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Short communication

Fast determination of saikosaponins in *Bupleurum* by rapid resolution liquid chromatography with evaporative light scattering detection

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ABSTRACT

A rapid resolution liquid chromatography coupled with evaporative light scattering detection method was established for simultaneous determination of six saikosaponins, namely saikosaponin a, saikosaponin c, saikosaponin d, 6″-*O*-acetylsaikosaponin a, 3″-*O*-acetylsaikosaponin d and 6″-*O*-acetylsaikosaponin d in *Bupleurum*. The analysis was performed by using an Agilent Zorbax SB-C18 column (1.8 μm, 3.0 mm × 50 mm i.d.) at gradient elution of water and acetonitrile, and the saikosaponins were well separated within 12 min, which provided about a fourfold reduction in analysis time by comparing a conventional high performance liquid chromatography method. Owing to their low ultraviolet absorption, the saikosaponins were detected by evaporative light scattering. The standard curves to quantify the saikosaponins were constructed by the log–log plot, which showed good linearity with the correlation coefficients exceeding 0.9954. The detection limits and quantification limits ranged in 8.38–25.00 μg/mL and 25.13–45.00 μg/mL, respectively. Satisfactory intra-day and inter-day precisions were achieved with the relative standard deviation (R.S.D.) less than 6.58%, and the average recoveries obtained were in the range of 96.9–100.4%. In addition, MeOH–1.0% (v/v) pyridine was found to be the best the extraction solvent when compared to MeOH and MeOH–1.0% (v/v) ammonia water. A total of 23 samples of roots of *Bupleurum* from different species or locations were examined with this analytical method, and their chemical profiles provided information for the chemotaxonomic investigation. The results demonstrated that the analytical method is highly effective for the quality evaluation of *Bupleurum* species.

1. Introduction

Radix bupleuri, one of the most popular Traditional Chinese Medicines (TCMs), is utilized to treat influenza, fever, malaria and menstrual disorders for approximately 2000 years in China [1]. As described in the Chinese Pharmacopoeia, *R. bupleuri* is derived from the dried roots of *Bupleurum chinense* DC. and *B. scorzonerifolium* Willd. (Umbelliferae). Besides, more than 10 other *Bupleurum* species are often locally used as *R. bupleuri* in China, including *B. yinchowense*, *B. marginatum* var. *stenophyllum*, *B. angustissimum*, *B. wenchuanense*, *B. rockii*, *B. komarovianum*, *B. smithii*, *B. smithii* var. *parvifolium*, *B. bicaule*, and so on [2].

The major bioactive compounds isolated from *R. bupleuri* are saikosaponins, which have been proved to possess significant biological activities, including anti-hepatitis [3], anti-inflammatory [4], anti-tumor [5,6] and immunoregulatory effects [7]. Among them, saikosaponin a (SSa), saikosaponin c (SSc) and saikosaponin d (SSd) and some acetyl saikosaponins, such as 6″-*O*-acetylsaikosaponin a (6″-acetyl-SSa), 3″-*O*-acetylsaikosaponin d (3″-acetyl-SSd) and 6″-*O*-acetylsaikosaponin d (6″-acetyl-SSd), are the main compounds existing in *Bupleurum* [2] (Fig. 1).

It has been found that even though herbal medicines come from different species within the same genera or different locations within the same species, the quality and efficacy are somewhat different according to their origins, growing conditions, and so on. Therefore, a rapid and sensitive analytical method to determine the distribution and the contents of the saikosaponins is essentially required for the quality control of the herbal material and products of *R. bupleuri*. However, presently developed method is limited to the detection of only a few active components, mainly focused on SSa, SSc and SSd, using analytical methods including thin layer chromatography (TLC) [8], high performance liquid chromatography (HPLC) [9–12], micellar electrokinetic capillary chromatography (MEKC) [13] and other methods [14,15]. Among them, HPLC on reversed-phase C18 columns with UV detection was the most frequently used technique for saikosaponin determination. The method, however, is limited due to

