ginsenoside Rh_1, notoginsenosides R_4, Fa and K(N-K) were provided by Prof. Katsuko Komatsu, Institute of Natural Medicine, University of Toyama, Japan. The purities of all the standard substances were tested using an area normalization method. The purities of Rg_1, Rb_1, Re, Rg_2, N-K, R_4 and Fa were all greater than 98%, and they were used as pure standards (i.e. purities considered as 100%) during the experiment (Table 1). Because the purities of R_1, Rd, Rf and Rh_1 were below 98%, their practical concentrations were calculated from their actual purities in the experiment. The structures of the 11 saponins are shown in Fig. 2. Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Deionized water was purchased from Wahaha Co. (Hangzhou, China). Formic acid (A.R.) was purchased from Tianjin Chemical Reagent Company (Tianjin, China). Methanol (A.R.) was purchased from Beijing Chemical Engineering Company (Beijing, China).

2.2. Plant materials

Twenty-four samples collected from Wenshan, Mengzi, and Yanshan Counties of the Yunnan Province in China were used in the present research (Table 2). The samples contained 10 three-year-old main root samples, 5 two-year-old main root samples, and 3 one-year-old main root samples, 3 rhizome samples (1 two-year-old sample and 2 three-year-old samples), and 3 fibrous samples (1 two-year-old sample and 2 three-year-old samples). All samples were identified as *Panax notoginseng* (Burk.) F.H. Chen by Prof. Shao-Qing Cai of Peking University, and their voucher specimens were deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Health Science Center, Peking University.

2.3. Chromatographic conditions

An Agilent 1100 (Agilent, USA) liquid chromatograph equipped with a binary gradient pump, a 10 μL sample loop and a diode array detector (DAD) system was used. The HPLC method from our previous study was used [10], which was developed using a reversed-phase column (Luna ODS-2, 250 × 4.6 mm, 5 μm). The binary gradient elution system consisted of solvent A (0.005% formic acid water solution) and solvent B (0.005% formic acid acetonitrile solution), and separation was achieved using the following gradient: 0–35 min, 21%B; 35–36 min, 21–30%B; 36–55 min, 30–40%B; 55–65 min, 40–85%B; and 65–80 min, 100%B. The column temperature was set at room temperature. The flow



	R_1	R_2
G-Rg_1	-Glc	-Glc
G-Re	-Glc	-Glc-Rha
N-R_1	-Glc	-Glc-Xyl
G-Rf	-Glc-Glc	-H
G-Rh_1	-Glc	-H

	R_1	R_2
G-Rb_1	-Glc-Glc	-Glc-Glc
G-Rd	-Glc-Glc	-Glc
N-R_4	-Glc-Glc	-Glc-Glc-Xyl
N-Fa	-Glc-Glc-Xyl	-Glc-Glc
G-Rg_2	-Glc-Rha	-H

	R_1	R_2
N-K	-Glc-Glc	-Glc

Fig. 2. Chemical structures of the 11 saponins (A) as well as chromatographs of the 11 components quantified in this study (B) and the notoginseng sample (C).

rate was 1 mL min^{-1}, and the injection volume was 10 μL. The UV detection wavelength was set at 200 nm.

2.4. Preparation of standard solution

Three stock solutions were prepared in methanol (HPLC grade) for the establishment of QAMS method and external standard method. Solution I consisted of R_1 (1 mg mL^{-1}), Re (1 mg mL^{-1}), and Rd (1 mg mL^{-1}). Solution II consisted of Rg_1 (3 mg mL^{-1}) and Rb_1 (3 mg mL^{-1}), and solution III consisted of R_4 (0.2 mg mL^{-1}), Fa (0.2 mg mL^{-1}), N-K (0.2 mg mL^{-1}), Rg_2 (0.2 mg mL^{-1}), Rh_1 (0.2 mg mL^{-1}), and Rf (0.2 mg mL^{-1}). The three stock solutions were then diluted step by step with methanol to the additional six concentration levels.

2.5. Sample preparation

The method of sample preparation established in our previous study was used [10]. The samples were pulverized and sifted through a sieve that contained 0.45 mm sieve holes. A total of 15 mL methanol (A.R.) was added to 0.5 g of the powder sample. Next, the suspension was ultrasonically extracted for 30 min and filtered. This extraction procedure was repeated once. The filter paper and residue were then washed with 10 mL methanol for 3 times, and the filtrate was evaporated to dryness at 50 °C under vacuum conditions. The residue was then dissolved in 10 mL methanol (HPLC grade) and filtered through a 0.45 μm filter membrane. Each sample was prepared in duplicate, and determined twice.

