A reproducible analytical system based on the multi-component analysis of triterpene acids in *Ganoderma lucidum*

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**Abstract**

Ultra-performance liquid chromatography (UPLC) and Single Standard for Determination of Multi-Components (SSDMC) are becoming increasingly important for quality control of medicinal herbs; this approach was developed for *Ganoderma lucidum*. Special attention was necessary for the appropriate selection of markers, for determining the reproducibility of the relative retention times (RRT), and for the accuracy of conversion factors (F). Finally, ten components were determined, with ganoderic acid A serving as single standard. Stable system parameters were established, and with successful resolution of those issues, this analytical method could be used more broadly.

**1. Introduction**

Lingzhi, *Ganoderma lucidum* (Leyss. ex Fr.) Karst (GL), is an important traditional Chinese medicine and food supplement. Among the major components are multiple triterpene analogues, and several high performance liquid chromatography (HPLC) methods have been reported for their analysis for quality control (Wang et al., 2006; Zhao et al., 2006; Chen et al., 2008). However, the use of conventional HPLC has a number of disadvantages, including the time- and solvent-consuming analysis, the inefficient separation of target peaks, and the use of a limited number of marker compounds. Application of ultra performance liquid chromatography (UPLC), however, achieved better separation of these triterpenes (Da et al., 2012). For their quantitative determination though, it was necessary to acquire multiple, rare, and expensive reference standards.

Because of the paucity and cost of reference substances, the Single Standard for the Determination of Multiple Components (SSDMC) method was applied for quantitative determination of multiple components in a plant-based medicine. HPLC determinations using SSDMC methods (HPLC–SSDMC) have been recorded in the USP monograph (The United States Pharmacopeia Convention, 2010) including, for example, *Echinacea angustifolia* DC. In addition, multiple anthraquinone derivatives in rhubarb (*Rheum rhabarbarum* L.) (Gao et al., 2009), Sanhuang tablets (Wang et al., 2012), and tanshinones in Chinese *Salvia* (Hou et al., 2011) were also investigated successfully using this SSDMC method.

The coupling of SSDMC and UPLC was anticipated to offer a feasible method to comprehensively enable analysis of individual natural products in complex matrices. Nevertheless, the combination of UPLC with SSDMC presents some new obstacles when applied in practice. A slight change of chromatographic conditions may have a notable impact on the effectiveness of the separation. Furthermore, chromatographic performance with different columns and instruments is expected to result in significant variations in the resulting chromatograms. Consequently, it became very challenging to determine the major parameters which were essential for an effective SSDMC method. As a result, the successful application of UPLC coupled with SSDMC is substantially more than a simple transformation, or an upgrade, of an established, conventional HPLC–SSDMC method.

In this present study, a relatively simple, economic, and accessible UPLC–SSDMC method was established. The most challenging issue, the ability to determine and reproduce stable system parameters, was resolved. The study design is shown in Fig. 1. A relatively long analysis time was necessary for the detection of...
the triterpenes due to the complex nature of the matrix and the structural similarities, and this afforded the opportunity to examine the significant variations of their chromatographic behavior.

2. Results and discussion

2.1. Selection of markers for quantitative determination based on fingerprints

2.1.1. The chromatographic fingerprints of G. lucidum

Higher peak capacity and better resolution were obtained with UPLC compared with conventional HPLC (Fig. S1). From their chromatographic profiles, the similarity of twenty batches of G. lucidum was in the range of 0.801–0.991. Common peaks were numbered according to their retention times, and identified using UPLC-Q/TOF. The relationship between the total area of these peaks, and all of the peaks recorded in the chromatogram was 82.57–95.40% for the twenty batches, with an average of 86.70%. Ten compounds were identified by comparison with the respective reference standards, and are shown in Fig. 2 and Tables S1–S3. Compound 1, ganoderenic acid C (peak 4); compound 2, ganoderic acid C2 (peak 6); compound 3, ganoderic acid G (peak 9); compound 4, ganoderenic acid B (peak 10); compound 5, ganoderic acid B (peak 11); compound 6, ganoderic acid A (peak 14); compound 7, ganoderic acid H (peak 15); compound 8, ganoderenic acid D (peak 17); compound 9, ganoderic acid D (peak 18); and compound 10, ganoderic acid F (peak 23). Other peaks were identified through their reported mass fragmentation patterns and cleavage rules and are shown in Tables S1–S3 (Yang et al., 2007; Cheng et al., 2010, 2011).
2.1.2. The selection of markers

