More accurate matrix-matched quantification using standard superposition method for herbal medicines

Ying Liu, Xiao-Wei Shi, E-Hu Liu, Long-Sheng Sheng, Lian-Wen Qi*, Ping Li*

State Key Laboratory of Natural Medicines (China Pharmaceutical University), Nanjing 210009, China

ARTICLE INFO

Article history:
Received 21 May 2012
Received in revised form 28 June 2012
Accepted 4 July 2012
Available online xxx

Keywords:
Quantitative analysis
Herbal medicine
Standard superposition
Standard addition
Matrix effect
Panax notoginseng
Saponins
LC–MS

ABSTRACT

Various analytical technologies have been developed for quantitative determination of marker compounds in herbal medicines (HMs). One important issue is matrix effects that must be addressed in method validation for different detections. Unlike biological fluids, blank matrix samples for calibration are usually unavailable for HMs. In this work, practical approaches for minimizing matrix effects in HMs analysis were proposed. The matrix effects in quantitative analysis of five saponins from Panax notoginseng were assessed using high-performance liquid chromatography (HPLC). Matrix components were found to interfere with the ionization of target analytes when mass spectrometry (MS) detection were employed. To compensate the matrix signal suppression/enhancement, two matrix-matched methods, standard addition method with the target-knockout extract and standard superposition method with a HM extract were developed and tested in this work. The results showed that the standard superposition method is simple and practical for overcoming matrix effects for quantitative analysis of HMs. Moreover, the interference components were observed to interfere with light scattering of target analytes when evaporative light scattering detection (ELSD) was utilized for quantitative analysis of HMs but was not indicated when Ultraviolet detection (UV) were employed. Thus, the issue of interference effects should be addressed and minimized for quantitative HPLC–ELSD and HPLC–MS methodologies for quality control of HMs.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Herbal medicines (HMs) are one of the oldest compositions in traditional medicine in oriental countries. As estimated by World Health Organization (WHO), nearly one third of the world’s population uses HMs as the primary form of healthcare. Because of their unique effects and relatively low side effects, HMs have been gaining increasingly popularity all over the world [1–3]. Quality control is a challenge to ensure the safety, efficacy and batch-to-batch consistency of HMs products due to the complexity of phytochemical constituents. Usually, determination of single or several marker compounds by a developed method is required for quality control purpose [2,4].

The coupling of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) have enormous potential for quantitative analysis due to its undeniable capabilities [5–9]. High sensitivity and selectivity make HPLC–MS a preferred choice for HMs quantitative analysis. As we know, accurate quantitative determinations of marker compounds in herbal preparations need not only sophisticated instrument but also calibration curves calculated with a series of proper concentrations of working standard solutions. However, in most case, blank samples without the target analytes are not available for HMs [10]. Calibration curves are obtained by neat solvents without considering the presence of matrix effect [11,12]. Matrix effect is an important issue that should be addressed for HMs analysis. The environments in which the ionization processes take place are different, the response of the desired analyte in neat solvent and extract may even differ [13,14]. Unpredictable interferences coexist with the marker components in the complicated matrix as HMs consist of complex mixtures of phytochemical constituents. The coeluting, undetected matrix components may interfere with quantitative analysis of the analytes and affect the reproducibility, selectivity, precision and trueness of the assay [15,16]. Thus, developing more accurate assay method for HMs is of great concern to obtain quantitatively accurate results.

In this paper, a standard superposition method (SSM) has been proposed for accurately quantitative analysis of HMs. To validate precision and trueness of the proposed method, a detailed comparison with other two calibration curve preparation methods was performed, i.e., traditional neat standard method, standard addition method with a target-knockout extract (TKE) in which all compounds are present except for the determined ones and their coeluting. Three most commonly used detectors including MS,
evaporative light scattering detector (ELSD) and Ultraviolet (UV) detector were employed and compared for quantitative analysis of HMs. As an illustrative case study, Panax notoginseng (Burk.) F.H. Chen, a well-known herbal medicine, was used for method validation [17].