2.6. Establishment of QAMS method based on a novel method for calculating relative correction factors

Generally, RCFs were calculated based on the positive correlation between chromatographic response (i.e. peak areas of internal referring substance—S_0 and the analyte—S_x) and the amount of the analyte (i.e. concentration of internal referring substance—c_0 and the analyte—c_x), which has been illustrated in the following equation:

$$c_x \times f_r = \frac{S_x}{(S_0/c_0)} \quad (2)$$

In the present study, a novel RCF calculating method was established based on Eq. (2). The new method, which was referred to as LRG (linear regression) method in this study, used the linear relationship between c_x and $(S_x \times c_0)/S_0$ to calculate RCFs by linear regression. And the LRG method was also compared with AVG method which has been fully illustrated in Hou's study [6] and applied in most of the QAMS method related studies [2–4,6,7].

A new QAMS method was established using RCFs obtained by LRG method (*LRG*-QAMS), and compared with the one established with RCFs calculated by AVG method (*AVG*-QAMS). And these two QAMS methods were also compared with external standard method, respectively, by standard method difference (*SMD*) calculated according to the following equation:

$$SMD = \frac{(C_{ES} - C_{QAMS})}{C_{ES}} \times 100\% \quad (3)$$

where C_{ES} and C_{QAMS} represent the concentrations of an analyte assayed by External Standard method and QAMS method, respectively.

Table 2
The contents (%, w/w) of Rd in 24 *Panax notoginseng* samples assayed by external standard method, QAMS method with relative correction factors calculated with the previous method—AVG method (*AVG*-QAMS) and the new method (*LRG*-QAMS), and the standard method differences (*SMD*s, %) of the two QAMS methods, using Rg_1 as internal referring substance.

Sample no.	Sample type	External standard	*AVG*-QAMS		*LRG*-QAMS		
		Content	Content	SMD	Content	SMD	*SMD*[a]
02002	3yrs' main root	**0.643**	**0.693**	−7.86	**0.650**	−1.20	*−1.18*
02005	3yrs' main root	**0.519**	**0.561**	−8.18	**0.526**	−1.45	*−1.43*
0208001	3yrs' main root	**0.688**	**0.742**	−7.78	**0.696**	−1.12	*−1.11*
0208003	3yrs' main root	**1.24**	**1.33**	−7.13	**1.25**	−0.68	*−0.68*
0208007	3yrs' main root	**0.615**	**0.664**	−7.91	**0.623**	−1.24	*−1.23*
0208008	3yrs' main root	**0.557**	**0.601**	−7.95	**0.564**	−1.36	*−1.34*
0208012	3yrs' main root	**0.450**	**0.487**	−8.31	**0.457**	−1.65	*−1.63*
0208020	3yrs' main root	**0.654**	**0.705**	−7.84	**0.661**	−1.18	*−1.16*
030108	3yrs' main root	**0.735**	**0.792**	−7.73	**0.743**	−1.06	*−1.05*
Y01016	3yrs' main root	**0.748**	**0.806**	−7.70	**0.756**	−1.04	*−1.04*
0208002	2yrs' main root	**0.588**	**0.635**	−7.99	**0.596**	−1.29	*−1.28*
0208004	2yrs' main root	**0.766**	**0.824**	−7.58	**0.774**	−1.02	*−1.01*
0208006	2yrs' main root	**0.347**	**0.377**	−8.76	**0.354**	−2.10	*−2.06*
0208009	2yrs' main root	**0.742**	**0.799**	−7.63	**0.750**	−1.05	*−1.04*
0208013	2yrs' main root	**0.635**	**0.684**	−7.79	**0.642**	−1.21	*−1.20*
0208016	1yr's main root	**0.084**	**0.097**	−15.10	**0.091**	−8.23	*−7.61*
030103	1yr' main root	**0.609**	**0.657**	−7.82	**0.617**	−1.25	*−1.24*
Y010129	1yr's main root	**0.215**	**0.237**	−10.04	**0.222**	−3.30	*−3.20*
0208003	Fibre	**0.363**	**0.394**	−8.58	**0.370**	−2.01	*−1.98*
0208009	Fibre	**0.184**	**0.204**	−10.69	**0.191**	−3.84	*−3.70*
0208020	Fibre	**0.126**	**0.142**	−12.52	**0.133**	−5.54	*−5.26*
0208003	Rhizome	**1.71**	**1.83**	−7.12	**1.72**	−0.53	*−0.53*
0208009	Rhizome	**0.969**	**1.04**	−7.47	**0.977**	−0.84	*−0.83*
0208020	Rhizome	**1.46**	**1.57**	−7.45	**1.47**	−0.60	*−0.60*

3yrs' main root—3 years old main root sample; 2yrs' main root—2 years old main root sample; 1yr's main root—1 year old main root sample; *SMD*s were calculated according to Eq. (3).