The common peaks reflected the characteristics of all of the visible peaks in the fingerprints (Fig. S2), and are listed in a decreasing order of average peak ratio and height ratio (Fig. S3). Compounds 1–10 were the main peaks, with a relatively larger peak area and height. The area percentage of each of the ten individual markers was more than 2% of the total area in the chromatogram. The percentage of the total area of the ten marker peaks to the overall twenty-four peaks was 58.69–73.01% for the twenty batches, with an average proportion of 67.18%. The ten marker peaks also accounted for over 50% of the total area and peak height (Fig. S4A). These twenty-four common components were divided into five structural classes (Fig. S4B), of which the ganoderic acids and ganoderenic acids were the main classes, representing in the range of 62.38–76.34% between their peaks and the twenty-four common peaks investigated in the twenty batches.

Compound 6 was chosen as the single reference substance due to its relatively high content, easier accessibility, better stability, and relatively lower cost to acquire. The relative peak area of compound 6 to the total peak area was 4.94–7.31% for the twenty different batches.

The structures of the ganoderenic and ganoderic acids are quite similar, however, their values of log $e$ and $\lambda_{max}$ were slightly and adequately different (Da et al., 2012). Despite the similarity in the chemical structures and characteristics of these triterpene components, this represents a UPLC–SSDMC method which can determine the two classes of triterpene component simultaneously.

2.2. Investigation of the sample preparation method

The sample preparation method was reported previously (Da et al., 2012), with extraction and purification as the major procedures. The solvent systems, extraction methods, frequency of extraction, volume of extraction solvent, and duration of the extraction were investigated, sequentially. The total content was the basis of selection. Purification was carried out through solid phase extraction in order to obtain a smoother baseline, and to remove impurities. In this way, the method of extraction conditions, application (solvent and rate), pre-elution, and elution were optimized.
2.3. Optimization of the chromatographic parameters

In order to optimize the chromatographic parameters, the retention time (RT), the tailing factor (T), and the resolution (R) between the markers and their neighboring peaks were examined. It was found that when neighboring peaks were enwrapped into the main peaks, inaccurate values of the resolution and tailing factor were obtained. In this case, univariate experiments were more appropriate. Investigations were sequentially performed on various chromatographic columns, mobile phase systems, the concentration of phosphoric acid in the mobile phase, different elution programs, flow rates, column temperatures, injection volumes, and the spectral resolution of the PDA detector.

2.4. Investigation of relative retention times (RRT)

2.4.1. Determination of RRT

For compounds 1–10, the RRTs were 0.36, 0.41, 0.56, 0.60, 0.66, 1.00, 1.05, 1.25, 1.33, and 1.54, respectively. These values were stable on different days (Table S4).

2.4.2. The necessity of a stable RRT

For a comprehensive and valid UPLC–SSDMC method, a comprehensive study on the RRTs was necessary. Firstly, the retention time (RT) originally obtained with UPLC was easily affected. The most substantial fluctuation of the retention time of compound 6 was about 8 min for different columns, and 10 min for different instruments (Fig. 3). Secondly, the RRT reflects the distribution of peaks in a chromatogram. The separation of those peaks might therefore be influenced by the change of RRT due to the higher peak capacity of UPLC. The resolution might decrease to less than 1.5, which potentially represents a serious issue. In such a case, the critical factors of peak alignment and prediction of retention time (Boswell et al., 2011) would be ineffective.