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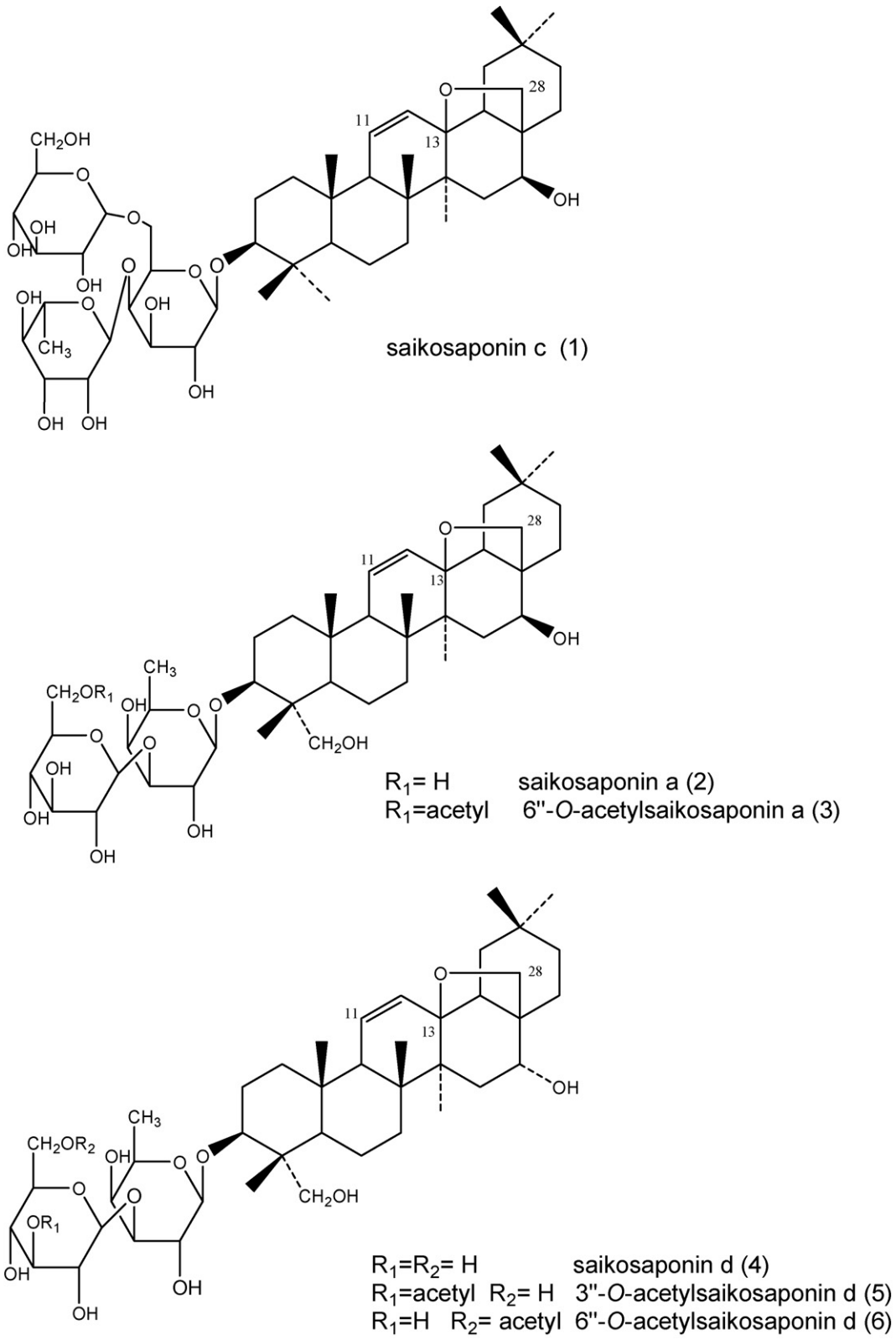

