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Short communication

Quality control of Chinese medicinal preparations LC/ESI(+)/MS/MS analyses of saikosaponins-a and -c as markers of Bupleuri radix samples

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Abstract

We have used LC/ion trap tandem MS analysis to determine saikosaponin-a and -c as target markers in crude 70% methanol extracts from three different species of Bupleuri radix and the 10 most-popular Chinese medicinal preparations containing "Chaihu" (B. radix) without any clean-up. The optimal ionization characteristics were obtained when using positive-ion electrospray ionization (ESI) with 50 μM sodium acetate as an additive in the mobile phase. We observed good linearity over the range from 0.02 to 2 μg/ml for saikosaponin-a and from 0.02 to 1 μg/ml for saikosaponin-c. The intra-day precisions varied between 3.3 and 8.8% for saikosaponin-a and 0.3 and 11.1% for saikosaponin-c. The limits of detection were 0.01 μg/ml for both markers. The recoveries of saikosaponin-a and -c from the extract of a medicinal preparation sample (Chai-Hu-Ching-Gan-Tang, No. 13 in the table of section Analysis on actual samples) were 97 and 100%, respectively, at a 1 μg/ml spiking concentration of each marker. The highest concentrations of saikosaponin-a and -c among the three B. radixes were found in *B. kaoi* Liu Chao & Chuang (10.1 mg/g) and in *B. falcatum* (3.4 mg/g), respectively. The amounts of these saikosaponins in the 10 Chinese medicinal preparations ranged between 0.11 and 1.22 mg/g for saikosaponin-a and between 0.01 and 0.33 mg/g for saikosaponin-c.

Keywords: Bupleuri radixes; Chaihu; Saikosaponin-a; Saikosaponin-c; LC/ESI(+)/MS/MS

1. Introduction

"Chaihu," the dried roots of plants of the *Bupleuri* species, is one of the most popular ingredients in many Chinese medicinal preparations; for example, it is used commonly as a herbal medicine for treating influenza and fever. The major components of Chaihu have been identified as a series of triterpenoid saponins, among which saikosaponin-a, -c, and -d (a C-16 epimer of saikosaponin-a) are present in the greatest amounts. Fig. 1 displays the chemical structures of saikosaponin-a and -c. The biomedical effect of saikosaponins has been observed as *in vivo* and *in vitro* anti-inflammatory activity, activation of anti-tumor effector cells, and hepatoprotective activity [1–3].

These saikosaponins have been determined previously in various species of Chaihu through the use of HPLC with UV detection at 210 nm [4] and the use of micellar electrokinetic capillary chromatography [5]. However, for quantitative analysis of saikosaponins in medicinal preparations containing other plants, interference is common and it is difficult to obtain good results. Hattori et al. [6] briefly described the use of LC/MS for the qualitative analysis of saikosaponins. Fifteen saikosaponin derivatives were identified and determined in the Chinese medicinal preparation "Xiaochaihu-tang" when LC/MS/MS was employed using the negative electrospray ionization (ESI) mode [7]. In contrast, in our present study, using an RP-HPLC system combined with an ion-trap (LCQ) tandem MS, we found that the positive ESI mode provided better performance than did the negative mode. In addition, in this study we investigated the 10 most-popular Chinese medicinal preparations containing Chaihu as an ingredient through the analysis of two target markers. Additional mass spectrometric information was obtained from saikosaponin-a and -c through their MS^3 fragmentations. For quantitative analyses of these two markers, we compared the effects of the selected ion monitoring (SIM) and selected