[a] The *SMD*s were speculated according to the linear regression curve of *SMD* and the reciprocal of the content of Rd ($SMD* = -0.6822/content - 0.1333$).

Table 3
Relative correction factors calculated by LRG method.

Saponin	Internal referring substances					
	Rg_1	Rb_1	R_1	Rd	R_4	Rg_2
Rg_1		1.35	1.20	1.22	1.44	0.94
Rb_1	0.74		0.88	0.90	1.07	0.69
R_1	0.83	1.12		1.01	1.20	0.78
Re	0.87	1.17	1.04	1.06	1.25	0.82
Rd	0.82	1.10	0.98		1.18	0.77
R_4	0.69	0.93	0.83	0.84		0.65
Fa	0.66	0.89	0.79	0.80	0.95	0.62
Rf	1.12	1.51	1.34	1.36	1.62	1.06
N-K	0.82	1.11	0.99	1.00	1.19	0.78
Rg_2	1.06	1.43	1.27	1.29	1.53	
Rh_1	1.19	1.60	1.43	1.44	1.71	1.11

2.7. Investigations of potential influencing parameters of QAMS method

The accuracy of *LRG*-QAMS method was evaluated with the difference between *LRG*-QAMS method and external standard method (i.e. *SMD*, Eq. (3)). In order to investigate the influence of different parameters on the accuracy of QAMS method, *SMD*s of 11 quantitative markers in 24 notoginseng samples with 6 of them used as internal referring substances individually (a total of 1584 data) were divided into several groups according to the criteria described as followed: (1) structure types of internal referring substances including Panaxadiol- and Panaxatriol-type; (2) structure types of analytes including Panaxadiol- and Panaxatriol-type; (3) contents of internal referring substances including high (0.5–6%, w/w), middle (0.2–2%, w/w), and trace (< 0.2%) contents; (4) contents of analytes including high (0.5–6%, w/w), middle (0.2–2%, w/w), and trace (< 0.2%) contents; (5) purities of standard substances including high (> 98%) and low (< 98%) purity. A scatter diagram and Pearson correlation analysis (SPSS 18.0, *SPSS China*) were used to investigate the mathematical relationship between these potential influencing parameters and *SMD*s.

2.8. Validation of the QAMS method in another laboratory

Five batches of notoginseng samples were also determined using the *LRG*-QAMS method established in laboratory B to evaluate its applicability across multiple laboratories.

3. Results and discussion

3.1. Evaluation of relative correction factors from the new calculation method

The RCFs of 11 components in notoginseng were calculated using both LRG calculation method and AVG method. And the differences between the RCFs calculated using these two methods when trace component R_4 or Rg_2 was used as the internal referring substance, were higher than those when the other four components were used as the internal referring substances (RCFs calculated with LRG method were listed in Table 3, more data could be found in Supplementary material). It implied that the content of the internal referring substance in the plant material would greatly influence the differences between the RCFs calculated using these two methods. Significant differences in the RCFs would lead to great differences in the contents determined by the *LRG*-QAMS or *AVG*-QAMS method. Additionally, there were significant fluctuations in the RCFs calculated using the previous method at different concentration levels of the

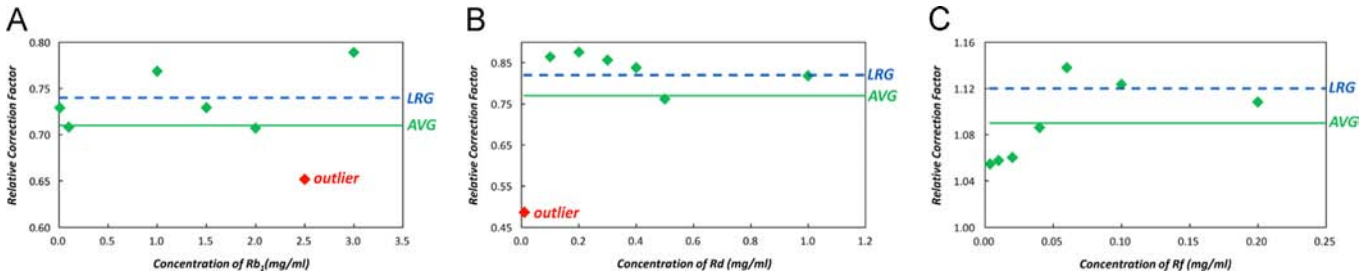


Fig. 3. Relative correction factors at different concentration levels of Rb_1 (A), Rd (B) and Rf (C) when Rg_1 was used as the internal reference substance. (◆ relative correction factors at different concentration levels; ◆ outlier (≥ 1.5 sd) of relative correction factors at different concentration levels; ▬ the final relative correction factors calculated by AVG method; ▬ ▬ relative correction factors calculated by LRG method; *more figures are available in the* Supplementary material).

