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Short communication

# Simultaneous quantification of active components in the herbs and products of Si-Wu-Tang by high performance liquid chromatography–mass spectrometry

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ABSTRACT

Si-Wu-Tang (SWT), comprising Paeoniae, Angelicae, Chuanxiong and Rehmanniae, is one of the most popular Traditional Chinese Medicine (TCM) formulae for woman's health. Data mining from the available Chinese and English literatures indicated that the major bioactive components of SWT consist of paeoniflorin, paeonol, gallic acid, ferulic acid, Z-ligustilide, ligustrazine, butylphthalide, senkyunolide A and catalpol. Since content determination of the marker compounds is generally considered as an initial step for quality control of TCM product, a high performance liquid chromatography–mass spectrometric method employing both positive and negative electrospray ionization was developed for the simultaneous determination of the nine identified compounds in the raw herbs and products of SWT. The LOQ of the developed assay method for the tested components was 10 ng/ml for ligustrazine, 200 ng/ml for catalpol, and 100 ng/ml for the other seven compounds. The intra-day and inter-day variations of the current assay were within 17.5%. Paeoniflorin, ferulic acid, gallic acid, Z-ligustilide and senkyunolide A were found in all SWT products investigated. Variations in the contents of the studied compounds were observed among batches of raw herbs and SWT products. The currently developed method provides a sensitive and rapid quantification approach that can be useful in the quality control of raw herbs and products of SWT.


## 1. Introduction

Si-Wu-Tang (SWT) is a Traditional Chinese Medicine (TCM) formula that is widely used for the treatment of women's disease such as relief of menstrual discomfort, climacteric syndrome, dysmenorrheic and other estrogen related diseases [1] despite of the lack of significant reduction in menstrual pain intensity of primary dysmenorrhoea reported from a pilot post-market clinical trial [2]. A more recent study demonstrated that the SWT formula can be integrated as an alternative therapy within Western medicine [3].

The SWT formula comprises four herbs, i.e. Radix Paeoniae Alba, Rhizoma Chuanxiong, Radix Angelicae Sinensis and Radix Rehmanniae Preparata [4]. The major bioactive components in these four herbs include phenolics, phthalides, alkaloids, terpene glycosides and iridoid glycosides. Among these classes of compounds, nine compounds have been identified from data mining of the available Chinese and English database. They are gallic acid (GA), paeoniflorin (PF) and paeonol (PO) from Paeoniae [5–8]; ferulic acid (FA), Z-ligustilide (Lig) and senkyunolide A (SA) from Angelica [9,10]; ferulic acid (FA), Z-ligustilide (Lig), ligustrazine (TMP), butylphthalide (Bu) and senkyunolide A (SA) from Chuanxiong [9,11–13]; and catalpol (Cat) from Rehmanniae [14–16]. Among these compounds, ferulic acid, paeoniflorin and Z-ligustilide in SWT were found to have antioxidative, antimutagenic, anti-inflammatory, vasodilation and antiallergic effects [17–19]. The structures of these reported active components are shown in Fig. 1.

In view of the popular use of SWT in Asian countries and lack of information on the contents of these active components in the marketed products, the quality of various SWT products is unknown. According to the Chinese Pharmacopeia (CP) 2005 [4], paeoniflorin is the only designated marker for the quantitative analysis of Si Wu He Ji (SWHJ, i.e. SWT in liquid dosage form). It is well known that therapeutic effect of TCM can be attributed from multiple rather than one component in the product. Thus, a rapid, sensitive, accurate analytical method for the simultaneous determination of multiple components will be helpful for initial comparison of the quality of the raw herbs as well as the SWT products.

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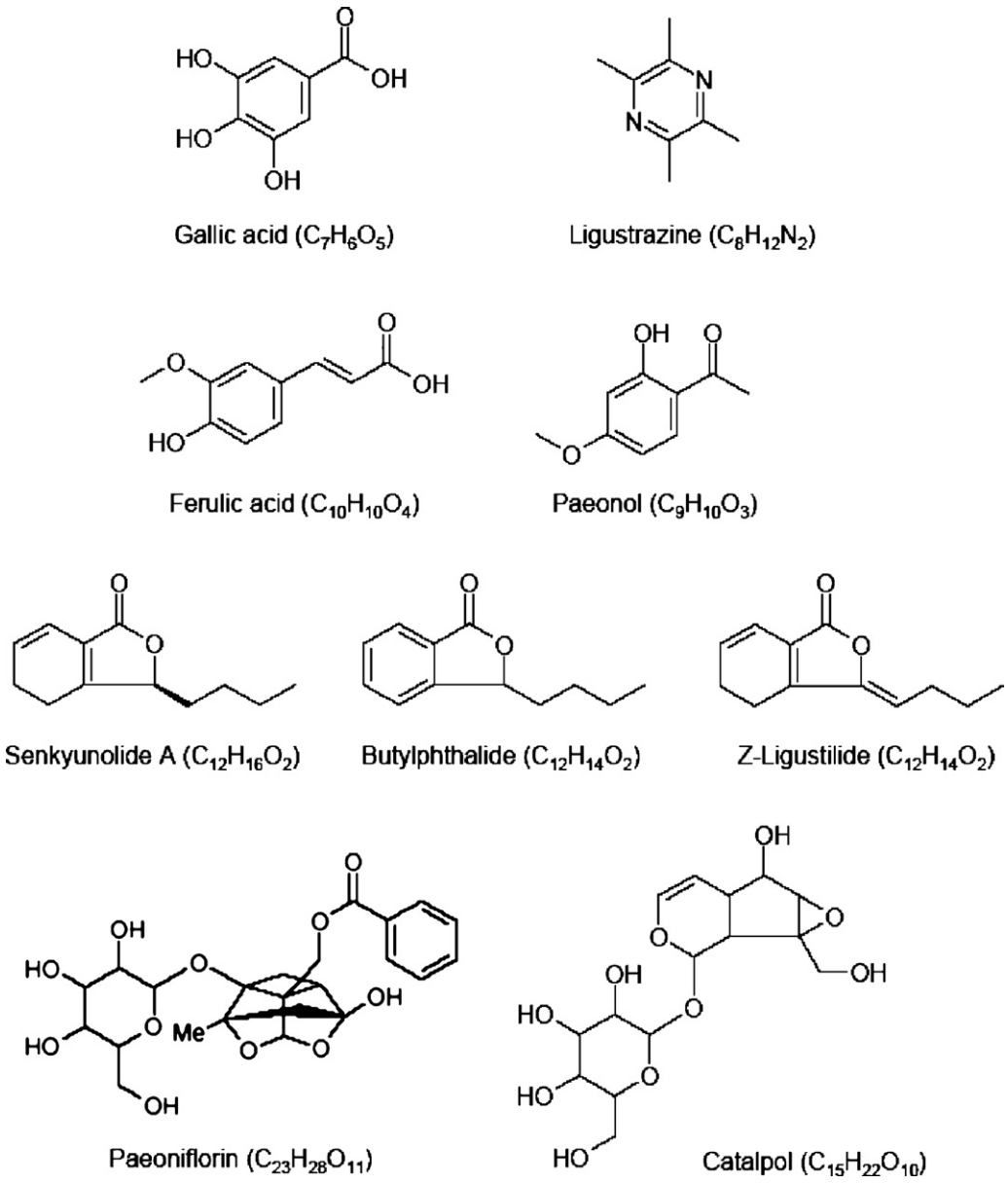


**Fig. 1.** Chemical structures of the nine studied compounds.

Up to now, there are only limited reports on the quantitative analysis of SWT. Li et al. determined the content of ferulic acid and paeoniflorin in the slice decoction and dispensing granule decoction of SWT by high performance liquid chromatography with diode array detector (HPLC-DAD) using two separate assays [20]. Simultaneous determination of the components (including gallic acid, paeoniflorin, ferulic acid and other components) of SWT and/or crude herbs were also reported using HPLC-DAD [5] for quantification and HPLC-DAD–MS [13] for quantification (DAD) and identification (MS). In general, DAD lacks specificity and is not sensitive enough for compounds in trace amount, which is usually encountered in the analysis of TCM products. Moreover, catalpol, the identified active component in Rehmanniae, cannot be determined by DAD due to its low UV absorptivity. In addition, most mass spectrometric studies on SWT were focused on qualification rather than quantification [21].

We here report a sensitive and specific high performance liquid chromatography–mass spectrometric method for the simultaneous quantification of nine bioactive components in SWT products. The developed method was applied to the determination of the nine components in various commercially available raw herbs of SWT and SWT products.