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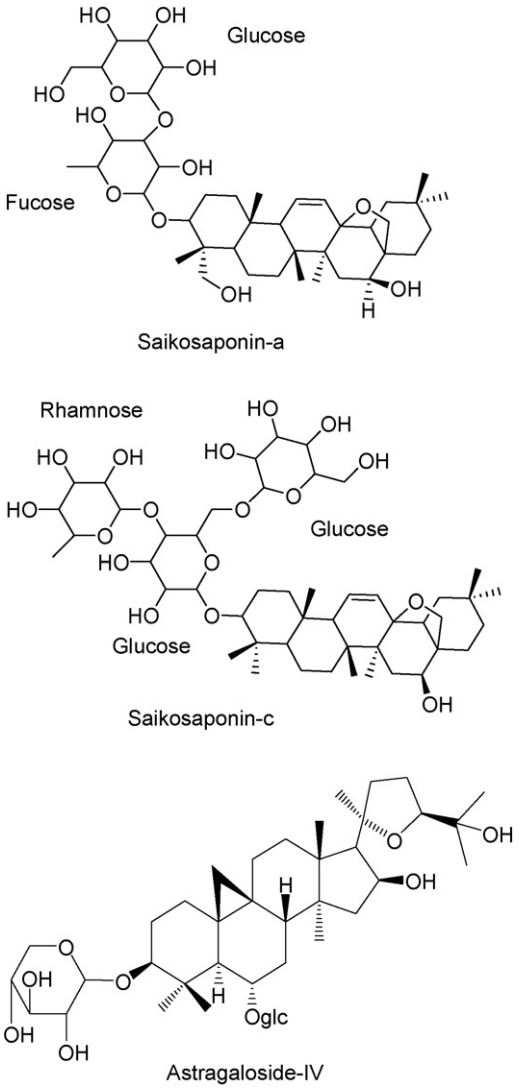

Fig. 1. Structures of saikosaponin-a, -c, and astragaloside-IV (internal standard).

reaction monitoring (SRM) mass scan modes. As a result, we have developed a simple and reliable routine analytical method for the quality control of these types of herbal medicine preparations. Using this approach, we have analyzed the saikosaponin-a and -c contents in three Bupleuri radix samples from different origins: Japan (*B. falcatum*), China (*B. chinense* DC.), and Taiwan (*B. kaoi* Liu Chao and Chuang).

2. Experimental

2.1. Standards and materials

Saikosaponin-a and -c were purchased from the Pharmaceutical Industry Technology and Development Center (Taipei). The three B. radix samples (*B. falcatum*, *B. chinense* DC., and *B. kaoi* Liu Chao & Chuang) and 10 medicinal preparations (Chai-Shiann-Tang, Dah-Chai-Hu-Tang, Sheau-Chai-Hu-Tang, Chai-Hu-Guey-Jy-Tang, Chai-Hu-Shu-Gan-Tang, Chai-Hu-Jiee-Ji-Tang, Chai-Hu-Guey-Jy-Ghyan-Jiang-Tang, Chai-Hu-Jia-Long-Guu-Muu-Lih-Tang, Jia-Wey-Shiau-Yau-Saan, and Chai-Hu-Ching-Gan-Tang) were obtained from Chuang Song Zong Pharmaceutical Co. Ltd., Ligang Shiang, Pingtung, Taiwan. Astragaloside IV, which was used as an internal standard (IS), was kindly provided by Prof. Hwang of the Chia-Nan University of Pharmacy and Science, Tainan, Taiwan.

2.2. Instrumentation

HPLC/MS was performed using a quadrupole ion trap instrument LCQ^{TM} (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization (ESI) source that was connected to a Surveyor LC pump. Xcalibur (v. 1.2) data acquisition software was employed. Helium was used as the damping and collision gas. Parent ions in the ESI were examined under negative and positive ion modes after the direct injection of a standard solution (1 μg/ml) in 70% methanol through a syringe at a flow rate of 3 μl/min. MS fragment analysis of saikosaponin-a and -c and MS^n experiments were also performed. All of the internal lens and octapole voltages were optimized through the instrument's automatic tune procedure using the $[M+Na]^+$ ions of saikosaponin-a and -c. For optimization of the parameters, 5 μl of the standard solution (0.1 μg/ml) was injected into the sample loop with the mobile phase at a flow rate of 200 μl/min; this procedure was performed five times for each set. The optimum conditions for the ESI-MS were as follows: sheath gas flow rate, 70 units (1.05 l/min); auxiliary gas flow rate, 20 units (6.0 l/min); spray voltage, 5 kV; capillary temperature, 275 °C; capillary voltage, 10 V; IT, 500 ms per microscan.

For LC/MS or LC/MS/MS chromatographic separation, a Supelco Supelcosil LC-18 column (5 μm; 150 mm × 2.1 mm i.d.) was used at ambient temperature with a flow rate of 0.2 ml/min. The solvent system consisted of 50 μM sodium acetate in water; 5 μl of sample was injected. The gradient was programmed as follows: starting at 30% (v/v) acetonitrile (ACN), increasing to 60% ACN over 10 min, increasing to 100% ACN over 5 min, holding at 100% ACN for 5 min, and then returning to 30% ACN over 5 min.