Table 4
Standard method differences (mean ± sd, %) of QAMS methods established using relative correction factors calculated by the new method—LRG method (*LRG*-QAMS) and the previous method (*AVG*-QAMS) when 6 components were used as internal referring substances individually.

Internal referring substances	Standard method difference (%)	
	***LRG*-QAMS**	***AVG*-QAMS**
Rg_1	0.16 ± 0.07–8.84 ± 2.85	1.16 ± 0.07–23.29 ± 3.23
Rb_1	0.10 ± 0.09–8.94 ± 2.85	1.20 ± 0.02–18.99 ± 3.12
R_1	0.20 ± 0.12–8.75 ± 2.85	1.22 ± 0.14–21.44 ± 3.18
Rd	0.12 ± 0.09–8.79 ± 2.85	3.11 ± 1.73–15.19 ± 3.02
R_4	0.11 ± 0.02–8.45 ± 2.84	3.55 ± 1.04–11.88 ± 0.12
Rg_2	0.25 ± 0.02–5.12 ± 4.45	4.79 ± 1.30–15.17 ± 0.12

The mean ± sd were calculated with the absolute value of Standard Method Difference which was calculated according to Eq. (3); the *SMD*s of Rh_1 (28.67% ± 23.27%-30.15% ± 23.54% for *LRG*-QAMS and 10.84% ± 15.02%–15.47% ± 19.90% for *AVG*-QAMS) were not included due to the low resolution (below 1.5) of ginsenoside Rh_1 peak and its adjacent chromatographic peak.

analytes (see Fig. 3), which made the *AVG*-QAMS method unreliable when the concentrations of the analytes differed.

In addition, the RSDs of the calculation formulas (see Table 1) established with RCFs calculated with LRG method were 1.08–2.08%, whereas those of AVG method were 4.44–7.01%. The result implied that LRG method was much more consistent compared to the previous method when different internal referring substances were used.

Standard method difference (*SMD*, calculated according to Eq. (3)), which represented the difference between the results assayed by QAMS method and external standard method, were used to evaluate the accuracy of the *LRG*-QAMS and *AVG*-QAMS method. As it showed in Tables 2 and 4, *SMD*s of *LRG*-QAMS method were mostly lower than those of *AVG*-QAMS method, which meant that the concentrations of 10 saponins (except for Rh_1, the contents of which in most notoginseng samples were out of the linear range of standard curve) assayed by *LRG*-QAMS method were much closer to those obtained by external standard method (more data could be found in Supplementary material). And *SMD*s of *LRG*-QAMS method were below 5% for Rg_1, Rb_1, Re, Rd, R_1 and Fa, and mostly below 10% for Rf, R_4, Rg_2 and N-K. These results implied that *LRG*-QAMS method had higher accuracy in comparison with *AVG*-QAMS method, and the *LRG*-QAMS method could be used as the substitutive method of external standard method in the quantitative determination of these 10 saponins in notoginseng using the chromatographic method described in this article.

It has been demonstrated that RCFs might fluctuate with the variations of some experimental conditions (e.g. detectors and peak measurement parameters) [6]. So it could be deduced that systematic and/or random error might also affect the results when using AVG method to calculate RCFs, especially when there were one or more outliers (≥ 1.5 sd). However, the influence of outliers could be diminished when using LRG method to calculate RCFs (see Fig. 3). Therefore, the *LRG*-QAMS method was more accurate and stable than *AVG*-QAMS method. And LRG method was more suitable than AVG method for the calculation of RCFs in QAMS method.

3.2. Investigation of influencing parameters of QAMS method

The influences of three parameters on the accuracies of the *LRG*-QAMS method were investigated, including the chemical structure types (Panaxadiol- and Panaxatriol-type), the purities of the standard substances (high— > 98; low— < 98%), and the content of each quantitative component in the plant materials (high—0.5–6%; middle—0.2–2%; low < 0.2%). A bi-variable correlation analysis was conducted to investigate the correlations between the *SMD*s and these potential influencing parameters with exception of the Rh_1 data because of the considerable influence of its adjacent chromatographic peaks on the quantitative determination. The results showed that the influences of the various parameters decreased in the following order: content of the analytes > purity of standard substance > chemical structure type > content of the internal referring substance (Table 5). As shown in Fig. 4A and B, the contents of the internal referring substances had minimal influence on the *SMD* values of the QAMS method, while a clear downward trend was observed when the contents of the analytes increased. The result implied that the low accumulation of a component in sample material was the main reason for the decrease of QAMS method's accuracy. It could also be further confirmed with the result of Pearson correlation analysis (Table 5). The coefficient between *SMD* and $1/c$ was 0.581 ($P < 0.001$) which meant that the accuracy of QAMS method increased as the content of the analyte rose.