## 2. Materials and methods

### 2.1. Materials

The reference standards of gallic acid, paeoniflorin, paeonol, ferulic acid, ligustrazine, catalpol were obtained from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Senkyunolide A, Z-ligustilide, butylphthalide were obtained from the Hong Kong Jockey Club Institute of Chinese Medicine Limited (Hong Kong, China). 2′-Hydroxyflavanone (internal standard for ions detected at negative ionization mode) and tolbutamide (internal standard for ions detected at positive ionization mode) were purchased from Sigma–Aldrich (St. Louis, USA). The purity of all these standards

**Table 1**
MS conditions for the nine studied compounds.

| Tested compound[a] | Scanning mode | Q1 (*m*/*z*) | Q3 (*m*/*z*) | DP[b] | EP[b] | CEP[b] | CE[b] | CXP[b] |
|---|---|---|---|---|---|---|---|---|
| Gallic acid | MRM | 168.9 $[M-H]^-$ | 124.7 | −31 | −9 | −10 | −22 | 0 |
| Paeoniflorin | MRM | 525.1 $[M+HCOO]^-$ | 120.8 | −31 | −7.5 | −22 | −40 | 0 |
| Ferulic acid | MRM | 193.0 $[M-H]^-$ | 133.7 | −80 | −10 | −12 | −22 | 0 |
| Catalpol | MRM | 407.0 $[M+HCOO]^-$ | 198.7 | −31 | −7 | −20 | −18 | −2 |
| Ligustrazine | SIM | 137.0 $[M+H]^+$ | NA[c] | 41 | NA | 10 | NA | NA |
| Paeonol | SIM | 167.0 $[M+H]^+$ | NA | 41 | NA | 12 | NA | NA |
| Senkyunolide A | SIM | 193.0 $[M+H]^+$ | NA | 51 | NA | 10 | NA | NA |
| Butylphthalide | SIM | 191.0 $[M+H]^+$ | NA | 41 | NA | 10 | NA | NA |
| Z-Ligustilide | SIM | 191.0 $[M+H]^+$ | NA | 51 | NA | 10 | NA | NA |

[a] Gallic acid, paeoniflorin, ferulic acid and catalpol were analyzed in negative ionization mode while the others were detected in positive ionization mode.
[b] DP: declustering potential; EP: entrance potential; CEP: collision cell entrance potential; CE: collision energy; CXP: collision cell exit potential.
[c] NA: not applicable.

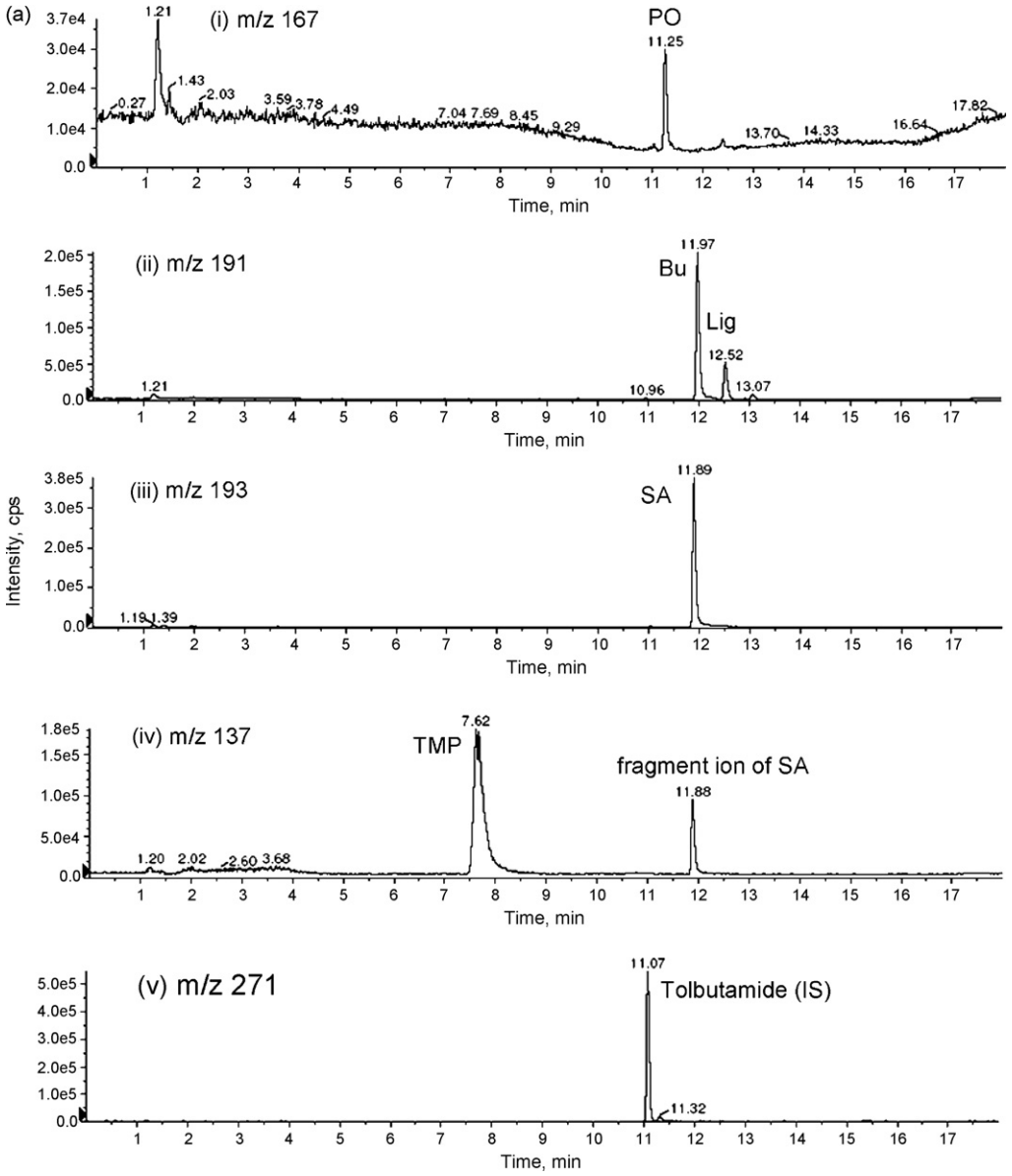


**Fig. 2.** Extracted ion chromatogram of calibration standard mixture obtained by (a) SIM scanning (positive mode). The concentrations of analytes in standard mixture are 1.5 μg/ml for paeonol (PO), butylphthalide (Bu), senkyunolide A (SA) and Z-ligustilide (Lig), 0.15 μg/ml for ligustrazine (TMP), and 20 μg/ml for tolbutamide (IS); (b) MRM scanning (negative mode). The concentrations of analytes in standard mixture are 1.5 μg/ml for gallic acid (GA), ferulic acid (FA) and paeoniflorin (PF), 3 μg/ml for catalpol (Cat), and 20 μg/ml for 2′-hydroxyflavanone (IS).