2.3. Preparation of samples

Dry powders of the three B. radix samples (0.1 g) and 10 medicinal preparations (0.5 g) were dissolved in 70% methanol (10 ml), sonicated for 30 min, filtered, and then centrifuged at 5000 rpm for 10 min. Seventy percent Methanol was added to the supernatant to obtain a final sample solution volume of 10 ml. The sample solutions were diluted 50 times (for single herbs) or 20 times (for mixed preparations) and the IS (1 μg/ml) was added (for calibration of the instrument only); the solutions were filtered through a syringe filter (pore size: 0.22 μm) when injected.

2.4. Calibration curves

A 1000 μg/ml standard stock solution of saikosaponin-a and -c was prepared and diluted to 10 μg/ml to provide the working solution. To determine saikosaponin-a and -c simultaneously, a mixture of saikosaponin-a and -c (1:1 ratio) was used to prepare

a series of standards having concentrations of 10, 20, 50, 100, 250, 500, 1000, 1500, and 2000 ng/ml in pure 70% methanol solution – and also in the extract from one of the medicinal preparations (Chai-Hu-Ching-Gan-Tang) – to allow calibration of the samples. As the IS, astragaloside IV (1 μg/ml) was added in each solution. A calibration curve was obtained by plotting the ratio of the peak areas of the analyte and the IS as a function of the analyte concentration (ng/ml). A weighted ($1/x^2$) linear regression line was fitted over the concentration range from 10 to 2000 ng/ml. The concentrations of saikosaponin-a and -c in the three B. radix samples and the 10 preparations were calculated from the ratios; they are expressed in units of micrograms per gram of preparation powder.

3. Results and discussion

3.1. MS^n behavior of saikosaponin-a and -c

MS, MS^2, and MS^3 spectra were recorded after the direct injection of standards. Saikosaponin-a exhibited its most intense adduct ions under the ESI positive ion mode; they appeared at *m/z* 781 $[M+H]^+$, 803 $[M+Na]^+$, 798 $[M+NH_4]^+$, and 819 $[M+K]^+$, with a fragment ion at *m/z* 763 $[M+H–H_2O]^+$. Saikosaponin-c displayed a similar mass pattern, with its corresponding peaks at *m/z* 927 $[M+H]^+$, 949 $[M+Na]^+$, 944 $[M+NH_4]^+$, 965 $[M+K]^+$, and 909 $[M+H–H_2O]^+$. The signal intensities were dependent on the temperature of the capillary: more $[M+H]^+$ and $[M+NH_4]^+$ ions formed at temperatures below 150 °C, while $[M+Na]^+$ ions were the most intense peaks observed at higher temperatures (>225 °C). In MS/MS experiments, when the $[M+H]^+$ and $[M+NH_4]^+$ ions were chosen as parent ions of saikosaponin-a, we observed only unstable fragmentations in the product ion scan. In contrast, by optimizing the energy for the collision-induced dissociation (CID) at 38%, the $[M+Na]^+$ (*m/z* = 803) ion cleaved into a major ion at *m/z* 331, corresponding to the mass of the sugar moiety (glucose and fucose) of saikosaponin-a plus a sodium ion, and a minor ion at *m/z* 641 $[M+Na-162]^+$ arising from the loss of a glucose moiety from the parent ion. The MS^3 spectrum of the ion at *m/z* 331 displays only the sodiated glucose ion at *m/z* 203, which arose through the loss of the fucose unit (see Fig. 2(a) and (b)). We observed similar fragmentation patterns for the $[M+Na]^+$ (*m/z* 949) ion of saikosaponin-c: product ions appeared at *m/z* 803 $[M+Na-146$ (rhamnose)$]^+$ and 511 [rhamnose + 2 glucose + Na]$^+$, with the MS^3 spectrum of the ion at *m/z* 803 exhibiting its strongest fragment ion at *m/z* 365 (see Fig. 2(c) and (d)), corresponding to the remaining sodiated di-glucose moiety.

3.2. Chromatographic analysis

We used LC/MS with a reverse-phase C18 column for the chromatographic separation of saikosaponin-a, -c, and astragaloside IV (a triterpenoid glycoside from *Astragalus membranaceus* Bunge var. mongholicus, used here as the IS because of its structural similarity with the target markers). The mobile phase additives that we tested separately were 1% acetic acid, 0.1% trifluroacetic acid, 50 mM ammonium acetate, and 50 μM sodium acetate. We observed no significant differences among these systems except that the addition of 50 μM sodium acetate slightly improved the sensitivity toward the $[M+Na]^+$ ions. When using a mobile phase of ACN/water (+50 μM sodium acetate) and gradient elution, saikosaponin-c, the IS, and saikosaponin-a appeared at retention times of 6.2, 6.8, and 8.3 min, respectively.