2. Experimental

2.1. Materials and reagents

Five standards of saponins, i.e., notoginsenoside R1 (1), ginsenoside Rg2 (2), Re (3), Rb1 (4), and Rd (5) were isolated previously from the dried roots of Panax notoginseng in our laboratory. The internal standard (IS), macranthoidine A, was also isolated previously from the flower buds of Lonicer a macranthoids in the authors’ laboratory. Their structures were elucidated by spectroscopic methods (UV, IR, MS, 1H NMR, and 13C NMR) and further confirmed by RRLC–TOF–MS (shown in Fig. 1) [18,19]. The purity of each compound was higher than 98%.

HPLC grade ACN was from Merck (Darmstadt, Germany). HPLC grade formic acid (>96% pure) was obtained from Tedia (Fairfield, OH, USA). Deionized water (18 MΩ cm−1) was prepared by distilled water through a Milli-Q system (Millipore, Bedford, MA, USA). All the chemicals used from commercial sources were of analytical grade or higher.

Twelve samples of Radix et Rhizoma Notoginseng (RRN) were collected from different places in Yunnan, China. All samples were authenticated by Dr. Xiuming Cui, professor of the Sanqi Research Institute of Wenshan Prefecture (Yunnan, China). The voucher specimens were deposited in the State Key Laboratory of Natural Medicines, China Pharmaceutical University.

2.2. Apparatus and chromatographic conditions

2.2.1. HPLC–MS analysis

Chromatographic analysis was performed on an Agilent 1100 Series (Agilent, Germany) LC system equipped with a binary pump, micro degasser, an auto plate-sampler, and a thermostatically controlled column apartment. Chromatographic separation was carried out at 30 ± 0.1 °C on an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 µm). The mobile phase was consisted of (A) water (0.1% formic acid, v/v) and (B) acetonitrile (0.1% formic acid, v/v) using a gradient elution of 20% B at 0–34 min, 20–29% B at 34–42 min, 29–38% B at 42–66 min. The injection volume was 10 µL and the flow rate was 1.0 mL/min. The post-column flow split ratio was 1:1. The mass spectrometer was Agilent 6530 single quadrupole mass spectrometer with an ESI source in negative mode. Conditions of MS analysis were as follows: drying gas flow rate, 10 L/min; drying gas temperature, 350 °C; nebulizer pressure, 35 psi; capillary voltage, 3000 V; quad temperature, 100 °C; fragmentor voltage, 100 V. The samples from HPLC column were analyzed in selective ion monitoring (SIM) mode by monitoring the molecular ions [M–H]− or [M+HCOO]− for saponins. SIM for each compound was restricted to specific retention time windows: 0–24 min at m/z 977.5, 24–40 min at m/z 845.5, 40–51 min at m/z 663.5, 51–65 min at m/z 1107.7 by channel 1; and 0–40 min at m/z 991.5, 40–51 min at m/z 663.5 and 51–65 min at 945.5 by channel 2.

2.2.2. HPLC–UV–ELSD analysis

The analyses were performed using an Agilent 1100 Series HPLC system, equipped with a binary pump, an auto-sampler, a column oven, a UV detection coupled with an ELSD (Alltech Associates, Deerfield, USA), a ChemStation Software Version A.10.02 (Agilent Technologies, USA) and the HPLC conditions were identical to those used for HPLC–MS analyses above-mentioned. The drift tube temperature for ELSD was set at 106 °C and the nitrogen flow rate was 3.0 L/min. The wavelength was set at 203 nm.

2.3. Sample preparation

The dried roots were powdered to a homogeneous size, and sieved through a No. 40 mesh. RRN (0.60 g) were extracted according to China Pharmacopoeia (2010 edition) [20]. An aliquot of 10 µL of the extract was injected into the HPLC system.

2.4. Target-knockout extract preparation

Target-knockout extract was prepared by authors’ laboratory previously developed fishing and knock-out method [21]. The fishing and knock-out experiments of determined components were performed using a HPLC analysis process and a valve-switching part. Firstly, different components of the extract were separated by the LC column, and the target compounds (determined compounds) were knocked out from the extract. Then, the residual part (without the target ones) was collected in a flask. Finally, the collections were evaporated and redissolved as the same concentration as the injection with methanol for the further study including standard solutions preparation and method validation.

2.5. Standard solutions and calibration curves

Mixed standard stock solution containing five accurately weighed reference compounds was directly prepared in 70% methanol (v/v).