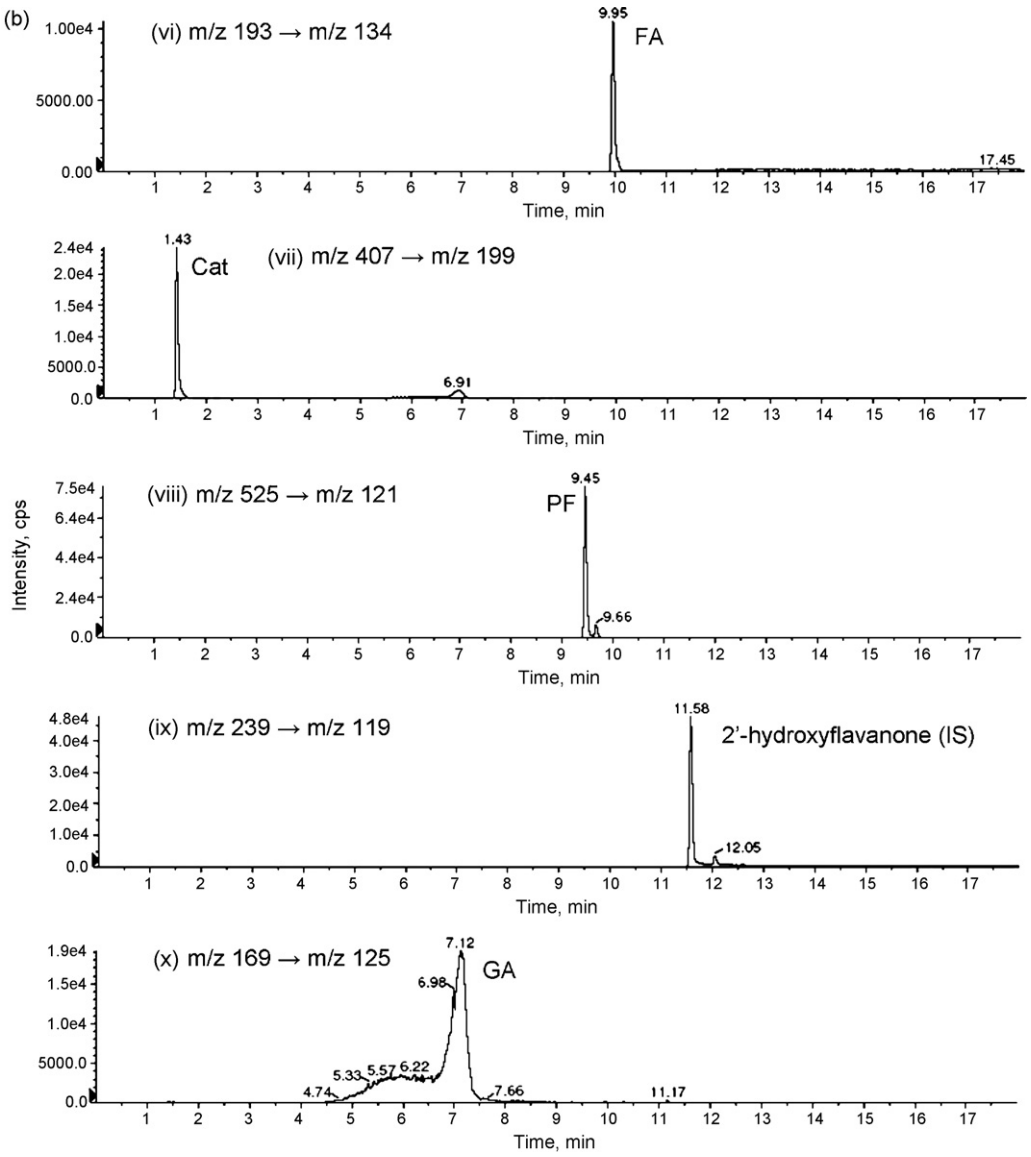


**Fig. 2.** (*Continued*).

was better than 98% (except Z-ligustilide which is >95% purity). Acetonitrile (HPLC grade), formic acid (reagent grade), dimethyl sulfoxide (reagent grade), ethanol and methanol (HPLC grade) were obtained from Labscan (Labscan Asia, Bangkok, Thailand). Unless specified elsewhere, all reagents were used without further purification. Distilled and deionized water (dd$H_2O$) was prepared from Millipore water purification system (Millipore, Milford, USA).

Crude herbs of SWT were purchased from five different vendors of the Mainland China and/or Hong Kong. These include Radix Paeoniae Alba obtained from Anhui and Zhejiang Provinces, Radix

**Table 2**
Linearity, LOQ and LOD of tested compounds determined by the current method.

| Tested compound | Slope | *y*-Intercept | $r^2$ | LOQ[a] (μg/ml) | LOD[b] (ng/ml) | LOD[c] (ng per injection) |
|---|---|---|---|---|---|---|
| Gallic acid | 1.5250 | 0.0041 | 0.9980 | 0.1 | 20 | 0.4 |
| Paeoniflorin | 0.6095 | 0.0141 | 0.9834 | 0.1 | 10 | 0.2 |
| Ferulic acid | 0.0555 | 0.0009 | 0.9937 | 0.1 | 20 | 0.4 |
| Catalpol | 0.0416 | 0.0007 | 0.9964 | 0.2 | 20 | 0.4 |
| Ligustrazine | 8.9546 | 0.0079 | 0.9974 | 0.02 | 5 | 0.1 |
| Paeonol | 0.0512 | −0.0003 | 0.9984 | 0.2 | 50 | 1 |
| Senkyunolide A | 0.6526 | 0.0032 | 0.9992 | 0.1 | 30 | 0.6 |
| Butylphthalide | 0.3907 | 0.0055 | 0.9958 | 0.1 | 30 | 0.6 |
| Z-ligustilide | 0.1357 | 0.0002 | 0.9992 | 0.1 | 30 | 0.6 |

[a] LOQ is defined as the concentration of analyte where the signal-to-noise of ≥10:1 and the signal is reproducible to a precision of 20% and accuracy of 80–120%.
[b] LOD is defined as the concentration of analyte where signal-to-noise ratio of ≥3:1.
[c] LOD per injection = LOD × 20 μl injection volume.

**Table 3**
Inter-day and intra-day precision and accuracy of the assay method.

| Tested compound | Nominal value (μg/ml) | Intra-day ($n$ = 5) | | Inter-day ($n$ = 4) | |
|---|---|---|---|---|---|
| | | % Accuracy | % RSD | % Accuracy | % RSD |
| Gallic acid | 0.4 | 85.7 | 17.5 | 97.1 | 14.1 |
| | 1.5 | 104.2 | 7.5 | 101.5 | 3.9 |
| | 4 | 105.8 | 10.3 | 108.3 | 4.0 |
| Paeoniflorin | 0.4 | 109.0 | 9.9 | 105.6 | 4.6 |
| | 1.5 | 102.1 | 8.1 | 98.7 | 3.4 |
| | 4 | 93.6 | 8.1 | 84.5 | 6.5 |
| Ferulic acid | 0.4 | 107.8 | 8.2 | 105.8 | 2.6 |
| | 1.5 | 99.1 | 6.3 | 97.3 | 3.0 |
| | 4 | 98.7 | 6.2 | 94.4 | 5.3 |
| Catalpol | 0.8 | 98.5 | 11.1 | 101.5 | 5.6 |
| | 3 | 94.5 | 4.8 | 102.3 | 10.8 |
| | 8 | 92.9 | 10.9 | 92.9 | 8.8 |
| Ligustrazine | 0.04 | 97.1 | 8.0 | 99.8 | 4.6 |
| | 0.15 | 92.7 | 6.0 | 96.7 | 5.0 |
| | 0.4 | 95.0 | 8.6 | 92.8 | 9.2 |
| Paeonol | 0.4 | 105.3 | 14.0 | 100.8 | 12.8 |
| | 1.5 | 92.3 | 7.8 | 98.9 | 1.8 |
| | 4 | 96.8 | 9.2 | 100.9 | 6.3 |
| Senkyunolide A | 0.4 | 111.4 | 1.6 | 100.4 | 8.0 |
| | 1.5 | 100.2 | 4.2 | 94.1 | 8.0 |
| | 4 | 89.4 | 9.2 | 80.8 | 8.0 |
| Butylphthalide | 0.4 | 102.1 | 7.6 | 111.0 | 9.1 |
| | 1.5 | 95.7 | 7.8 | 98.0 | 8.2 |
| | 4 | 92.6 | 16.7 | 83.5 | 13.8 |
| Z-ligustilide | 0.4 | 98.1 | 11.5 | 102.8 | 6.0 |
| | 1.5 | 94.8 | 6.6 | 100.4 | 1.2 |
| | 4 | 90.5 | 9.6 | 94.0 | 2.8 |

Angelicae Sinensis from Gansu Province, Rhizoma Chuanxiong from Sichuan Province and Radix Rehmanniae Preparata from Henan Province. Three commercially available SWT products, assigned as SWT1, SWT2 and SWT3, were purchased from the Mainland China and/or Hong Kong markets. SWT1 contains a packet of 4 raw herbs to be decocted before use. SWT2 and SWT3 are SWT products in solid (granules) and liquid dosage forms, respectively. In addition, a SWT product (assigned as CU-SWT), in solid dosage form, was manufactured by the Hong Kong Institute of Biotechnology Limited (Hong Kong, China) according to the Chinese Pharmacopeia 2005 [4] with modification.

### 2.2. Apparatus

The high performance liquid chromatography (HPLC)–mass spectrometric system consisted of an ABI 2000 Q-Trap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, USA) equipped with an electrospray ionization (ESI) source, two Perkin-Elmer PE-200 series micro-pumps and an auto-sampler (PerkinElmer, Norwalk, CT, USA). The chromatographic separation of the analytes was achieved by using a Zorbax Eclipse XDB-$C_{18}$ column (2.1 mm × 150 mm, 3.5 μm, Agilent) connected with a Zorbax Eclipse XDB-$C_8$ guard cartridges (2.1 mm × 12.5 mm, 5 μm, Agilent).