Additionally, a linear relationship between *SMD* and $1/c$ was discovered in our study as well (detailed discussion was presented in the next section), which also implied that, for a specific analyte, its concentration in sample solution was the major influencing parameter of the accuracy of QAMS method.

A previous review [8] mentioned that the purities of standard substances influenced the accuracy of the QAMS method, and that the purities of the standard substances should be greater than 98% to maintain the high accuracy of the QAMS method in practical applications. However, in our present study, the purities of the standard substances were demonstrated to have no significant influence (correlation coefficient < 0.3, Table 5) on the accuracy of the QAMS method when purities were between 92.3% and 100%. As long as the exact purities of the standard substances were known, their influence on the accuracy of the QAMS method could be disregarded during the establishment and practical application.

3.3. Establishment of applicable concentration ranges of the analyte in samples for the QAMS method

Due to the strong correlation between the accuracy of QAMS method and the concentrations of the analytes in sample solutions,

Table 5
Results of bi-variable correlation analysis between standard method differences (*SMD*s) and the six potential influencing parameters factors, including chemical structure types, purities of the standard substances, and concentration of each quantitative marker in the plant materials, using SPSS 18.0.

Factors parameters	Concentration		Chemical structure		Purities of standard substance	
	IRS	Analyte	IRS	Analyte	IRS	Analyte
Pearson correlation coefficient	−0.031	**−0.354[a)]/0.581[b)]**	0.059	−0.065	−0.139	0.180
Significance	0.135	**0.000**	0.018	0.010	0.000	0.000

IRS—internal referring substance; [a)] Correlation between *SMD* and the concentration of the analytes in the plant materials; [b)] Correlation between *SMD* and the reciprocal of the concentration of the analytes in the plant materials.