Process A: Traditional standard method. Working standard solution was prepared by diluting the mixed standard stock solution with methanol to a series of proper concentrations. In detail, the working standard solutions were prepared by placing 3 µL of the mix standard stock solution, 1 µL IS and 57 µL neat solvent into 0.2 mL centrifuge tubes.

Process B: Standard addition method with TKE. The mixed standard stock solution was diluted with TKE instead of neat solvent to a series of proper concentrations. The samples were prepared by placing 3 µL of the mix standard stock solution, 1 µL IS and 57 µL target-knockout extract into 0.2 mL centrifuge tubes.

Process C: SSM with extract. In the last method, the mixed standard stock solution was diluted with the extract of RRN to a series of proper concentrations instead of neat solvent. In another word, working standard solution was prepared by spiking mixed standard stock solution to the extract. The samples were prepared by placing 3 µL of the mix standard stock solution, 1 µL IS and 57 µL the extract into 0.2 mL centrifuge tubes. The samples were prepared in double with two batches of extract of RRN originating from two different sources in Yunnan. As the control, only the 1 µL IS was added separately to 60 µL similar extract.

The final concentration of IS was 11.4 µg/mL for all working standard solutions. The standard stock and working solutions were all stored at 4 °C until use. The working standard solution of 10 µL was injected into HPLC for the construction of calibration curves. In Processes A and B, at least six concentrations in triplicate were analyzed and the calibration curves were calculated by linear regression of the plots of the ratio of the peaks area (analyte/IS) versus the concentration of the analyte. For Process C (see Fig. 2), six concentrations were prepared and analyzed three times. The control was also analyzed three times. Calibration curves were constructed as calibration curves were constructed as Eq. (5).

Please cite this article in press as: Y. Liu et al., J. Chromatogr. A (2012), http://dx.doi.org/10.1016/j.chroma.2012.07.020
2.6. Precision, repeatability and recovery

Intra- and inter-day variations were chosen to determine the precision of the developed method. For intra-day variability test, the working standard solutions (at low, medium and high levels of concentration) were analyzed in triplicate three times within one day, whereas for inter-day variability test, the working solutions were examined in triplicate for consecutive three days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD).

For repeatability test, five independent analytical sample solutions from the same batch of sample (No. 9) were prepared in the same procedures noted in Section 2.3. RSD (%) values of the obtained contents of each analyte were used to estimate repeatability.

Recovery was performed to evaluate the trueness of this method. Herein, spike blank recovery was used for determining the trueness of this method. The procedure was summarized as follows: 0.3 g of RRN was added into a flask, and different amounts (80%, 100%, 120%) of neat standard solution was then spiked, the residue was subsequently extracted and analyzed as described in Section 2.3. The recovery was determined by the formula: recovery (%) = (observed amount – original amount)/spiked amount × 100%.