### 2.3. Instrumental conditions

The separation of nine compounds was achieved by a linear gradient elution using a mobile phase containing acetonitrile (A) and 0.1% formic acid (B). Due to the difference in physicochemical properties of the nine compounds, both positive and negative ionization mass spectrometric analyses were conducted. For positive ion analysis, the gradient began with 90% A for 5 min, changed linearly to

**Table 4**
Extraction recoveries of the nine compounds from herbs.

| Analytes | Spiked concentration (mg/g) | Extraction recovery (%) |
|---|---|---|
| Ligustrazine | 0.1 | 94.6 ± 10.9 |
| | 2 | 88.1 ± 2.2 |
| | 5 | 86.5 ± 3.9 |
| Paeonol | 0.5 | 101.8 ± 10.5 |
| | 1 | 96.7 ± 4.3 |
| | 5 | 104.8 ± 2.9 |
| Senkyunolide A | 0.5 | 92.5 ± 7.9 |
| | 1 | 89.0 ± 1.1 |
| | 5 | 90.3 ± 0.9 |
| Butylphthalide | 0.5 | 90.5 ± 4.8 |
| | 1 | 89.8 ± 2.6 |
| | 5 | 90.4 ± 2.3 |
| Paeoniflorin | 0.5 | 90.3 ± 8.1 |
| | 1 | 89.5 ± 2.1 |
| | 5 | 80.5 ± 2.8 |
| Ferulic acid | 0.5 | 102.3 ± 5.9 |
| | 1 | 99.3 ± 6.8 |
| | 5 | 90.4 ± 4.3 |
| Z-ligustilide | 0.5 | 88.9 ± 6.7 |
| | 1 | 86.8 ± 2.6 |
| | 5 | 98.9 ± 3.1 |
| Catalpol | 0.5 | 91.1 ± 5.2 |
| | 1 | 88.1 ± 2.2 |
| | 5 | 83.4 ± 2.3 |
| Gallic acid | 0.5 | 121.0 ± 0.6 |
| | 1 | 90.7 ± 0.6 |
| | 5 | 92.0 ± 3.2 |

20% A in 5 min and remained for 5 min before changing it back to 90% A and equilibrating for 3 min before next sample injection. The elution profile for negative ion analysis was the same as that in positive mode, except the gradient started at 95% A. In both cases, flow rate was 0.3 ml/min, and the temperatures of auto-sampler and the analytical column were set at 4 °C and room temperature, respectively. Injection volume of 20 μl was used.

All the liquid chromatographic eluent was then introduced into the ESI source. Typical mass spectrometric conditions were: gas 1, nitrogen (30 psi); gas 2, nitrogen (40 psi); ion spray voltage, 5500 V for positive mode and −4500 V for negative mode; ion source temperature, 300 °C; curtain gas, nitrogen (20 psi). Selected ion monitoring (SIM) and multiple reaction monitoring (MRM) scanning were acquired in positive and negative ionization modes, respectively. All other settings were analyte-specific and are summarized in Table 1.

### 2.4. Preparation of standard solutions and calibration standards

Individual stock solutions of Cat, SA, Lig, Bu and PO were prepared by dissolving the appropriate amount of each standard compound in dimethyl sulfoxide, while GA, FA, PF and TMP were

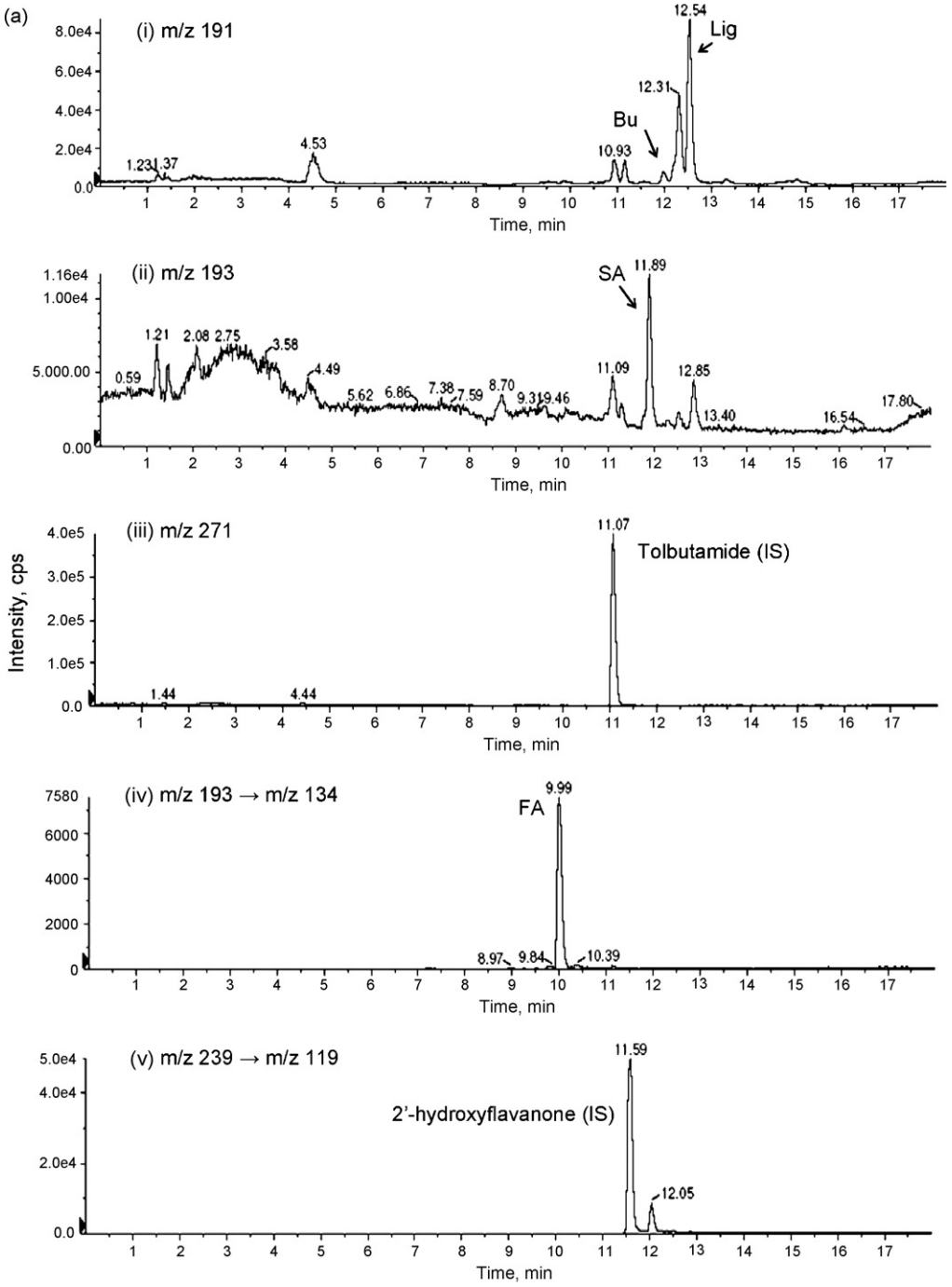


**Fig. 3.** Extracted ion chromatogram of (a) Angelicae Sinensis obtained by (i to iii) SIM scanning (positive mode) and (iv to v) MRM scanning (negative mode); (b) Chuanxiong obtained by (i to iv) SIM scanning (positive mode) and (v to vi) MRM scanning (negative mode); (c) Paeoniae Alba obtained by (i to ii) SIM scanning (positive mode) and (iii to v) MRM scanning (negative mode); (d) Rehmanniae Praeparata obtained from MRM scanning (negative mode). Lig: Z-ligustilide; Bu: butylphthalide; FA: ferulic acid; SA: senkyunolide A; TMP: ligustrazine. PO: paeonol; PF: paeoniflorin; GA: gallic acid; Cat: catalpol.