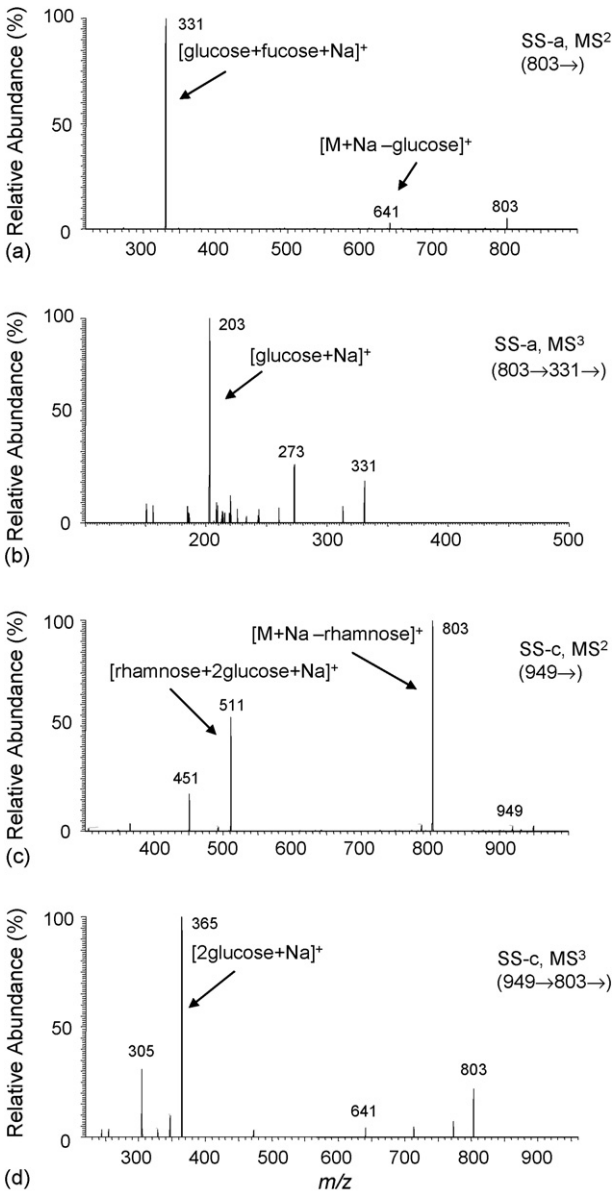

Fig. 2. MS^2 and MS^3 fragmentations of (a and b) saikosaponin-a (SS-a) and (c and d) saikosaponin-c (SS-c).

3.3. Comparison of LC/MS and LC/MS/MS

To compare the results of quantitative analyses using LC/MS (SIM) or LC/MS/MS (SRM), we tested a sample from the 70% methanol extract of *B. falcatum*. Fig. 3(b) and (c) display the SIM chromatograms of saikosaponin-a and -c, respectively. In the SRM experiments (see Fig. 3(e)–(g)), three events

Table 1
Linear ranges, calibration curves, linearities, limits of detection, and recoveries of saikosaponin-a (SS-a) and -c (SS-c) from a Chai-Hu-Ching-Gan-Tang matrix solution determined using LC/ESI(+)/MS/MS

Analyte	Linear range (ng/mL)	Calibration curve	Linearity (R^2)	LOD (S/N = 3; ng/mL)
SS-a	20–2000	$y = 0.0036x + 0.1782$	0.997	10
SS-c	20–1000	$y = 0.0092x + 0.3938$	0.997	10

Analyte	Spiked concentration (ng/mL)	Recovery[a] (%)	R.S.D. (%)
SS-a	20	70	14.6
	500	98	2.9
	1000	97	4.6
SS-c	20	74	13.7
	500	97	7.3
	1000	100	6.2

[a] Standards were spiked into the extract of a medicinal preparation; $n = 3$.