Fig. 1. Chemical structures of the six standard compounds in the study.

that most saikosaponins not possessing any chromophore necessary for UV detection. Although a small number of studies have reported the determination of saikosaponins using HPLC coupled with mass spectrometry (MS), the instrument is more expensive [12,16,17]. There is a trend to determine the non-chromophoric compounds in TCMs with evaporative light scattering detection (ELSD) and this detector was successfully applied for saponin determination in *Flos lonicerae* [18], *Radix astragali* [19], *Panax ginseng* [20], and so on. ELSD can detect all analytes less volatile than the solvent. The baseline can be maintained stable regardless the

Table 1
Summary for the tested samples roots of *Bupleurum*.

No.	Code	Species	Site of collection
1	CH-1	*B. chinense*	Jilin province
2	CH-2	*B. chinense*	Shanxi province
3	CH-3	*B. chinense*	Shanxi province
4	CH-4	*B. chinense*	Hebei province
5	CH-5	*B. chinense*	Shanxi province
6	CH-6	*B. chinense*	Henan province
7	SC-1	*B. scorzonerifolium*	Anhui province
8	SC-2	*B. scorzonerifolium*	Gansu province
9	SC-3	*B. scorzonerifolium*	Shanxi province
10	YI-1	*B. yinchowense*	Henan province
11	YI-2	*B. yinchowense*	Shaanxi province
12	YI-3	*B. yinchowense*	Gansu province
13	YI-4	*B. yinchowense*	Inner Mongolia autonomous region
14	ST-1	*B. marginatum* var. *stenophyllum*	Shaanxi province
15	AN-1	*B. angustissimum*	Inner Mongolia autonomous region
16	AN-2	*B. angustissimum*	Shanxi province
17	WE-1	*B. wenchuanense*	Sichuan province
18	RO-1	*B. rockii*	Yunnan province
19	KO-1	*B. komarovianum*	Jilin province
20	SM-1	*B. smithii*	Inner Mongolia autonomous region
21	SM-2	*B. smithii*	Shaanxi province
22	PA-1	*B. smithii* var. *parvifolium*	Liaoning province
23	BI-1	*B. bicaule*	Heilongjiang province

solvent's UV absorbance and the slope of the gradient conditions.

Due to the fact that plant extract usually occurs as a complex mixture, their fully separation needs long analysis time by HPLC. To accelerate the analysis process, there has been substantial focus on high-speed chromatographic separations. Recently, rapid resolution liquid chromatography (RRLC) has proven to be one of the most promising developments in the area of fast chromatographic separations, which allows satisfactory separation, good resolution and sensitivity, and high-speed detection with complex biological samples, such as herbal medicine [21]. The application of RRLC coupled to ELSD was able to separate target analytes in a short period of time and was specific and sensitive for the quantification of saikosaponins.

In this study, a fast RRLC–ELSD approach for the simultaneous determination of six saikosaponins, namely SSa, SSc, SSd, 6″-acetyl-SSa, 3″-acetyl-SSd and 6″-acetyl-SSd was developed, which was also compared with the conventional HPLC method. The validated method was then applied for the determination of the analytes in 23 samples of roots of *Bupleurum* from different species or locations.

2. Experimental

2.1. Chemicals reagents and standards

6″-Acetyl-SSa (**3**), 3″-acetyl-SSd (**5**) and 6″-acetyl-SSd (**6**) were isolated from the roots of *B. chinense* by our laboratory. SSa (**2**) and SSd (**4**) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SSc (**1**) was purchased from National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine (Jiangxi, China). Their structures were elucidated by comparison of their spectral data (UV, MS, ^{1}H NMR, and ^{13}C NMR) with the literature values [22–24] (Fig. 1). Their purities were all determined to be over 98% by HPLC–ELSD analysis. HPLC-grade acetonitrile and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ultra pure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA). Other organic solvents and chemical reagents used were of analytical grade and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China).