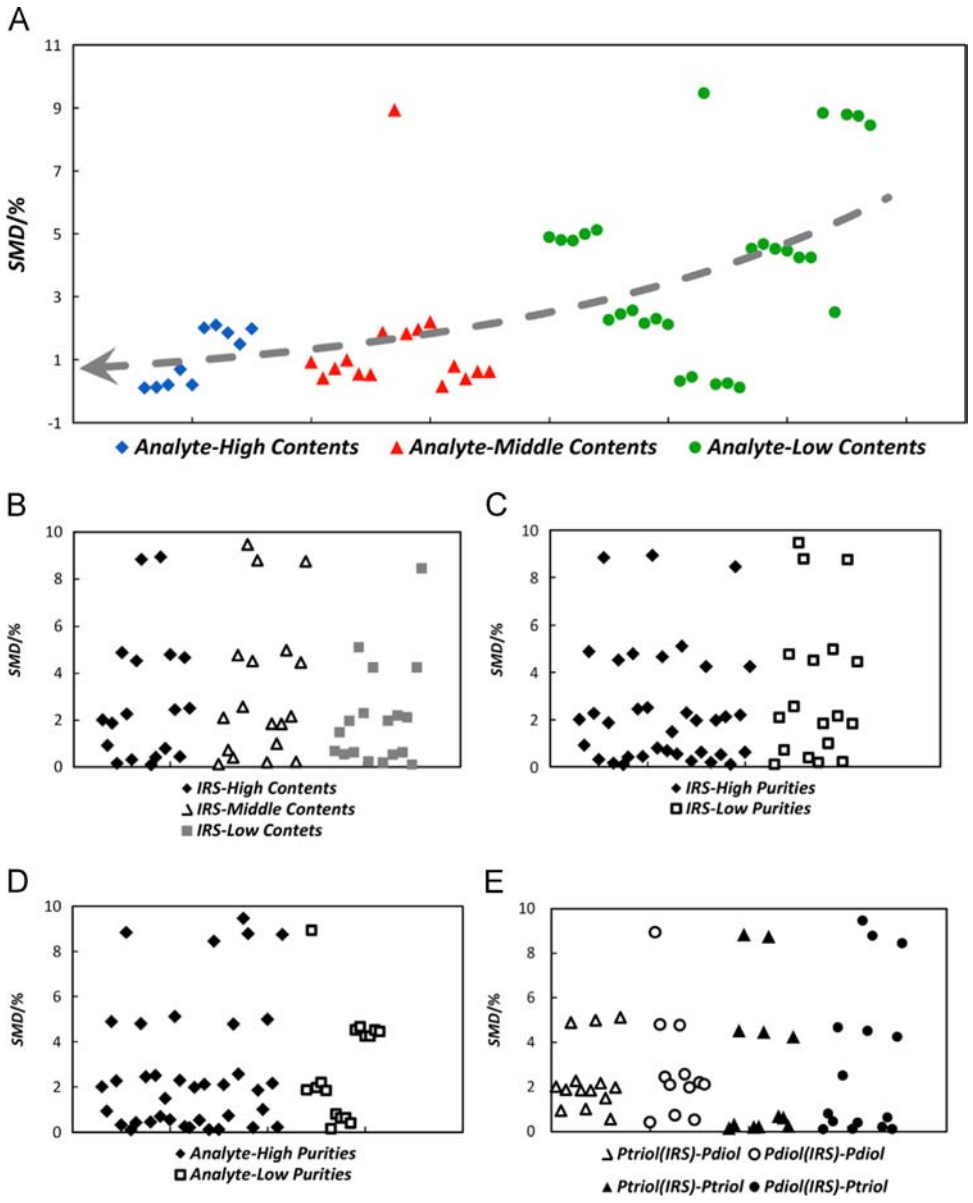


Fig. 4. The average standard method differences (*SMD*s) of the concentrations of ten saponins (Rh_1 not included) in twenty-four batches of notoginseng samples assayed by the QAMS method using relative correction factors calculated by the new method—LRG method with six saponins as internal reference substances (IRSs) separately at different concentrations of analytes (A) and internal referring substances (B), at different purities of the standard substances of internal referring substances (C) and analytes (D), and different chemical structure types of the internal referring substances and analytes (E).

a method for the establishment of the applicable concentration ranges of the analyte in samples in the QAMS method is especially necessary.

In the present study, a linear relationship was discovered between *SMD* and the reciprocal of the concentration of the analyte determined using external standard method (see Fig. 5), which could be used to calculate the concentration limits of QAMS method with a specific requirement of accuracy. According to Eq. (3), the linear relationship was:

$$SMD = \left(1 - \frac{k_x}{f_r \times k_0}\right) - \frac{b_x}{f_r \times b_0} \times \frac{1}{\boldsymbol{c_x}}. \quad (4)$$