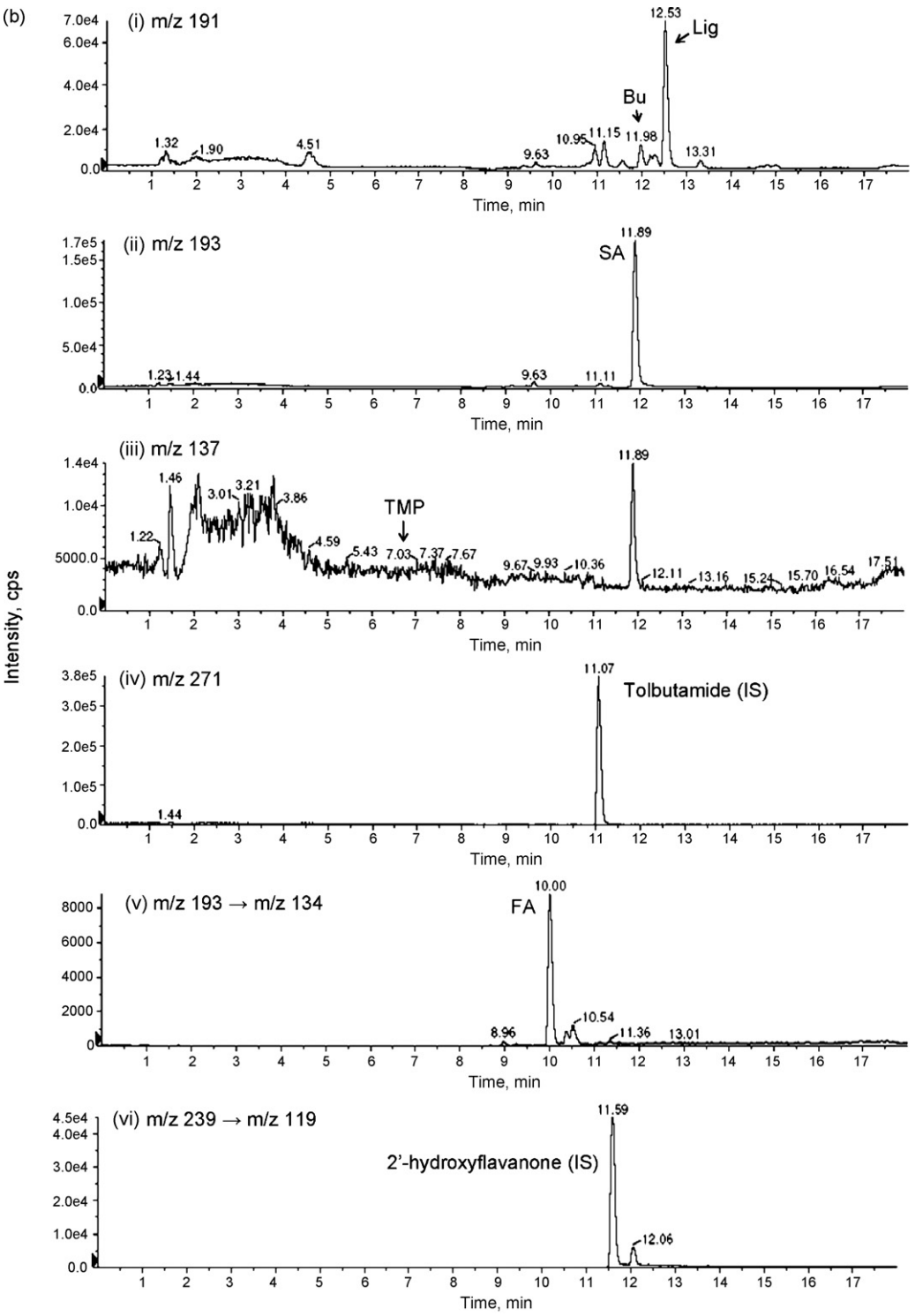


**Fig. 3.** (*Continued*)

dissolved in 50% (v/v) methanol to yield a concentration of 1 mg/ml. 2′-Hydroxyflavanone and tolbutamide were the internal standards (IS) for negative and positive ionization mass spectrometric analysis, respectively, and were prepared by dissolving the appropriate amount of the standard compounds in methanol to yield a concentration of 1 mg/ml.

Calibration standard mixture (for positive ion detection) was prepared by serial dilution of the stock standard solutions with 50% (v/v) methanol to yield concentrations of 0.02–0.5 μg/ml for TMP, 0.2–5 μg/ml for PO, 0.1–5 μg/ml for SA, Bu and Lig, and 20 μg/ml for tolbutamide. Similarly, calibration standard mixture (for negative ion detection) contains 0.2–10 μg/ml for Cat, 0.1–5 μg/ml for FA, PF and GA, and 20 μg/ml for 2′-hydroxyflavanone. Each calibration curve contained at least five different concentrations and was conducted in triplicate. Calibration curves were constructed by plotting the peak area ratio of analyte/IS versus the analyte concentration and fitted by weighed linear least-squares regression analysis.

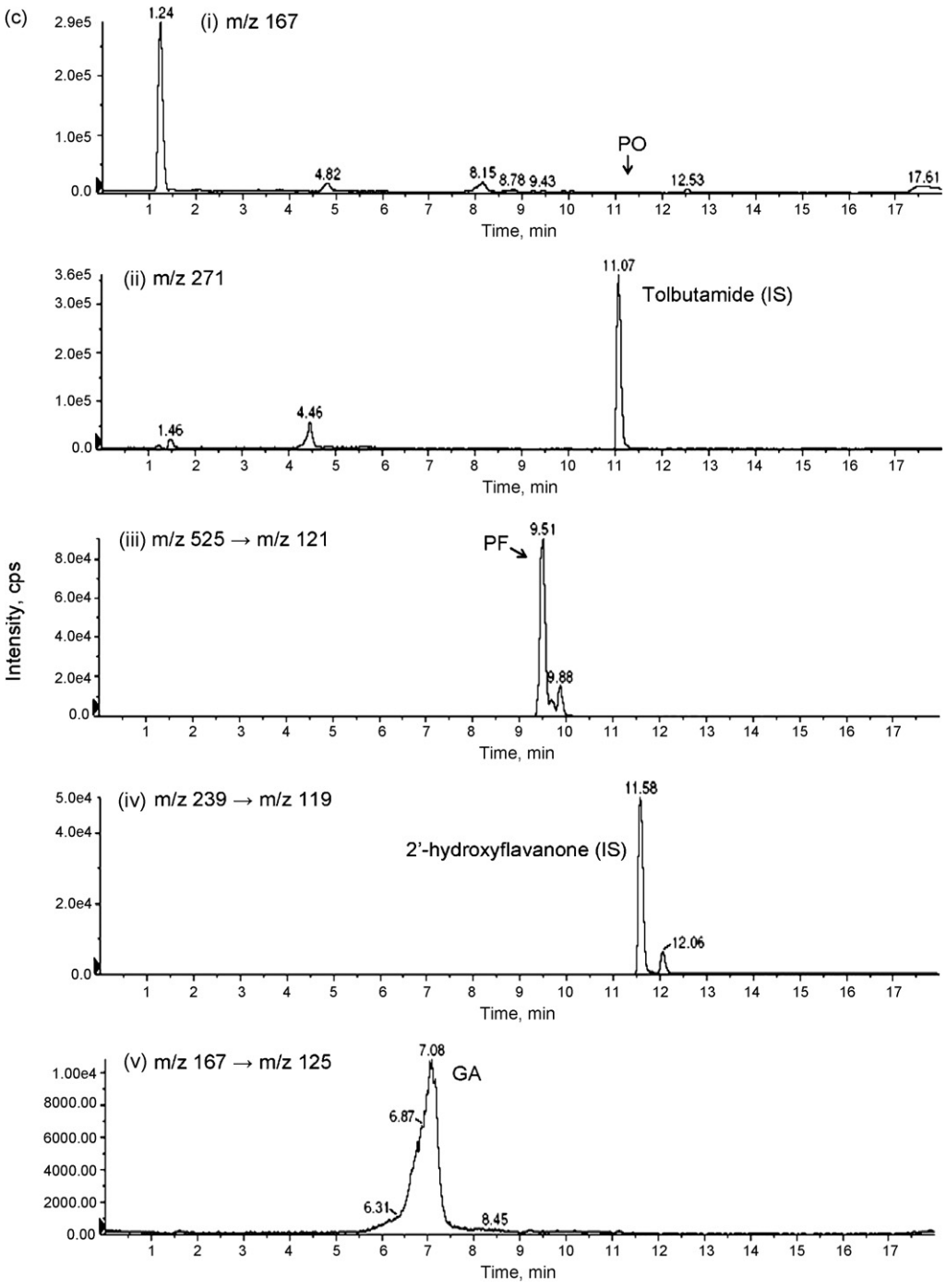


**Fig. 3.** (*Continued*)

### 2.5. Limit of detection and limit of quantification

The limit of detection (LOD) was defined as the concentration of each analyte where its signal-to-noise ratio is ≥3. The limit of quantification (LOQ) was defined as the concentration of each analyte where the signal-to-noise ratio is ≥10 and the signal is reproducible to a precision of 20% and accuracy of 80–120%.

### 2.6. Precision and accuracy

The intra-day and inter-day precision and accuracy of the developed method was determined by validating quality control samples at low, median and high concentrations. Each sample was analyzed in five replicate in the intra-day reproducibility test, and the inter-day test was conducted on four separate days with each sample analyzed in triplicate. The precision is presented as a measure of relative standard deviation (RSD) and the accuracy is assessed as the percentage bias from the nominal concentration (%).