Compared with conversion factors, the relative peak area of each marker to compound 6 could better reflect the influence of changed RT, because minor peaks were not involved in the test of conversion factors. Through the manual alteration of chromatographic conditions (Table 1), the relationship between RT and relative area (RA) of each marker to Ganoderic acid A in sample solution was studied (Fig. 4, Column A and Instrument I). The results were divided into three categories. Firstly, it was determined that the RRTs and RAs were relatively stable. That was found for different concentrations of H₃PO₄ in the mobile phase (B), injection volume, and a slight change in the third part of elution program. An exception was represented by the RA of compound 7 obtained with 0.01% H₃PO₄, when compound 7 was not efficiently separated, and its peak area was decreased. In the second situation, both the RRTs and RAs changed synchronously, which was determined in the investigations of the first and second parts of the elution program. When a greater change of the RRTs appeared, the change in the RAs was also more notable. In the third situation, the change in the RAs was more obvious than that of the RRTs. In the instance of using different flow rates and column temperatures, almost all of the RAs were affected, albeit to different degrees. In addition, the fluctuation of the larger RRTs was more apparent than for the smaller ones. In conclusion, the fluctuation of the RRTs could reflect variation in other important parameters. However, a stable RA could not be guaranteed by having stable RRTs (Fig. S5).

2.4.3. The method to obtain a relatively stable RRT on different instruments and columns

As the existing methods for peak alignment and RT prediction could not avoid fluctuation of RA, adjustment of chromatographic conditions was necessary and feasible. To eliminate the effect of different instruments, chromatographic specialists often suggest that the addition of a dwell time should be employed ahead of the formal chromatographic conditions. In this study, for instruments I, III and IV the dwell volume was 160–180 μL; for instrument II, it was 460 μL. The dead time of columns A–G was in the range of 0.77–0.87 min. This range was so small that a significant difference caused completely by changing the dwell time and dead time was unlikely to occur. Meanwhile, a significant variation of retention times took place when isocratic elution was conducted on the different instruments. For example, a variation of 7 minutes were shown when CH₃CN–H₂O (26.5:73.5, v/v) was used. This effect might be attributed to the different types of pumps, or a slight deviation within certain pumps. Unfortunately, when the diversity in chromatographic behavior is caused by the instruments themselves, nothing can be done to modulate the situation. Typically, there is an improved opportunity to obtain a more stable RRT with isocratic elution rather than gradient elution. However, under these conditions, the retention times of compounds 8–9 would then be very long. Thus, it was determined that an elution pattern which combines isocratic elution with gradient elution was the most suitable. The separation of the peaks with a shorter retention time might be not ideal, consequently, adjust-

![Fig. 3. Chromatograms obtained with different columns and instruments. (A) Chromatogram from Column B, Instrument III; (B) chromatogram from Column B, Instrument I; (C) chromatogram from Column G, Instrument I.](image-url)
ments were also necessary for the first section of the gradient profile. Through multiple attempts, a simple and rapid method was found for the adjustment of the elution program: 0/3/time of the end of elution of compound 6|(the end of elution of compound 6 + 12 min), 0%/26.5%/26.5%/38.5% (B). The proposed gradient elution program was divided into three sections. Concerning the second section, the end time was not fixed; it depended on the elution of 6, and also represented the beginning of the third section of the program.

In this way, the RRT for each marker obtained from the different columns (with RSD in the range of 0–2.19%) and from the instruments (RSD in the range of 0–2.76%, Table S5), was not significantly different. In addition, for the change of RRT caused by an inaccurate flow rate or column temperature, the above mentioned adjustment was still useful (Fig. S6).

2.5. Determination and validation of the conversion factors (F)

The correlation coefficients of the standard curves of all of the analytes were 0.9998−0.99997 (Table S6). For compounds 1–10, the conversion factors were 0.51, 1.05, 1.18, 0.45, 1.10, 1.00, 1.54, 0.51, 1.08, and 1.45, respectively. Compared with the ganoderic acids, the conversion factors of the ganoderenic acids were smaller. Further study on the relationship between the larger F of compounds 7 and 10, and the β-OAc on R4 in the chemical structure of the triterpenes, was thus necessary.