2.7. Evaluation of matrix effects

Changes in the calibration curves generated by a series of standard solutions were used to evaluate the matrix effect contributed by extract. Different amounts (80%, 100%, and 120%) of neat standard solutions were prepared by spiking into three solvents (neat solvent, target-knockout extract and extract). An exact determination of matrix effects was obtained by relative recoveries: relative recoveries = (sample contents after adding–original contents)/contents of standard solutions for adding × 100%.
Table 1
Calibration curve, LODs and LOQs of the five analytes obtained in neat solvent (A), target-knockout extract (B) and extract (C) using LC-MS.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Calibration curve</th>
<th>R²</th>
<th>Test range (µg/mL)</th>
<th>CV [%]</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( A' ) ( y = 0.0062x - 0.0013 )</td>
<td>0.9992</td>
<td>3.62–231.52</td>
<td>AB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.34</td>
<td>34.64</td>
</tr>
<tr>
<td></td>
<td>( B' ) ( y = 0.0065x + 0.0095 )</td>
<td>0.9997</td>
<td>AB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C' ) ( y = 0.0065x + 0.0041 )</td>
<td>0.9997</td>
<td>AB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.34</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>( A ) ( y = 0.0339x + 0.0129 )</td>
<td>0.9955</td>
<td>9.53–609.90</td>
<td>AB</td>
<td>0.42</td>
<td>7.58</td>
</tr>
<tr>
<td></td>
<td>( B ) ( y = 0.0337x - 0.0848 )</td>
<td>0.9862</td>
<td>AC</td>
<td>AC</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C ) ( y = 0.0342x + 0.0248 )</td>
<td>0.9976</td>
<td>AC</td>
<td>AC</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>( A ) ( y = 0.0079x - 0.0030 )</td>
<td>0.9996</td>
<td>0.82–104.39</td>
<td>AB</td>
<td>4.39</td>
<td>79.69</td>
</tr>
<tr>
<td></td>
<td>( B ) ( y = 0.0081x - 0.0008 )</td>
<td>0.9992</td>
<td>AC</td>
<td>AC</td>
<td>8.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C ) ( y = 0.0088x - 0.0037 )</td>
<td>0.9994</td>
<td>AC</td>
<td>AC</td>
<td>8.52</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>( A ) ( y = 0.0140x - 0.0172 )</td>
<td>0.9954</td>
<td>1.50–96.21</td>
<td>AB</td>
<td>12.59</td>
<td>45.72</td>
</tr>
<tr>
<td></td>
<td>( B ) ( y = 0.0087x - 0.0018 )</td>
<td>0.9967</td>
<td>AC</td>
<td>AC</td>
<td>22.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C ) ( y = 0.0075x - 0.0267 )</td>
<td>0.9969</td>
<td>AC</td>
<td>AC</td>
<td>22.91</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>( A ) ( y = 0.0157x - 0.0039 )</td>
<td>0.9998</td>
<td>2.07–132.27</td>
<td>AB</td>
<td>8.47</td>
<td>20.05</td>
</tr>
<tr>
<td></td>
<td>( B ) ( y = 0.0177x + 0.0059 )</td>
<td>0.9995</td>
<td>AC</td>
<td>AC</td>
<td>9.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C ) ( y = 0.0179x + 0.0012 )</td>
<td>0.9894</td>
<td>AC</td>
<td>AC</td>
<td>9.26</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> \( y \) is the peak area ratio (analyte/IS), \( x \) is the concentration injected (µg/mL).
<sup>b</sup> \( y \) is the difference of peak area ratio (Peak area ratio<sub>pre-spiked</sub> – Peak area ratio<sub>blank</sub>), \( x \) is the concentration injected (µg/mL).
<sup>c</sup> Comparison of slope of each calibration obtained in Process B and in Process A.
<sup>d</sup> Comparison of slope of each calibration obtained in Process C and in Process A.

3. Results and discussion

3.1. Proposal of standard superposition method

The precision and trueness of quantitative results of HMs depends on the calibration. Unlike chemical medicine, HMs consist of complex mixtures of phytochemical constituents with diverse structures and physicochemical properties. The determined target compounds in HMs coexist with the complicated matrix in the extract. The coeluting, undetected matrix components may interfere with quantitative analysis of the target analytes and affect the reproducibility, selectivity, precision and trueness of the assay. The matrix effect phenomenon was described by Villagrasa and Ferrer, who showed that electrospray responses of one compound decreased with an increase in concentrations of other ones [22,23].

Many different calibration methods were used to compensate for matrix effects when matrix suppression phenomena cannot be eliminated, such as quantification with stable isotope labeled internal standards method [24], matrix-matched calibration method [25], and standard addition method [26]. For quality control of HMs, determination of multiple components is usually required. However, it is commercially unavailable to obtain isotopically labeled standards for each individual analytes of interest. A standard matrix similar to that of the samples to be investigated but free from analytes is difficult to be prepared for matrix-matched calibration method. Though standard addition method can compensate for precision and trueness losses by matrix compounds, it is achieved at the expense of sacrificing sensitivity.