### 2.7. Extraction of studied components from raw herbs and products of SWT

The extraction of raw herbs Angelicae and Paeoniae was performed according to the methods described in the CP (2005) [4]. Briefly, 20 ml of 70% (v/v) methanol was added to 200 mg Angelica powder. The mixture was refluxed for 30 min. After cooling

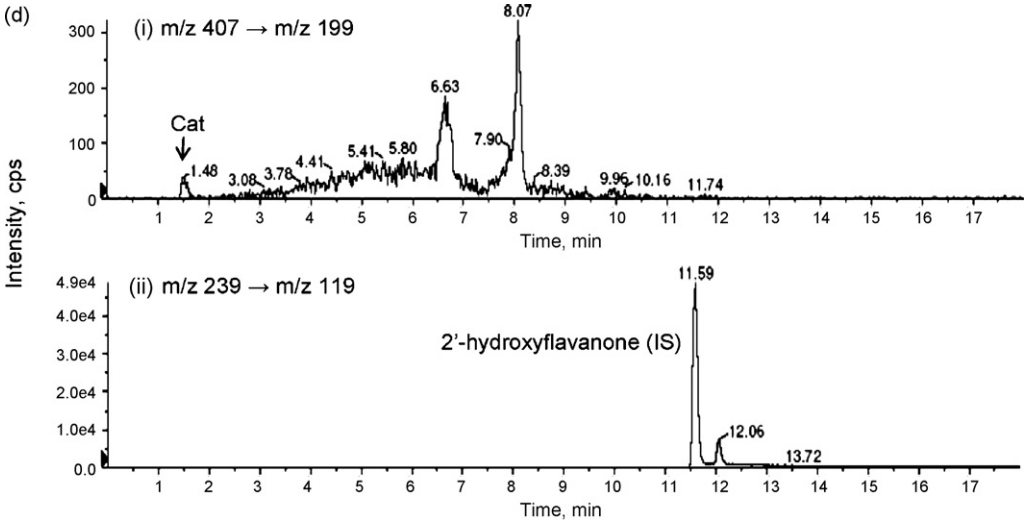


**Fig. 3.** (*Continued*).

down, the mixture was adjusted to the original weight by 70% (v/v) methanol and filtered through a 0.45 μm nylon filter. For the extraction of Paeoniae, 35 ml of 50% (v/v) ethanol in water added to 100 mg crude herb powder in a 50-ml volumetric flask and the mixture was sonicated for 30 min. After cooling, the volume was then adjusted to 50 ml with 50% (v/v) ethanol in water followed by filtration using a 0.45 μm nylon filter.

For Chuanxiong and Rehmanniae, due to the lack of well recognized standard extraction method, different conditions were tried in order to optimize the extraction efficiency. To extract the active components from Chuanxiong, 200 mg herb powder (40 mesh, pulverized by a pulverizer (Type A10, IKA-Werke, Germany)) was refluxed with 20 ml methanol (20%, 50% or 80%, v/v) for 30 min. After cooling down, the mixture was adjusted to the original weight by the extraction solvent and filtered through a 0.45 μm nylon filter. Raw herb Rehmanniae was cut into small pieces (2 mm × 2 mm) and 35 ml methanol (20%, 50% or 80%, v/v) was added to 100 mg herb pieces in a 50-ml volumetric flask followed by sonication for 30 min. After cooling down, the volume was adjusted to 50 ml with the original extraction solvent. In addition to optimization of the extraction solvent, different extraction period (30 and 60 min) was also investigated.

The nine components in commercially available SWT products were extracted according to their preparation methods as described in the product insert. For SWT1, the raw herb pieces were decocted together in 1.5 L water to a final volume of 500 ml. For SWT2, which is in granule form, 10 ml water was added to 100 mg granules and the mixture was extracted in 60 °C water bath for 30 min. For SWT3, which is in liquid dosage form, the sample was diluted to 100 times by water.

CU-SWT, our manufactured product prepared according to CP (2005) [4] with slight modification, was extracted by adding 10 ml 50% (v/v) methanol to 100 mg SWT powder followed by sonication for 30 min. The extraction solvent was then passed through a 0.45 μm nylon filter.

To 200 μl of each extracted sample, 20 μl of IS and 200 μl methanol was added, vortex mixed and 20 μl was taken for liquid chromatography–mass spectrometric analysis.

### 2.8. Evaluation of the extraction recoveries of the active components from raw herbs of SWT

Extraction recovery was conducted by spiking known quantities (at low, medium and high concentration ranges) of selected analytes to the corresponding herb powders and the resulted mixture was extracted and analyzed as described above. The extraction recovery was calculated as follows:

Extract recovery (%) = 100 × (amount of each compound measured − amount of each compound already existed in the studied herb)/(amount of each compound spiked).

## 3. Results and discussions

### 3.1. Liquid chromatography–mass spectrometric method development and validation

Due to different chemical nature of the nine compounds (their structures are shown in Fig. 1), both positive and negative ionization mass spectrometric analyses were conducted. Under negative ionization mode, deprotonated molecular ions $[M-H]^-$ of FA and GA, and adducted molecular ions $[M+HCOO]^-$ of PF and Cat were generated in abundance and upon collision-induced dissociation, produced specific fragment ions that can be used for MRM detection. In the positive ionization mode, abundant protonated molecular ions $[M+H]^+$ were observed for TMP, SA, PO, Bu and Lig. Although MRM is more specific than SIM, not all compounds can produce abundant fragment ion(s) for MRM monitoring. For the nine analytes investigated in this study, no apparent fragment ions were detected for TMP, PO and Lig. Therefore, the SIM scanning on the protonated molecular ions of TMP, SA, PO, Bu and Lig were monitored. The extraction ion chromatograms of each analyte were presented in Fig. 2 and the mass spectrometric conditions were listed in Table 1.

The linearity, LOD and LOQ values of each analyte are presented in Table 2. Precision and accuracy of the developed method are shown in Tables 2 and 3. The calibration curves for the nine compounds displayed good linear ($r^2 > 0.98$) relationships under the present chromatographic conditions. The LOQ was 0.02 μg/ml for TMP, 0.2 μg/ml for Cat and PO and 0.1 μg/ml for the rest. The LOD ($S/N \geq 3$) ranged from 5 to 50 ng/ml for the nine analytes, which corresponds to 0.1–1 ng analyte per injection. The overall intra-day and inter-day variability (shown in Table 3) were 1.6–17.5% RSD and 1.2–14.1% RSD, respectively, and the accuracy ranged from 85.7 to 111.4% and 83.5 to 111.0% for intra-day and inter-day assay, respectively. The extraction recoveries of the nine compounds investigated were found to be in the range of 80.5–121.0% (Table 4).

In the current study, we reported the first quantitative analysis of SWT components using high performance liquid chromatogra-

phy–mass spectrometry. The present mass spectrometric method has several advantages over the previous reported methods. Firstly, this method allows the simultaneous quantitative analysis of nine bioactive compounds in SWT and/or its raw herbs. These nine bioactive compounds were selected from each of the four herbs in SWT and should be more representative markers for SWT products than any designated single chemical marker. Secondly, by using mass spectrometric detection, LOD (per injection) of gallic acid, paeoniflorin and ferulic acid can be achieved at 0.2–0.4 ng, which is 2.5–25 times more sensitive than the reported HPLC-DAD–MS method [13]. Such sensitivity is crucial for the analysis of related bioactive components which are usually present in trace level in the products. Thirdly, catalpol, the active compound found in Rehmanniae and exhibited low UV absorptivity, can only be detected by mass spectrometry with LOD down to 20 ng/ml level. Fourthly, the current method significantly shortens the assay time by using both positive and negative ionization modes (total 36 min analysis time) in comparison to the reported HPLC-DAD–MS method (140 min if both positive and negative ionization modes are acquired) [13].

### 3.2. Optimization of the sample extraction methods for Chuanxiong and Rehmanniae

In selecting the extraction method for Chuanxiong, we followed similar extraction procedures from Angelicae, as both herbs shared common components (e.g. FA and Lig). It is expected that concentrations of methanol could affect the extraction efficiency of each component. Therefore, various methanol compositions (i.e.