A total of 23 batches of roots of *Bupleurum* species were collected from different provinces of China (Table 1). All the samples were authenticated by Prof. Shengli Pan, School of Pharmacy, Fudan University. The voucher specimens were deposited in the Herbarium of Second Military Medical University, Shanghai, PR China.

2.2. RRLC–ELSD analysis

The chromatographic separation was performed on an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany), equipped with a microvacuum degasser, a binary pump, a high performance autosampler, a column compartment, a diode array detector and an evaporative light scattering detector. The samples were separated on a Zorbax SB-C18 column (1.8 μm, 3.0 mm × 50 mm i.d., Agilent Technologies, USA) at a column temperature of 25 °C and flow rate of 0.8 mL/min using acetonitrile (solvent A) and water (solvent B) as mobile phase with a linear gradient: 0–6.5 min (30–40%, A), 6.5–9 min (40–50%, A), 9–10 min (50–100%, A), 10–12 min (100–100%, A). The drift tube temperature for ELSD was set at 40 °C and the pressure set at 3.5 bar. Date was acquired using Agilent ChemStation software B03.02 version (Agilent Technologies, USA).

2.3. HPLC–ELSD analysis

The Chromatographic separation was performed on an Shimadzu (Kyoto, Japan) LC2010 *AHT* HPLC system, equipped with a quaternary pump, an autosampler, a degasser and column heater–cooler coupled with a Sedex 75 ELSD (Sedere, Alfortville, France). The samples were separated on a TSKgel ODS-100V C18 column (3 μm, 150 mm × 4.6 mm i.d., Tosoh Co., Tokyo, Japan) at a column temperature of 25 °C and flow rate of 1 mL/min using acetonitrile (solvent A) and water (solvent B) as mobile phase with a linear gradient: 0–25 min (30–40%, A), 25–35 min (40–50%, A), 35–45 min (50–100%, A), 45–50 min (100–100%, A). The drift tube temperature for ELSD was set at 40 °C and the air flow rate was produced by a WYK-2 air pump (Lanketech, Tianjin, China) with the pressure set at 3.5 bar. Date was acquired using Class-VP software (Shimadzu, Kyoto, Japan).

2.4. Sample preparation

The standard stock solution of the mixture of the six reference compounds was prepared by dissolving accurately weighted

portions of the standards in MeOH, transferring it to a 5 mL volumetric flask, and then adding MeOH to make up the volume, the concentration (mg/mL) of each compound is, SSc (2.0), SSa (3.35), 6″-acetyl-SSa (0.6), SSd (2.9), 3″-acetyl-SSd (2.4) and 6″-acetyl-SSd (1.2), respectively. A series of working standard solutions with gradient concentration was obtained by diluting the mixed standard stock solution. All the solutions were stored in a refrigerator at −4 °C.

Finely powdered dried plant materials (0.5 g) were sonicated in 8 mL of MeOH (1% pyridine) for 30 min followed by centrifugation for 10 min at 3000 rpm. The procedure was repeated 3 times. Respective supernatants were combined and concentrated in a rotary evaporator. MeOH was used to dilute the concentrated solution under sonication, and the volume was made up to exactly 10 mL. Prior to use all samples were filtered through a 0.22 μm nylon membrane filter.

3. Results and discussion

3.1. Optimization of extraction conditions

In order to obtain quantitative extraction, variables involved in the procedure, including extraction solvent, extraction repetitions and extraction time were optimized.