When matrix effects are indicated or are to be expected and matrix-matched calibration samples are not available, a SSM is proposed to be the choice for calibration. Mechanism of the SSM is shown in Fig. 2. A reference HM extract or the test sample itself is first profiled by HPLC-MS. The peak area of the target analyte and internal standard was recorded as \( A'_{\text{pre-spiked}} \) and \( A_{\text{IS}} \), respectively. Working standard solutions were subsequently spiked into the reference extract. Their peak areas were recorded again as \( A_{\text{post-spiked}} \) and \( A_{\text{IS}} \). \( A_{\text{pre-spiked}} \) includes the contribution of the original extract (peak area as the “\( A'_{\text{pre-spiked}} \)”) and the spiked one (peak area as the “\( A_{\text{spiked}} \)”). Since the original analyte and the internal standard are present in the same solution, their ratios should remain constant, see Eq. (1). According to Eq. (1), we can calculate the area contribution of the original analyte \( A'_{\text{pre-spiked}} \) in spiked standard solution, see Eq. (2). Then, the peak area corresponding to the spiked concentration \( A_{\text{spiked}} \) can be calculated from Eq. (3). Calibration curves are constructed by plotting the ratio of the analyte spiked to the internal standard signal as a function of the analyte concentration of the standards, see Eqs. (4) and (5).

\[
\frac{A_{\text{pre-spiked}}}{A_{\text{IS}}} = \frac{A_{\text{pre-spiked}}}{A_{\text{IS}}} \quad \text{(1)}
\]

\[
\frac{A'_{\text{pre-spiked}}}{A_{\text{IS}}} = \frac{A'_{\text{pre-spiked}}}{A_{\text{IS}}} \quad \text{(2)}
\]

\[
A_{\text{spiked}} = A_{\text{post-spiked}} - A_{\text{pre-spiked}} = A_{\text{IS}} \cdot \frac{A_{\text{IS}}}{A_{\text{IS}}} - A_{\text{pre-spiked}} \quad \text{(3)}
\]

\[
A_{\text{spiked}} = \frac{A_{\text{IS}}}{A_{\text{IS}}} - A_{\text{pre-spiked}} \quad \text{(4)}
\]

\[
\frac{A_{\text{spiked}}}{A_{\text{IS}}} \quad \text{(5)}
\]

As a comparative case, a standard addition method with TKE was also studied. In TKE, target analytes were fished out according to their accurate retentions times in chromatographic resolution. It differed from the blank matrix samples since coeluting interferences were simultaneously knocked out. However, the comparison analysis of data from SSM and TKE could show the matrix effects from coeluting or other mechanisms for ion suppression/enhancements.

3.2. Calibration plots built through neat standard solvent, TKE and SSM

The performance of calibration curves was determined by ES-MS. Five standard lines were prepared by neat standard by SSM, TKE, and SSM, respectively. As shown in Table 1, no significant differences were observed in calibration curves by ANOVA test with calculated \( p \)-values > 0.05, the correlation coefficients (\( R^2 \)), LODs and LOQs for all the analytes by different methods. All calibration curves showed good linear regression (\( R^2 > 0.9954 \)) within the test ranges. The LODs and LOQs of the five saponins were in the range of 7.58–79.69 ng/mL and 30.95–318.75 ng/mL, respectively.

The matrix effect can be evaluated by the comparison of the slopes of the calibration plots built for neat standard analytes and for the TKE or SSM on the sample. The slope and the intercept in different plots should correspond to one another within the acceptable experimental error deviation. A relatively lower slope in the plot of TKE or SSM for compound 4 suggests possible ion signal suppression while higher slopes for compounds 1–3 and 5 indicate possible ion signal enhancement. Compared with the
Table 2
Calibration curve of the five analytes obtained in two batches of extract, respectively (C and C').

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Calibration curve*</th>
<th>CV (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Process C</td>
<td>Process C</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>y = 0.0065x + 0.0041</td>
<td>y = 0.0067x + 0.0068</td>
<td>2.14</td>
</tr>
<tr>
<td>2</td>
<td>y = 0.0342x + 0.0248</td>
<td>y = 0.0351x + 0.0335</td>
<td>1.84</td>
</tr>
<tr>
<td>3</td>
<td>y = 0.0088x – 0.0037</td>
<td>y = 0.0091x – 0.0060</td>
<td>2.37</td>
</tr>
<tr>
<td>4</td>
<td>y = 0.0075x + 0.0267</td>
<td>y = 0.0075x + 0.0152</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>y = 0.0179x + 0.0012</td>
<td>y = 0.0181x + 0.0010</td>
<td>0.79</td>
</tr>
</tbody>
</table>

* y is the difference of peak area ratio (Peak area ratio_{analyte} – Peak area ratio_{pre-spiked}), x is the concentration injected (μg/mL).

coefficient variability (CV) of the slopes between SSM and neat solvent, CV of the slopes between TKE and neat solvent was smaller. This phenomenon showed that the matrix effect mainly result from coeluting interferences rather than near components. Among the five analytes, the matrix effects were greater for compounds 4–5 with CV at 16.43% and 7.11%, respectively.