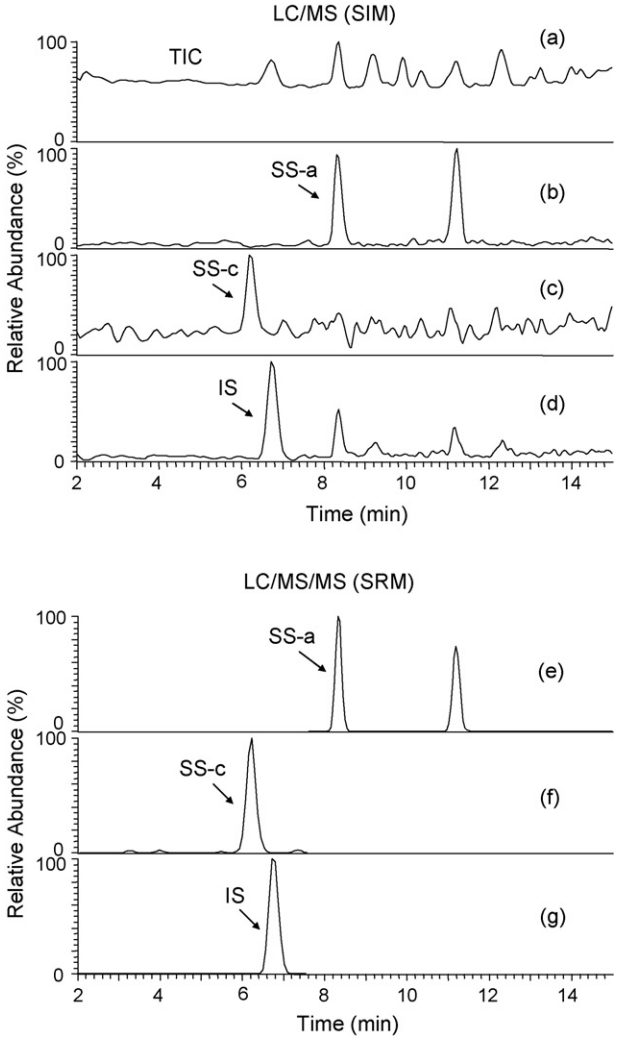

Fig. 3. (a–d) SIM and (e–g) SRM chromatograms of the extract of *Bupleuri falcatum* (single herb): (a) TIC, (b) SS-a (*m/z* 803), (c) SS-c (*m/z* 949), (d) IS (*m/z* 807), (e) SS-a (*m/z* 803 → 331), (f) SS-c (*m/z* 949 → 803), and (g) IS (*m/z* 807 → 627).

were performed in two segments of each run to obtain optimum sensitivity. In segment-I (0–7.5 min), only saikosaponin-c and Astragaloside IV (IS) were monitored for the two events *m/z* 949 → 803 and 807 → 627, respectively. In segment-II (7.5–20 min), saikosaponin-a was monitored for the process *m/z* 803 → 331. In Fig. 3, we find that the LC/MS/MS (SRM) method provided sensitivity that was superior to that of SIM at the same sample concentration. The extra peak at 11.2 min in both Fig. 3(b) and (e), having analogous precursor and product ions as those of saikosaponin-a, was assigned to saikosaponin-d, an isomer of saikosaponin-a, based upon its retention time and abundance as reported previously [4].

3.4. Calibration curves, limits of detection, precisions, and recoveries

We observed good linearity over the range from 10 to 2000 ng/ml for saikosaponin-a and from 10 to 1000 ng/ml for saikosaponin-c, with coefficients of determination (R^2) of 0.9997 and 0.9989, respectively, after analyzing the standards dissolved in 70% methanol. Matrix interference led to calibration curves exhibiting lower slopes, while maintaining good linearity, when we measured the standards in the extract from a complicated medicinal preparation (Chai-Hu-Ching-Gan-Tang; see Table 1). The linear-range experiment provides the information necessary to estimate the limit of detection (LOD), which is based on the lowest detectable peak that has a signal-to-noise ratio of 3. We calculated the values of LOD for both saponins to be 10 ng/ml.

We established the precision of our method by analyzing the results of six consecutive identical injections of a mixed sample of saikosaponin-a and -c (each at 50 ng/ml) and the IS (1000 ng/ml) under the optimal conditions. The reproducibilities, expressed as the relative standard deviation (R.S.D.), for saikosaponin-a and -c were 9 and 8%, respectively.