The conversion factor F was investigated through five experiments: (a) a different method for the calculation of F; (b) different standard curves; (c) comparison of the composition obtained with a conventional external standard (ES) method and the SSDMC method; (d) comparison of the composition acquired with the SSDMC method based on analysis at 257 nm and the corresponding maximum absorption wavelength (λmax); and (e) different columns and instruments.

The results are as follows: (a) two methods have been reported for the calculation of F, using either the slope of the standard curve, or the ratio of response to concentration (U/k) (Gao et al., 2009; Hou et al., 2011), and the results are shown in Table 2(a). Some issues might become apparent when the F value is used. The fact that the response includes background absorption leads to a significant concern. The smaller peak areas resulted in a larger variance of r/C. This, in turn, would influence the average value of those ratios, namely the F value. Because each concentration point has different levels of deviation, when the numerator and the denominator correspond to different concentration points, the F value obtained would be different. On the other hand, a calculation using the slope method was capable of avoiding the influence caused by the variance of concentration. For instance, the standard curve of compound 7 included seven points, namely S1–S7, with increasing concentration. The r/C of S1 and S2 were obviously different from S3–S7 (RSD was 17.00% for S1–S7 and 4.94% for S2–S7), but the slope values obtained with S1–S7 and S3–S7 were similar (RSD 0.83%). As a result, for S3–S7, the RSD of F calculated by the different methods was 1.89%.

However, the peak areas of the samples still included the background absorption, which was required to be relatively low in order to ensure the accuracy of the constituent determination. Fortunately, this is a requirement of SSDMC, and of the conventional external standard method. The peak area of the sample solution was much larger than the peak area of the standard solution with low concentrations in a standard curve, so there were no additional constraints in the application of the proposed method. The constituent determination obtained from the SSDMC and ESM was compared in (c).

(b) The F values obtained from the different standard curves were similar (Table 2(b), Column B, Instrument I).

(c) The content of GL (No. 1) was calculated using Formula 1 (SSDMC method) and Formula 2 (external standard method), and the results are shown in Table 2(c).

For different batches, if ESM was performed at the λmax of compound 6 (257 nm) for the simultaneous determination of the ten markers, the Contenti,1/Contenti,2 ratio would be a constant value (Formula 3). On the other hand, if the determination was performed at the λmax of each marker, the ratio would associate with the ti,1,2 and ti,1,2 of each batch (Formula 4).

For Formulae 1–4, “i” represents the peak area, “C” represents concentration; the subscript “U” represents the analyte (marker), and subscript “S” represents the reference standard, subscript “I” represents the λmax of each marker, and subscript “I” represents each marker.

When the values of F were simplified for a quick determination, as 0.5 for compounds 1, 4, and 8, 1.0 for compounds 2, 3, 5, 6, and 9, and 1.50 for compounds 7 and 10, and then used, the total content was affected, with a percentage of 95.79–97.31% for the twenty batches.

(d) Compound 6 (λmax = 257 nm) was the single reference standard used to determine ten components. Thus, the wavelength of the UV detector was set at 257 nm, and all of the conversion factors were obtained from the peak response at 257 nm. However, since different triterpenes have different λmax values, it was necessary to investigate the conversion factors under the corresponding λmax of each component. Comparison was carried out to evaluate the SSDMC method with F obtained at 257 nm, and at λmax, according to the Formulae 1, 5, and 6.

For Formula 6, the “Slope” represents the slope of the relevant standard curve at 257 nm; the “Slopei,2” represents the slope of the relevant standard curve at corresponding λmax of each component. The results are shown in Table 2(d). The percentage for compound 8 was 103.72%, indicating the potential presence of an impurity. However, the average percentage of compound 8 in the total content was 3.88%, thus the influence of the impurity on the total content was only about 0.14%, and this was considered acceptable. For the other markers, including the ganoderic and ganoderenic acids, the percentage was in the range of 99.08–100.12%, indicating that determination at the single λmax of 257 nm was feasible.