For high-throughput sample determination, one reference batch extract will be applicable instead of each batch extract for accurate calibration assays. To validate the method, we compared the instrumental response obtained for: (C) the sample to be tested for calibration; (C') another sample from different location for calibration. As shown in Table 2, the slopes of five saponins obtained from two different sources of extract are similar with CV < 2.37%. Calibration curve developed by one batch of extract can be used for quantitative analysis of other RRN commercial samples. Moreover, the CV of slopes of five saponins obtained for different target-knockout extract was smaller (data not shown). Thus, the target-knockout extract was also prepared using one batch of sample extract.

3.3. Precision, repeatability and recovery of the SSM

The intra- and inter-day variations of the SSM with ESI–MS detector were less than 6% and repeatability test shown in Table 3 demonstrated that the developed assay was reproducible (RSD < 5%). The results of the percentage recoveries were in the range of 91–110% with RSD less than 5% (Table 3).

3.4. Evaluation of matrix effect

The assessment of matrix effect is critical when homologues rather than stable isotope labeled analytes are utilized as internal standards. Based on the method of LC–MS, as indicated in Table 4, the average relative recoveries of three concentrations (the low, medium and high concentrations) for five analytes the using the neat solvent were 108.08%, 99.00%, 110.21%, 68.77% and 111.79%, respectively. While these determined by Process B and Process C were improved to some degree. The average relative recoveries of SSM were close to 100%, i.e., 98.18%, 97.36%, 99.54%, 98.17% and 96.34% for five saponins, indicating the matrix effects were decreased significantly. Compared with the data from SSM, the relative recoveries using TKE were compensated fewer. This phenomenon also indicated that the matrix effect mainly arise from coeluting interferences. By examining the ratio analyte/IS in different solvents, it was shown that the differences of the relative recoveries were due to significant variability in MS response when the same amount of standard solution and IS was prepared in three process. The differences in MS response could be explained as the competition between an analyte and a number of other undetected, especially the coeluting components for ion formation in the HPLC–MS interface. The ion formation is highly dependent on the relative ionization energies, proton affinities, or both of the molecules present in the MS interface at any given time. It is obvious that the formation efficiency of the desired ions can be matrix-dependent.

In neat solvents, the formation efficiency of the [M-H]− or other ion is not affected by the presence of matrix coeluting with the analyte, and the ionization efficiency is high. However, when an analyte with the same amount is present in the extract, a number of other compounds having lower and/or higher ionization potentials, proton affinities, etc., may affect the ionization efficiency of the analyte. Thus, SSM is done to compensate for the loss of analytes signal response. Using an internal extract conforms to the environment, will improve precision, trueness and sensitivity of quantitative analysis. Because of no additional cost, simplicity, precision and trueness, SSM can act as a very practical approach to overcome matrix effects when the quantitative analysis of HMIs is carried out.

An efficient chromatographic separation of analytes from matrix interferences can also minimize the matrix effect. As shown in Fig. 3b, the separation of notoginsenoside R1, ginsenoside Rg1, and Re was better than ginsenoside Rb1. The data obtained confirmed that the reliability and selectivity of quantitative method can be improved through more efficient HPLC separation (Tables 4 and 5). The results presented in this paper demonstrated the importance of studying and minimizing matrix effect during the development of quantitative methods.

3.5. Application of three calibration method to quantitative analysis of five saponins in 12 batches of RRN commercial samples

The proposed methods determined by ESI–MS detection were successfully applied to simultaneous determination of five saponins in 12 batches of RRN from different sources in China. Fig. 3b shows the representative TIC of the extracts of RRN by HPLC–MS. Five saponins in samples were identified in comparison to retention time, characteristic fragment ions and accurate mass data of reference standards (see Fig. 3a). The content comparison between the neat standard solution and SSM or TKE was illustrated in Table 5. Because of matrix interferences, high RSD variations of 4.80–9.71%, 14.60–31.18% and 8.12–9.32% were observed for the content of notoginsenoside R1, ginsenoside Rb1 and Rd, respectively.