As described previously, the plant materials were usually extracted with organic solvent under slight alkalinity (pyridine or ammonia water) [25–28], because most of saikosaponins contain the unstable 13, 28-oxide linkage, which might be hydrolyzed during the process of the extraction by organic acid or on heating [3] (Fig. 1). In this study, three types of extraction organic solvent, including (A) MeOH, (B) MeOH–1.0% (v/v) pyridine and (C) MeOH–1.0% (v/v) ammonia water were evaluated for their extraction efficiency using ultrasonication method (Fig. 2). The results showed that the highest extraction yield was obtained by using MeOH–1.0% (v/v) pyridine (Fig. 2C). On the other hand, with the inclusion of 1.0% (v/v) ammonia water in the extraction, most acetyl saikosaponins (compounds **3**, **5** and **6**) were undetectable, but the levels of SSa (**1**), SSc (**2**) and SSd (**4**) were shown much higher (Fig. 2D). This observation is in harmony with other study which found the elimination of acetyl groups from acetyl saikosaponins when extracts treated with 5% (v/v) KOH in MeOH [29]. From these results, MeOH–1.0% (v/v) pyridine was chosen as a preferred extraction solvent.

Other experimental factors were also tested and evaluated, which included extraction repetitions (1–3 or 4 times) and extraction time (15 min, 30 min, 60 min or 90 min). All the results suggested that ultrasonication with MeOH–1.0% (v/v) pyridine for 30 min 3 times was simple and effective for saikosaponin extraction.

All investigated compounds were identified by comparing the retention times of the peaks with those of the reference compounds eluted under the same conditions and spiking the sample with stock standard solutions of saikosaponins (Fig. 2A).

3.2. Optimization of chromatographic conditions

Both of ELSD and UV detections were applied to evaluate the levels of saikosaponins in the extract of *Bupleurum*. It was found that RRLC–ELSD showed high sensitivity in the analysis of these saikosaponins compared to RRLC–UV method. Due to the absence of chromophores, saikosaponin allows non-specific UV detection at 210 nm, which results in poor sensitivity and high baseline noise. With the ELSD detection, however, the method provided clean chromatograms with a strong response and low baseline noise that were perfectly suitable for the determination of saikosaponins in *Bupleurum* (Fig. 2C). In addition, the important parameters that affect the sensitivity of ELSD, including the flow rate of nebulizer gas (pressure) and drift tube temperature, were evaluated at different drift tube temperatures from 30 °C to 100 °C and the pressure from 2.0 bar to 3.5 bar by the injection of SSa. Finally, the drift tube temperature of 40 °C and pressure of 3.5 bar were selected for detecting the analytes by comparing peak area values. These optimized parameters allow a complete solvent evaporation and produce negligible baseline noise.