(e) Conversion factors obtained with different columns and instruments.
The conversion factors obtained with different columns and instruments were relatively stable (RSD < 2%, Table S7). Some details were, however, found to be significant. Firstly, for the different instruments, the wavelength of the detector was an important factor. The RSD of \( F \) on different instruments was in the range of 0.33–3.07% when the determination was performed only at 257 nm, but decreased to 0.19–1.09% when the determination was performed at the \( \lambda_{\text{max}} \) of compound 6 on each instrument, due to the very small difference of those detectors (about 1 nm). Secondly, when the resolution of compounds 6 and 7, which represent the two peaks with the least resolution of the ten markers, was only 1.5 instead of 2.0, the integration method started to per-

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**Fig. 4.** The change of RRT and RA under different chromatographic conditions. (A) Concentration of the aqueous solution of \( \text{H}_3\text{PO}_4 \) in the mobile phase B. (B) Gradient elution program. (C) Flow rate. (D) Column temperature. (E) Injection volume.
form. Integration with the baseline at the peak valleys might bring a decrease to the peak area of compound 6, and further result in a smaller F value of the ten markers (Column F). As a result, the F value obtained from the different instruments would vary (for Column F, the RSD of the ten markers was 0.32–3.89%). Baseline integration was therefore preferred when calculating the total content.

2.6. Method validation

Method validation was performed according to the method performance requirements, inclusive of precision (repeatability and intermediate precision), recovery, specificity, stability, linearity, and robustness. All of the above parameters were acceptable, as previously reported (Da et al., 2012), and are presented in Tables S8 and S9.

2.7. Content obtained with different columns, instruments, and a different laboratory

In this laboratory, the contents of a randomly selected sample solution obtained using different columns and instruments are shown in Fig. 5. The results may be summarized as: (a) For the different batches of columns containing the same packing materials (Columns A–D), the RSD of compound 8 was 13.29%, due to the fact that the small peak preceding was enwrapped when eluted on Column D. However, the total content varied only within 0.1%, and this was deemed acceptable. The content of the other compounds, and the total content, as investigated on four different columns, was similar (RSD 0.72%).

(b) It is rather challenging to perform an UPLC method using columns containing various packing materials. Columns B, F, and G were selected as an example (instrument I). Due to different selectivity of the columns, and the inherent complexity of GL, the peak shapes of compound 5 (RSD 10.58%), compound 8 (11.72%), and compound 10 (8.54%) were vulnerable, sometimes due to the neighboring peaks. However, these columns still managed to achieve a relatively stable content assessment (RSD = 2.65%).

(c) When the method was performed on different instruments with the same column, better results were obtained. For Column B, the main variance was principally caused by compound 8. However, the content of the other components, and the total content assessment were not influenced (RSD 1.15%). For Column E, the RSD of the total content was 2.72%.

(d) Further applications of this SSDMC method were also conducted on columns with a length of 100 mm (Columns H, I, and J), using the data from Column B as a comparison. The first and third sections of the elution program were changed into two-thirds of the original time length. The RRTs of Columns B, H, and I were similar (RSD 0.15–2.87% for the ten markers). For Column J, which contained packing materials made with the Core–Shell technique, the RRTs were significantly different (RSD for compound 1 was 11.41%). The F value was not significantly influenced by the decrease in column length, but the content of the sample could not be accurately obtained due to insufficient separation of many of the markers, resulting in a somewhat larger apparent total content (RSD 4.65%). However, the experiment indicated that the value of F might be applied on shorter columns in the case of testing less complex samples, and then the retention time would be shortened.

(e) If the elution program was not adjusted, the total content could be significantly increased. The RSD values of Column G and Column B were 8.21% and 5.36%, respectively.