The quantification results by SSM were summarized in Table 6. Ginsenoside Rg1, with an average concentration of 50.04 mg/g, was the predominant constituent among the five saponins in RRN, followed by ginsenoside Rb1, notoginsenoside R1, ginsenoside Rd, and Re. The total level of five ginseng saponins ranged from 97.21 to 152.60 mg/g among twelve batches of commercial samples. This change can be ascribed to several factors like environmental conditions, harvesting time, collecting and processing. Thus, establishment of an accurate quality control method is important to ensure the efficacy and safety of HMIs.
Table 3
Precisions, repeatabilities and recovery of the five analytes in Process C.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precisiona (n = 3)</th>
<th>Inter-day (RSD%)</th>
<th>Recoveryb (n = 3)</th>
<th>Recoveryc (n = 3)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High Med Low</td>
<td>High Med Low</td>
<td>Mean content (mg/g)</td>
<td>RSD (%)</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>0.25 0.79 4.90</td>
<td>0.56 0.14 4.72</td>
<td>23.10</td>
<td>1.01</td>
<td>102.50</td>
</tr>
<tr>
<td>2</td>
<td>0.94 0.32 0.36</td>
<td>2.04 0.69 2.28</td>
<td>60.75</td>
<td>1.63</td>
<td>96.19</td>
</tr>
<tr>
<td>3</td>
<td>1.41 4.92 2.37</td>
<td>2.24 1.98 3.76</td>
<td>8.48</td>
<td>4.61</td>
<td>98.03</td>
</tr>
<tr>
<td>4</td>
<td>0.97 1.23 3.06</td>
<td>4.37 5.18 6.00</td>
<td>50.22</td>
<td>3.51</td>
<td>100.87</td>
</tr>
<tr>
<td>5</td>
<td>1.50 3.07 2.47</td>
<td>1.11 2.64 11.14</td>
<td>10.05</td>
<td>4.62</td>
<td>97.87</td>
</tr>
</tbody>
</table>

a The low, medium and high concentrations of working standard solutions were 0.06, 0.25, and 0.5-fold of stock solution, respectively.
b Sample no. 9 was analyzed, and contents of each analyte were used to access repeatability.
c Recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of RRN 0.6 mg alone within the linearity range were taken.

Table 4
Matrix effect4 data in three different processes using LC-MS.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Process A</th>
<th>Process B</th>
<th>Process C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High Med Low</td>
<td>High Med Low</td>
<td>High Med Low</td>
</tr>
<tr>
<td>1</td>
<td>104.57 105.41 114.27</td>
<td>98.69 92.11 75.27</td>
<td>99.39 97.67 97.48</td>
</tr>
<tr>
<td>2</td>
<td>100.38 103.10 93.53</td>
<td>91.39 96.01 97.29</td>
<td>99.39 101.75 90.94</td>
</tr>
<tr>
<td>3</td>
<td>111.10 107.90 111.54</td>
<td>104.88 101.05 102.70</td>
<td>100.00 97.46 101.17</td>
</tr>
<tr>
<td>4</td>
<td>72.10 70.90 63.31</td>
<td>84.51 81.40 68.95</td>
<td>101.18 100.73 92.59</td>
</tr>
<tr>
<td>5</td>
<td>111.69 113.85 109.84</td>
<td>98.67 97.79 91.04</td>
<td>97.76 98.22 93.05</td>
</tr>
</tbody>
</table>

4 Matrix effect studies were carried out by adding different amounts (80%, 100%, and 120%) of RRN 0.6 mg alone within the linearity range were taken.

3.6. Performance of standard superposition method in ELSD and UV detector

ELSD and UV are also commonly used for quantitative analysis of HMs. Their detection mechanisms are different from the MS. An precision and trueness comparison of the three processes was also done using these two detectors. For ELSD, the slopes of two calibration curves of the five saponins developed by neat solvent and TKE were almost the same, but the results developed by SSM were different (see Fig. S1 in Supplementary Data). These results indicated that the coeluting interference compounds rather than others interfered the peak signal of the analytes in ELSD. The comparison among the content of the three calibration curves obtained in ELSD is illustrated in Fig. S2. It is clear that the high RSD values were highly variable for content of notoginsenoside R1, ginsenoside Rg1, Re, Rb1 and Rd in neat solvents and extract. The content of analytes determined by Process A is always higher than that measured by Process C. The SSM was capable of eliminating the interference of the coeluting molecules to guarantee the selectivity of quantitatively analysis of the targets.