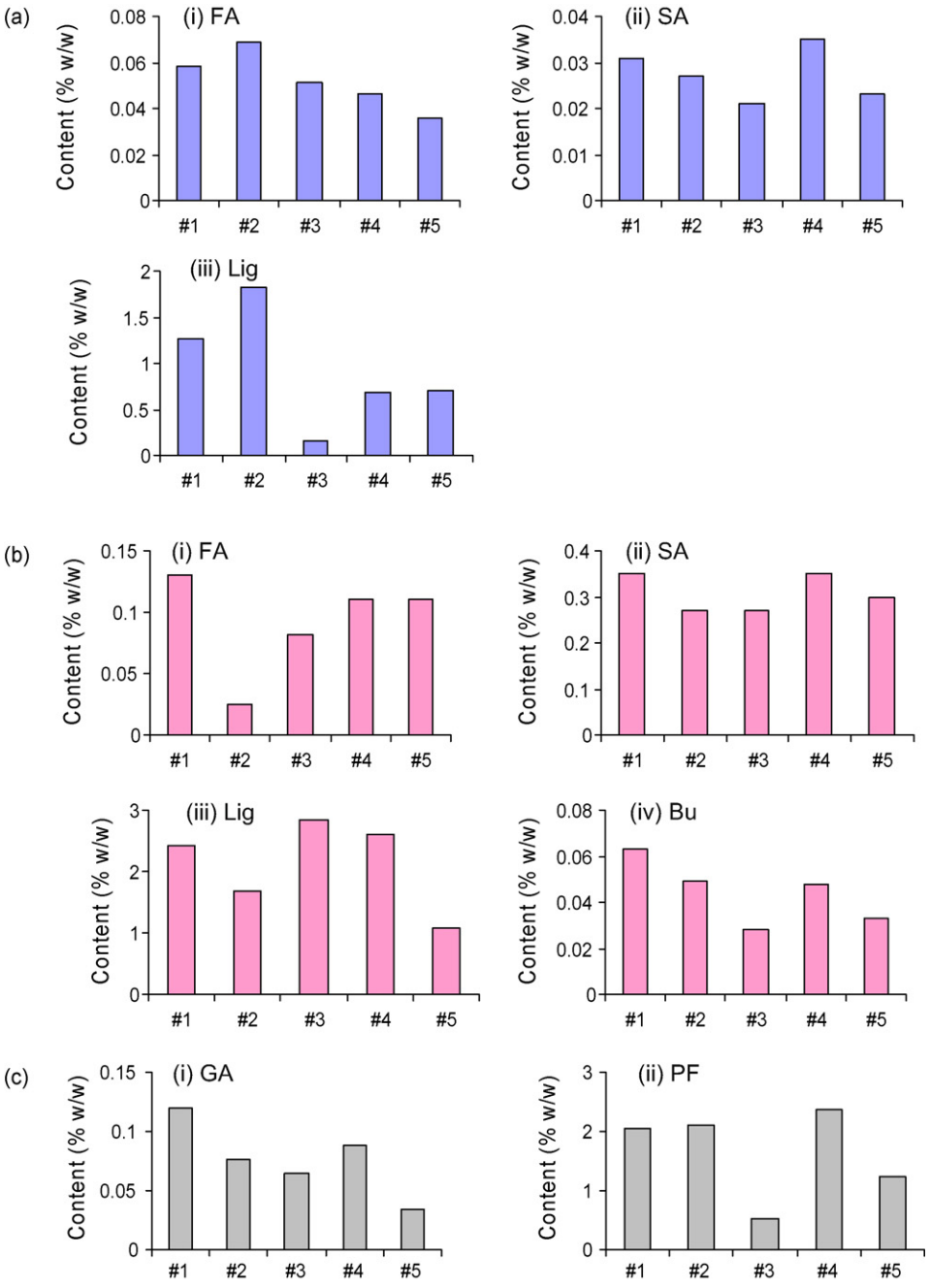


**Fig. 4.** Contents of tested compounds in the five batches of (a) Angelicae Sinensis; (b) Chuanxiong; (c) Paeoniae Alba. Lig: Z-ligustilide ; FA: ferulic acid; SA: senkyunolide A; Bu: butylphthalide; PF: paeoniflorin; GA: gallic acid.

20%, 50% and 80%, v/v) were tried. The contents of FA, Lig and SA, the major components in Chuanxiong, were found to be 0.13%, 2.41%, 0.35% (w/w), respectively, when extracting with 50% (v/v) methanol. Similar results (i.e. 0.11%, 2.42%, 0.35%, w/w) for FA, Lig and SA, respectively) were obtained when 80% methanol was used for extraction. On the other hand, the extraction efficiency of Lig and SA reduced (2.10% and 0.20%, w/w) when 20% (v/v) methanol was used. For the extraction of Rehmanniae, the content of Cat was found to be around 0.004% (w/w) when 50% (v/v) and 80% (v/v) methanol were used but was almost undetectable when extracting with 20% (v/v) methanol. In order to achieve the maximal extraction efficiency for all studied components in Chuanxiong and Rehmanniae, 50% (v/v) methanol was eventually used to as the extraction solvent for both herbs.

In addition to the selection of extraction solvent, the effect of extraction duration on the extraction efficiencies was also investigated. It is found that no significant difference in extraction efficiency of all studied compounds between the extraction period of 30 or 60 min. Thus, extraction period of 30 min was chosen for the analysis.

### *3.3. Contents of active components in single herbs of SWT and SWT products*

The extracted ion chromatograms of Angelicae, Chuanxiong, Paeoniae and Rehmanniae were shown in Fig. 3a, b, c and d, respectively, and the contents of representative analytes in the corresponding herbs of all batches were presented in Fig. 4. As shown in Fig. 4a, FA, Lig and SA were detected in all five batches of Angelicae, with Lig being the major component. Although the herb was cultured from the same province (Gansu), large variation on the content FA (0.04–0.07%, w/w) and Lig (0.16–1.82%, w/w) among batches were observed. Moreover, two (Batch #4 and Batch #5) out of five batches of Angelicae examined could not comply with the 0.05% (w/w) FA requirement as specified by CP (2005) [4].

The contents of FA, SA, Lig and Bu in Chuanxiong were presented in Fig. 4b. Similar to Angelicae, Lig in Chuanxiong contributes to the highest content among the compounds tested. In general, the contents of Lig, FA and SA in Chuanxiong were higher than those in Angelicae. Furthermore, variation of FA and Lig among batches (all from Sichuan Province) was found. Bu was detected in trace