(f) Content assessment obtained in different laboratories was compared with three randomly selected batches of G. lucidum (Table S10). The variance regarding the total content of the

![Fig. 5. Content assessment obtained with different columns and instruments.](image-url)
samples was small (RAD was in the range of 0.16–2.12%), although the content of some markers was occasionally larger, especially for the ganoderenic acids. Possible reasons include the small amount of these components in the matrix, and the different wavelengths of the detectors. The storage conditions of GL could also influence the content, due to the instability of ganoderenic acid compared with ganoderic acids.

(g) The difference between the total content obtained with ESM and SSDMC was very small, with the percentage of content in the range of 99.58–100.80%, for the different columns and instruments.

3. Conclusions

In this study, combination of the SSDMC method with UPLC technology enabled the quantity control of multiple triterpenoid constituents in G. lucidum based on their fingerprints. The most significant issue, the ability to obtain stable major chromatographic parameters, was overcome. A variety of the main aspects and procedures were carefully investigated, including the selection of the marker compounds to be used, the determination of RRT and F values, and the wider application of the analytical method on different columns, instruments, and in different laboratories. A stable RRT was considered necessary for different columns and instruments, and this was successfully achieved with a new concept for a gradient elution program. It was necessary to pay attention to the wavelength of the \( \lambda_{\text{max}} \) of compound 6 when performing the analysis on different instruments. In order to assure the improved quality control of natural food supplements, this method may be regarded as a model for the future application of the UPLC–SSDMC technique.

4. Experimental

4.1. Chemicals and standards

CH3CN (HPLC grade, Lot K4WA1H) and MeOH (HPLC grade, Lot k2BG1H) were purchased from Honeywell (USA). Phosphoric acid (HPLC, Lot 911254) was obtained from Tedia (USA). Reference chemicals were prepared in this laboratory and identified to be of not less than 98% purity. All other reagents and solvents were of either analytical or HPLC grade.

4.2. Materials

Twenty samples of G. lucidum were collected from seven major medicinal plant distribution centers in China, including Nos. 1–11 (Anhui), Nos. 12–13 (Shandong), Nos. 14–15 (Jiangsu), Nos. 16–17 (Jilin), No. 18 (Fujian), No. 19 (Henan), and No. 20 (Guangxi). All of the acquired samples were identified by one of the co-authors (Professor De-an Guo) and are retained in the Shanghai Research center for Modernization of Traditional Chinese Medicine. Voucher specimen numbers for No. 1–20 were: GL09330101, GL09010202, GL09020303, GL09070504, GL09100705, GL09110806, GL09120907, GL09131008, GL09141109, GL09181310, GL09191411, GL09080612, GL09341813, GL09351914, GL09362015, GL09151216, GL09241517, GL09030418, GL09271619, GL09321720.

4.3. Preparation of experimental solutions

4.3.1. Standard solution

The stock solution for each component, the stock solution for standard curve, and standard curve solutions were prepared sequentially. The concentrations used in the experiments are shown in Table S11.

4.3.2. Sample solutions

Sample solutions were prepared according to the method reported in the previous study (Da et al., 2012).

4.4. HPLC and UPLC procedure

HPLC separations were performed on a Waters HPLC system (Waters Co., Milford, MA, USA) with an Agilent Zorbax SB-C18 Column (5 \( \mu \text{m}, 4.6 \times 250 \text{ mm} \)) according to the method described previously (Wang et al., 2006).

Two laboratories, four UPLC systems, and ten columns were involved in the series of experiments (Table S12). The mobile phase consisted of CH3CN (A) and a 0.075% aqueous solution of H3PO4 (B). Three sections were included in the elution program: section I: 0–3 min, 20–26.5% (A); section II: from 3 min to the end of the peak of compound 6, 26.5% (A); section III: the ratio of (A) rose from 26.5% to 38.5% in 18 min. Other parameters were: injection volume: 5 \( \mu \text{L} \); flow rate: 0.4 \( \text{mL min}^{-1} \); detection wavelength: 257 nm; column temperature: 25°C (Da et al., 2012).

4.5. Similarity evaluation

Similarity was evaluated using the “Similarity evaluation system for chromatographic fingerprint of TCM, 2004 edition”.