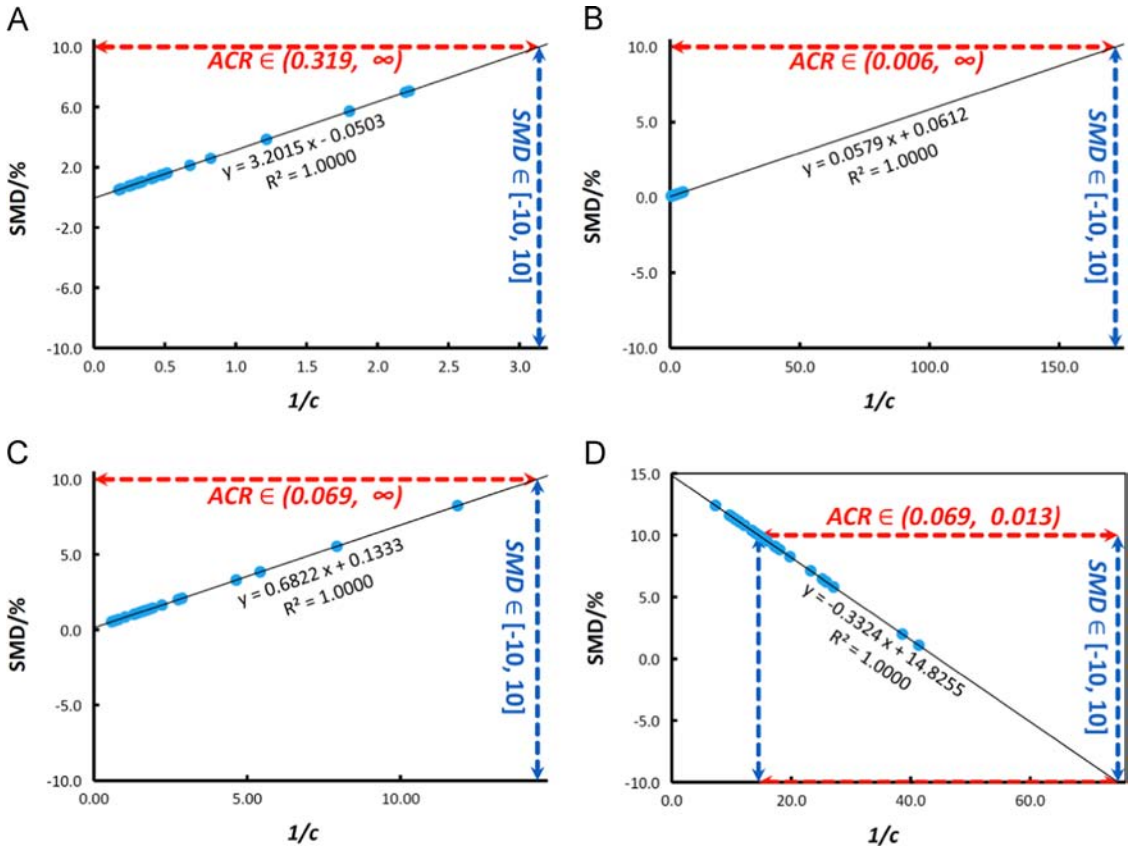


Fig. 5. A schematic diagram of the method for the calculation of applicable concentration ranges (ACRs, m0067 mL^{-1}) using the linear relationships between the standard method differences (*SMD*s, %) and the reciprocals of the concentrations (*c*, mg mL^{-1}) of Rb_1 (A), R_1 (B), N-K (C), and Rg_2 (D) determined by QAMS method. Rg_1 was used as the internal reference substance, and the applicable range of *SMD* was set between −10% and 10% (*more figures are available in* Supplementary material).

where *x* and 0 represented the analyte and the internal referring substance, respectively.

For specific analyte and internal referring substance, the RCF (f_r) between them, the slopes (k), and the intercepts (b) of their standard curves were all constants, which made the concentration of the analyte (c_x) the only influencing parameter of *SMD* in Eq. (4). In practice, the mathematical relationship could be obtained using a linear regression model when taking *SMD* and $1/c$ as dependent and independent variables, respectively. Then, the range of the concentration within a certain range of *SMD* (e.g. between −10% and +10%), called the applicable concentration range, could be calculated according to Eq. (4). If the acceptable range of *SMD* is set to between −10% and +10%, the corresponding concentration range can be calculated by inserting the lowest and highest value of *SMD*s (i.e., −10 and +10) into the linear curves of *SMD* and $1/c$. In practice, the applicable concentration ranges of the quantitative components should also be included in their linear ranges. Because the same equation (Eq. (1)) was used to calculate the content of an analyte, the method for determining the applicable concentration ranges discussed above was suitable for both *LRG*-QAMS and *AVG*-QAMS method.

The applicable concentration ranges of the 11 saponins determined in this study were calculated according to the method described above when the ranges of *SMD*s were set at either from −10% to +10% or from −5% to +5%, respectively (detailed data could be found in Supplementary material). And the detection results of Rg_1, Rb_1, Re, Rd, R_1 and Fa in 24 notoginseng samples were all in the applicable concentration ranges with *SMD*s between −10% and +10%. And the detection results of Rg_1, Re, Rd, R_1, Fa in all the 24 samples, and Rb_1 in 21 samples were in the applicable concentration ranges with *SMD*s between −5% and +5%. There were only 3 samples (Sample no.: fibrous roots 208,020f, 208,009f and 1 year-old main root 208,016), in which the detection results of Rb_1 had exceeded the applicable concentration range with *SMD*s between −5% and +5%. In practical application, *SMD* could be speculated using the detection result of an analyte by QAMS method according to the linear regression curve of *SMD* and $1/c$ (see *SMD*⁎ in Table 2). And the sample solution could be concentrated or diluted subsequently and tested again to make the *SMD* fit the requirement.

3.4. Establishment and application validation of QAMS method for the simultaneous determination of eleven saponins in notoginseng

In the present study, a QAMS method was established for the quantitative determination of 11 saponins in notoginseng samples (the contents of Rd referring to Rg_1 were listed in Table 2, more data could be found in Supplementary material). The RCFs of the 11 quantitative components were all calculated using Rg_1, Rb_1, R_1, Rd, R_4 and Rg_2 as internal referring substances, respectively, which meant that the quantitative analysis could be conducted with the standard referring substance of any one of the six saponins. And this was also the first report to provide detailed experimental data to demonstrate that different components could be used as internal referring substances in regardless to their contents.

Five batches of notoginseng samples were also tested in another lab (lab B), and the concentrations of Rb_1, Rg_1, Rd, Rg_2 and Rf were calculated using both the external standard method (c'_{ES}) and the *LRG*-QAMS method (c'_{QAMS}) established in this lab. The value of the c'_{ES}/c'_{QAMS} ratio was used to evaluate the accuracy of the *LRG*-QAMS method when used in different labs. The c'_{ES}/c'_{QAMS} ratios were 97.24–97.70% for Rb_1, 95.94–96.60% for Rg_1, 89.64–93.43% for Rg_2, and 84.65–88.89% for Rf when Rd was used as the internal referring

Table 6
The content (%, w/w) of 4 saponins in five batches of notoginseng samples that were assayed by both the external standard method (c'_{ES}) and the *LRG*-QAMS method (c'_{QAMS}) using Rd as internal referring substance.