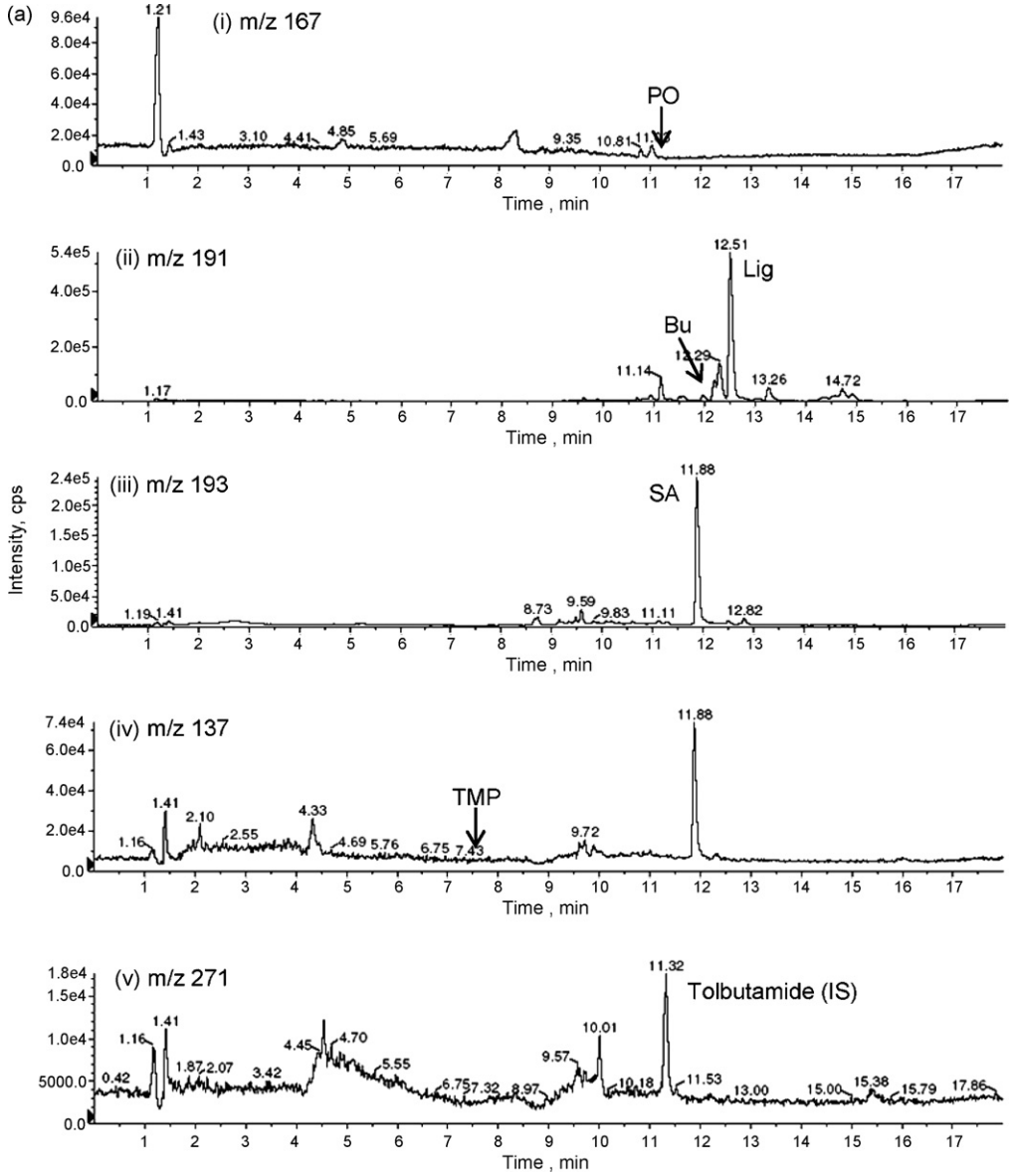


**Fig. 5.** Extracted ion chromatogram of CU-SWT obtained by (a) SIM scanning (positive mode) and (b) MRM scanning (negative mode). PO: paeonol; Lig: Z-ligustilide; Bu: butylphthalide; SA: senkyunolide A; TMP: ligustrazine; FA: ferulic acid; Cat: catalpol; PF: paeoniflorin; GA: gallic acid.

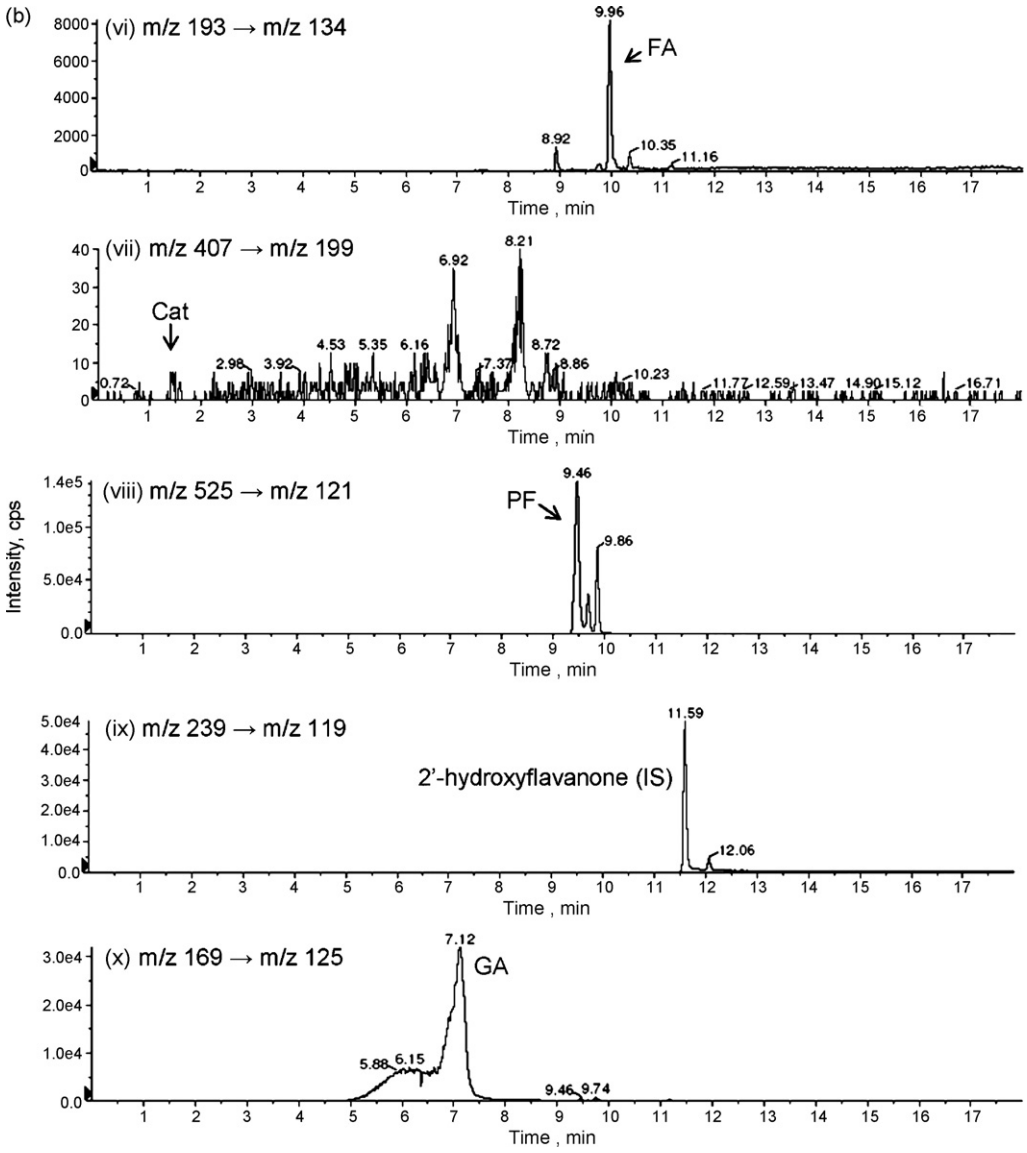


**Fig. 5.** (*Continued*).

amount and TMP was undetectable in all batches of Chuanxiong. The absence in TMP in Chuanxiong is consistent with the literature [13].

PF was the most dominant component in Paeoniae (Fig. 4c), followed by GA. Batch #3 contained the lowest PF content and Batch #5 also below the 1.6% (w/w) requirement set in CP (2005) [4]. PO was not detectable in all tested Paeoniae samples. Due to the complexity TCM, the co-existing compounds with same molecular weight or structural isomers are also common. It was noticed that a structural isomer is present in the herb Paeoniae. Based on the previous report by Dong et al. [22], the compound is suggested to be albiflorin. Both PF and albiflorin contain same benzoic acid

**Table 5**
Contents of the nine studied compounds[a] in SWT products.

| Product | Content (mg/g) ± SD ($n$ = 3) | | | | | | |
|---|---|---|---|---|---|---|---|
| | GA | SA | PF | FA | Lig | Bu | TMP, PO, Cat |
| (a) Products in solid dosage form | | | | | | | |
| CU-SWT | 0.987 ± 0.089 | 0.075 ± 0.002 | 8.651 ± 0.240 | 0.525 ± 0.015 | 1.127 ± 0.224 | ND[b] | ND |
| SWT1 | 0.131 ± 0.009 | 0.044 ± 0.002 | 0.054 ± 0.004 | 0.020 ± 0.002 | 0.037 ± 0.002 | ND | ND |
| SWT2 | 0.506 ± 0.070 | 0.059 ± 0.001 | 1.930 ± 0.143 | 0.154 ± 0.041 | 0.034 ± 0.003 | ND | ND |
| Product | Content (μg/ml) ± SD ($n$ = 3) | | | | | | |
| | GA | SA | PF | FA | Lig | Bu | TMP, PO, Cat |
| (b) Products in liquid dosage form | | | | | | | |
| SWT3 | 32.7 ± 1.5 | 2.07 ± 0.04 | 5795 ± 366 | 1.62 ± 0.47 | 1.02 ± 0.04 | 0.42 ± 0.03 | ND |

[a] GA: gallic acid; PF: paeoniflorin; FA: ferulic acid; Lig: Z-ligustilide; SA: senkyunolide A; Bu: butylphthalide; Cat: catalpol; TMP: ligustrazine; PO: paeonol.
[b] ND: not detectable.

backbone structure and could produce the benzoate ion ($m/z$ 121) with high abundance in MS/MS. Thus, the large peak next to PF in Fig. 5b is expected to be arisen from albiflorin, which could be well resolved from the peak of PF.

Cat is the only monitoring compound for Rehmanniae. Results show that very low content (<0.005%, w/w) of Cat was determined in all batches.

The contents of the nine tested compounds in CU-SWT and three commercial SWT products were listed in Table 5. Totally five of the nine components, namely GA, FA, PF, Lig and SA, were identified in quantified amount. Cat, PO and TMP, which are either not found in the crude herbs or of very low content, were not detectable in all SWT products. It has been reported that the content of Cat decreases greatly during the processing of raw herbs [23]. Bu was only detected in SWT3 (liquid dosage).

## 4. Conclusion

A sensitive, specific and rapid liquid chromatography–mass spectrometric method has been developed for the simultaneous detection of nine major active components in raw herbs and products of SWT. Application of the current method in the evaluation of raw herbs and/or SWT products showed a wide variation in the content of the identified active compounds in these products. Such results can form the basis for improvement of quality of this widely used product in the future.

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