Contrary to the results obtained using the MS and ELSD, the slopes of three calibration curves of the five saponins in UV were
Table 5
RSD value of content for five analytes in RRN using LC–MS.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>RSD (%)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACb</td>
<td>5.86</td>
<td>6.20</td>
<td>6.01</td>
<td>5.70</td>
<td>6.11</td>
<td>5.20</td>
<td>4.91</td>
<td>6.46</td>
<td>4.80</td>
<td>5.16</td>
<td>4.95</td>
<td>4.85</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>5.00</td>
<td>4.81</td>
<td>5.16</td>
<td>4.87</td>
<td>4.49</td>
<td>5.46</td>
<td>5.00</td>
<td>4.98</td>
<td>5.15</td>
<td>5.24</td>
<td>5.50</td>
<td>5.55</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>0.93</td>
<td>0.96</td>
<td>0.92</td>
<td>0.95</td>
<td>1.00</td>
<td>0.88</td>
<td>0.93</td>
<td>0.94</td>
<td>0.92</td>
<td>0.91</td>
<td>0.87</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>1.03</td>
<td>0.08</td>
<td>1.01</td>
<td>0.93</td>
<td>0.39</td>
<td>0.49</td>
<td>1.71</td>
<td>0.61</td>
<td>1.59</td>
<td>1.96</td>
<td>1.62</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>3.33</td>
<td>1.45</td>
<td>3.30</td>
<td>3.18</td>
<td>3.92</td>
<td>2.41</td>
<td>4.51</td>
<td>0.56</td>
<td>4.31</td>
<td>4.94</td>
<td>4.35</td>
<td>3.71</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>15.46</td>
<td>15.65</td>
<td>16.23</td>
<td>15.44</td>
<td>15.98</td>
<td>15.17</td>
<td>15.30</td>
<td>16.39</td>
<td>14.66</td>
<td>14.50</td>
<td>15.20</td>
<td>14.83</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>29.21</td>
<td>29.62</td>
<td>30.84</td>
<td>29.17</td>
<td>30.31</td>
<td>28.59</td>
<td>28.89</td>
<td>31.18</td>
<td>27.50</td>
<td>27.39</td>
<td>28.68</td>
<td>27.87</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>8.32</td>
<td>8.25</td>
<td>8.24</td>
<td>8.50</td>
<td>8.27</td>
<td>8.53</td>
<td>8.12</td>
<td>8.18</td>
<td>8.70</td>
<td>8.72</td>
<td>8.56</td>
<td>8.62</td>
<td></td>
</tr>
</tbody>
</table>

a Comparison of content of each investigated compound obtained in Process B and in Process A.
b Comparison of content of each investigated compound obtained in Process C and in Process A.

4. Conclusions

Accurate quantitation of HMs should constitute an important part of quality control. However, "unseen" endogenous compounds in HMs coeluting with the analytes might cause matrix effect, which has deleterious impact on the reliability and selectivity of quantitative determination of analytes. The extent of interference effect seems to be highly dependent on the mechanism of the detector. Under otherwise identical sample extraction and chromatographic conditions, the relative interference effect for compounds studied in this paper was not observed when the UV was utilized but was indicated when MS and ELSD were employed. When a significant interference effect was observed during the development of a valid determination for marker compound in HMs, standard superposition method is proposed to be a choice for calibration. It is good to conform to the environment by the greatest extent in which the ionization, the light scattering and light absorption of the extract processes take place. The calibration curves were more accurate as the interference, which generated by the presence of any other endogenous materials in the coeluting with the analyte, could be deducted in advance. Therefore, the validity quantitative data of target compound in HMs could be obtained by the absence of interference effect. The strategy described in this paper may provide guidance for establishing accurate quantitative methods of HMs.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Nos. 81130068 and 81173497), PhD top-notch talent incubation program (No. 2010BPBY01) and Program sponsored for Scientific Innovation Research of College Graduate in Jangsu Province (No. CXLS11_0789).

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.jchroma.2012.07.020.

References