Sample	Rb_1		Rg_1		Rg_2		Rf	
	c'_{ES}	c'_{QAMS}	c'_{ES}	c'_{QAMS}	c'_{ES}	c'_{QAMS}	c'_{ES}	c'_{QAMS}
1	3.78	3.70	2.60	2.50	0.09	0.08	0.08	0.07
2	3.05	2.97	3.08	2.96	0.11	0.11	0.06	0.05
3	2.46	2.40	3.02	2.91	0.07	0.06	0.07	0.06
4	2.20	2.14	3.57	3.44	0.12	0.11	0.06	0.05
5	3.17	3.10	3.04	2.93	0.07	0.07	0.08	0.07

substance (see Table 6). All the results implied that the established *LRG*-QAMS method could be applied well in different labs for the quantitative analysis of saponins in notoginseng.

Zhu et al., [12] has published a QAMS method for the quantitative analysis of 9 saponins in ginseng, which could also be applied in the quantitative analysis of 4 saponins (including Rg_1, Rb_1, Rd and Rh_1) in notoginseng with Rb_1 as internal referring substance. Although most of the saponins in notoginseng and ginseng had similar structure types (i.e., Panaxadiol- and Panaxatriol-type), the contents of some saponins were quite different (e.g., R_1 was the major component in notoginseng, but it could hardly be detected in ginseng). Therefore, in our previous study [11], a method for quantitative analysis of 5 major saponins (including Rg_1, Rb_1, Re, Rd and R_1) by QAMS with a short chromatographic analysis time (less than 40 min) was established, for the quality control of notoginseng and its slices. However, neither the theory nor advancement of LRG method was discussed in our previous study. And the influencing parameters of QAMS method were not investigated either. Therefore, our present study was mainly focused on those aspects, as well as the establishment of a novel QAMS method for quantitative analysis of 11 saponins (including 5 major components and 6 trace components) in notoginseng. As a supplemental verification of our present study, the raw data in our previous study [11] could also be used to conduct the comparison of *LRG*-QAMS and *AVG*-QAMS method. And *SMD*s were $0.24\% \pm 0.29\%$–$5.95\% \pm 1.65\%$ for *LRG*-QAMS method, and $0.80\% \pm 0.97\%$–$13.75\% \pm 0.39\%$ for *AVG*-QAMS method, which was consistent with the result gained in present study. And the method for determining applicable concentration ranges of QAMS method could also be successfully applied in our previous study.

4. Conclusion

In the present study, a QAMS method was established and applied for the simultaneous determination of 11 saponins in notoginseng, as well as to study the influence of several parameters (including chemical structure, concentrations of quantitative components, and purities of the standard substances) on the accuracy of the QAMS method. The results indicated that the concentration of the analyte in the sample was the major influencing parameter, whereas the other parameters did not significantly influence the accuracy of the QAMS method. Due to the importance of the relative correction factor in the QAMS method, a new method (LRG method) for the calculation of the relative correction factor was established in order to increase the accuracy of QAMS method. And the QAMS method established using relative correction factors calculated by LRG method (*LRG*-QAMS) had low difference (*SMD*s below 5% for Rg_1, Rb_1, R_1, Re, Rd and Fa, and mostly below 10% for Rf, R_4, Rg_2, and N-K) with external standard method, which implied that *LRG*-QAMS method could be used as a substitute of external standard method when lack of standard substances. Additionally, in order to ensure the high accuracy of the QAMS method, the concept of applicable concentration range of the analyte in samples in QAMS method was introduced for the first time, which was derived according to the mathematical relationship between the standard method difference (*SMD*) and the reciprocal of the concentration of the analyte ($1/c$) in sample solution. A method for determination of the applicable concentration range of an analyte was elaborated in the present study, which could also be used for other TCMs.

The results of the present study were conclusive proof to the practicability of the application of QAMS method for the quantitative analysis of multi-components in TCMs and TCM prescriptions. Because only one standard substance is needed for the QAMS method, which solves the problems associated with the absence of some rare standard substances (e.g., notoginsenoside Fa) during quantitative analysis, the QAMS method will play an important role in the quality control of TCMs in the future. Of course, like external standard method, this method can be used only if the peaks are well resolved, and there is not any previous issue with baseline drifts or other issues that might compromise the reproducibility of the chromatogram.

Conflict of interest statement

The authors confirm that there were no conflicts of interest in performing this study.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.11.028.

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