4.6. UPLC-Q/TOF MS method

Identification of the triterpene constituents was performed with a Synapt Q-TOF high-resolution mass spectrometer (Waters Co., Milford, MA, USA) connected to a Waters Acquity TM Ultra Performance LC. The MS detection was performed in the negative ion electrospray (ES–ve) mode, with \( \text{N}_2 \) and Ar as API and collision gas, respectively. Data were acquired from 80 to 1000 Da, with a source temperature of 100°C, a desolation temperature of 350°C, and a cone voltage of 40 V. Data were centroided during acquisition using an internal reference (leucine enkaphalin solution 400 ng mL\(^{-1} \), 5 \( \mu \text{L min}^{-1} \)) generating a reference ion in the ES–ve mode at m/z 554.2615. Data acquisition was achieved with MS\(^8\), which had two separate scan functions that were programmed with independent collision energies. A low collision energy scan (transmission collision energy, 2 eV; trap collision energy, 3 eV) were immediately followed by a high energy scan (transmission collision energy, 15 eV; trap collision energy, 20–35 eV) to induce fragmentation of the ions transmitted through Q1. The UPLC-Q/TOF MS system was operated using MassLynx 4.1 software.

4.7. Detection and validation of relative retention time (RRT)

RRT was calculated using Formula 7, in which “RRT\(_i\)” represents the RRT of relevant marker, and “RRT\(_E\)” represents the retention time of relevant marker. “RT\(_{ Xi}\)” represents the retention time of compound 6. Validation of RRT was carried out through: (a) different columns; (b) different instruments; and (c) fluctuation of chromatographic conditions (Column A, Instrument I).

4.8. Detection and validation of conversion factor (F)

Conversion factors were obtained with solutions of reference standards, and the F value was calculated using Formula 8, in which “F” represents the F of the relevant marker, “Slope” represents the slope of the relevant standard curve, and “Slope\(_{ Xi}\)” represents the slope of the standard curve of compound 6.
4.9. The relationship between RRT and RA

Through manually changing the chromatographic conditions, the influence on RRT and its sequential effect on other parameters, especially F, were investigated. F values were obtained from the standard curves, thus could not reflect the influence of minor constituent peaks on the content. The relative peak area of each marker to compound 6 (RA) in the sample solution was used as an alternative.

RA was calculated using the Formula 9, in which “RA” represents RA of the relevant marker; “RRT” represents the peak response of the relevant marker in the sample solution, and “RA” represents the peak response of compound 6 in the sample solution.

**Formulae**

**Formula 1:** 
\[ \text{Content}_{1j} = \left( \frac{\text{RT}_{1j}}{\text{RT}_{1}} \right) \cdot \left( \frac{C_{Sj}}{C_{O}} \right) \cdot F_{1} \cdot 100 \]

**Formula 2:** 
\[ \text{Content}_{2j} = \left( \frac{\text{RT}_{2j}}{\text{RT}_{1}} \right) \cdot \left( \frac{C_{Sj}}{C_{O}} \right) \cdot 100 \]

**Formula 3:** 
\[ \text{Content}_{1j}/\text{Content}_{2j} = \frac{\text{RT}_{1j} \cdot C_{Sj}}{\text{RT}_{2j} \cdot C_{Sj}} \cdot F_{1} \]

**Formula 4:** 
\[ \text{Content}_{1j}/\text{Content}_{2j} = \frac{\text{RT}_{1j} \cdot C_{Sj}}{\text{RT}_{2j} \cdot C_{Sj}} \cdot F_{1} \]

**Formula 5:** 
\[ \text{Content}_{1j} = \left( \frac{\text{RT}_{1j}}{\text{RT}_{1}} \right) \cdot \left( \frac{C_{Sj}}{C_{O}} \right) \cdot F_{1j} \cdot 100 \]

**Formula 6:** 
\[ \text{R}_{RA} = \frac{\text{Slope}}{\text{Slope}} \]

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.007.

**References**


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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.007.