Considering the high concentrations of the two marker compounds in the sample powders, we performed recovery tests by spiking saikosaponin-a and -c at three concentrations (20, 500, and 1000 ng/ml), together with the IS (1000 ng/ml) directly into the diluted methanol extract of a multi-herb sample of Chai-

Table 2
Concentrations (ng/mL), R.S.D. (%), and actual amounts (μg/g) of saikosaponin-a (SS-a) and -c (SS-c) in three Bupleuri radix samples from different origins and in 10 Chinese preparation samples

Entry	Sample	SS-a (R.S.D., %) (ng/mL)	SS-a (μg/g)[a]	SS-c (R.S.D., %) (ng/mL)	SS-c (μg/g)[a]
1	*B. falcatum*	1172 (3.6)[b]	5859.7	674 (3.7)[b]	3371.2
2	*B. chinense* DC	1852 (3.9)[b]	9261.6	512 (2.9)[b]	2559.5
3	*B. kaoi* Liu Chao & Chuang[c]	1012 (6.7)[b]	10116.1	125 (1.6)[b]	1252.6
4	Chai-Shiann-Tang	1602 (6.8)[d]	640.7	445 (0.3)[d]	177.9
5	Dah-Chai-Hu-Tang	1106 (7.9)[d]	442.2	313 (4.5)[d]	125.2
6	Sheau-Chai-Hu- Tang	272 (8.8)[d]	108.6	50 (9.1)[d]	19.9
7	Chai-Hu-Guey-Jy-Tang	530 (5.5)[d]	212.2	113 (7.1)[d]	45.1
8	Chai-Hu-Shu-Gan-Tang	305 (6.9)[d]	121.8	29 (11.1)[d]	11.4
9	Chai-Hu-Jiee-Ji-Tang	1472 (3.3)[d]	588.7	376 (5.6)[d]	150.4
10	Chai-Hu-Guey-Jy-Ghyan-Jiang-Tang[e]	1524 (3.4)[d]	1219.2	418 (4.6)[d]	333.4
11	Chai-Hu-Jia-Long-Guu-Muu-Lih-Tang	553 (4.6)[d]	221.4	129 (7.5)[d]	51.4
12	Jia-Wey-Shiau-Yau-Saan	296 (7.1)[d]	118.3	64 (3.3)[d]	25.4
13	Chai-Hu-Ching-Gan-Tang	459 (3.8)[d]	183.8	82 (1.6)[d]	33.0

[a] Actual amount (μg/g) in original herb.
[b] Measured concentrations; $n = 3$; each sample (0.1 g) was dissolved in 70% $MeOH_{(aq)}$ (10 ml) of, then diluted 50 times.
[c] Sample diluted 100 times.
[d] Measured concentrations; $n = 3$; each sample (0.5 g) was dissolved in 70% $MeOH_{(aq)}$ (10 ml) of then diluted 20 times.
[e] Sample diluted 40 times.

Hu-Ching-Gan-Tang. We used the calibration curve established for the Chai-Hu-Ching-Gan-Tang matrix solution to determine that the recoveries of saikosaponin-a and -c were 70–98 and 74–100%, respectively, with values of R.S.D. of 2.9–14.6 and 6.2–13.7%, respectively (Table 1).

3.5. Analysis on actual samples

We tested the effectiveness of this method in determining the amounts of saikosaponin-a and -c in samples of Chinese medicinal preparations by analyzing the 10 most-popular preparations used in Taiwan in addition to three B. radix samples from Japan (*B. falcatum*), China (*B. chinense* DC.), and Taiwan (*B. kaoi* Liu Chao & Chuang). The SRM chromatograms of the extracts from single herbs and medicinal preparations presented well-separated and -shaped peaks from which the determination of saikosaponin-a and -c could be quantified clearly.

Three injections of each sample were performed to obtain mean values. The measured concentrations were all within the ranges of the calibration curves; the values of R.S.D. were between 3.3 and 8.8% for saikosaponin-a and 0.3 and 11.1% for saikosaponin-c from the measurement of complex preparations (Table 2, entries 4–13), with more-acurate results (1.6–3.7%) being obtained from the measurement of single species (entries 1–3). Table 2 presents the final converted contents of saikosaponin-a and -c in each preparation sample and in each single species.

4. Conclusions

Quality control of Chinese medicinal preparations – based upon the analysis of markers – can be achieved readily through the use of LC/ESI/ion trap tandem mass spectrometry. In this paper, we describe a quantitative approach toward the analyses of saikosaponin-a and -c and demonstrate the applicability of this method for the determination of these markers in crude Chaihu roots and in multiherb remedies.

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