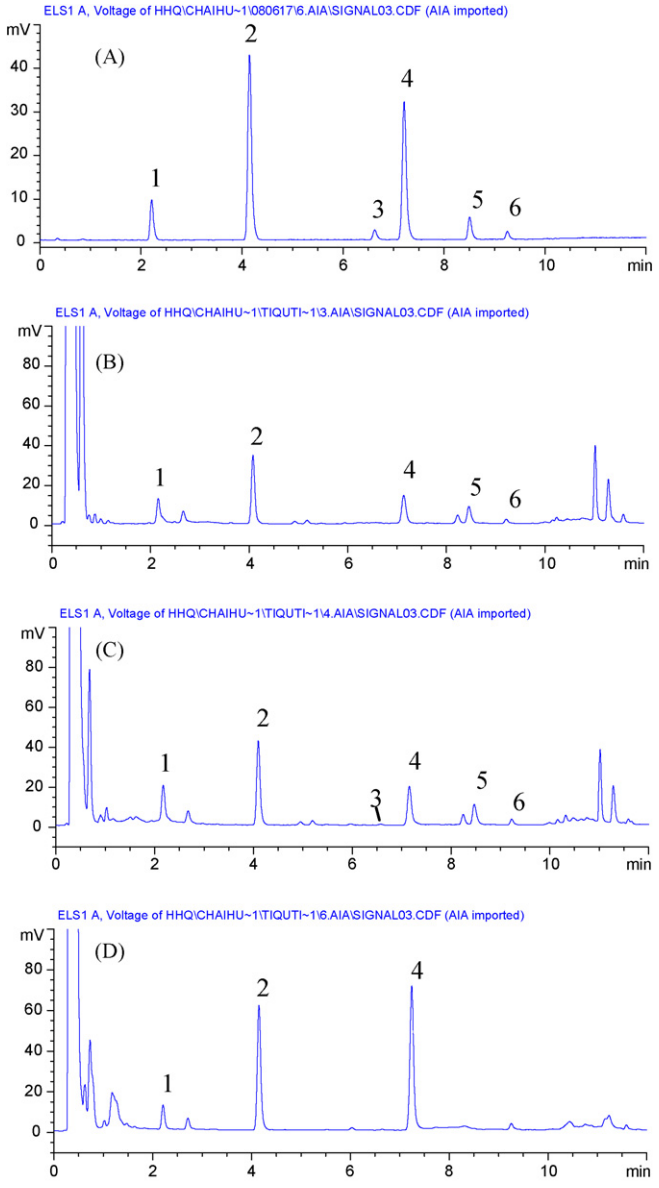

Fig. 2. RRLC–ELSD chromatograms of (A) mixed standards and roots of *B. scorzonerifolium* extracted by different solvent. (B) MeOH; (C) MeOH–1.0% pyridine; (D) MeOH–1.0% ammonia water. 1 = SSc, 2 = SSa, 3 = 6″-acetyl-SSa, 4 = SSd, 5 = 3″-acetyl-SSd and 6 = 6″-acetyl-SSd.

In order to quantify the investigated compounds, full separation of major saikosaponins is necessary. However, saikosaponins usually occur in plants as a mixture of structurally related forms with very similar polarities, and the separation is rather a difficult task. Thus, slowly gradient elution with different elution systems, including acetonitrile–water, MeOH–water, acetonitrile–acetic acid buffer and MeOH–acetic acid buffer in various proportions, were all investigated. The results suggested that a linear gradient elution of acetonitrile–water system gave the best resolution and the majority of saponin constituents could be efficiently separated within 12 min (Fig. 2C).

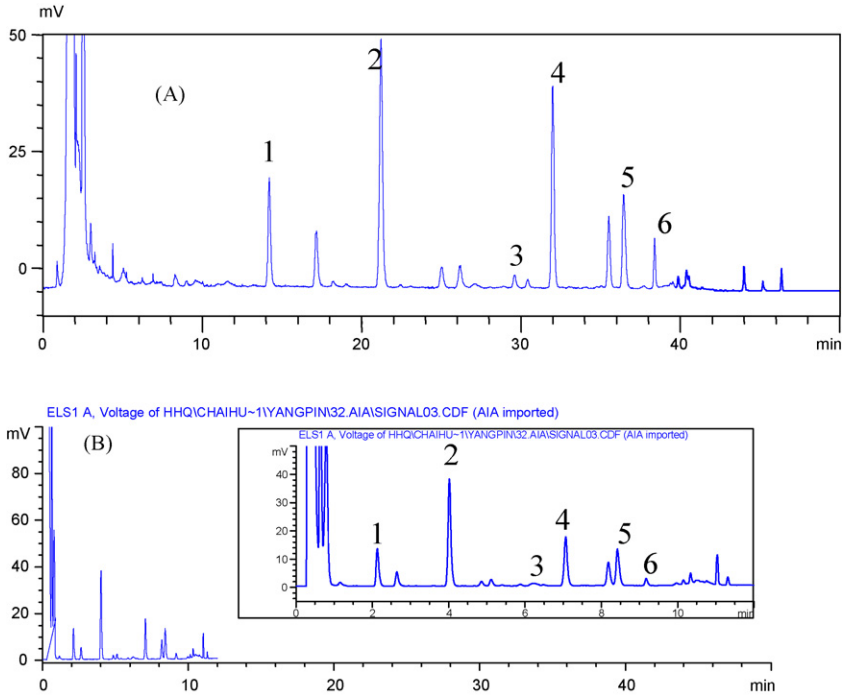

Fig. 3. Comparison of chromatograms of roots of *B. scorzonerifolium*. (A) HPLC–ELSD and (B) RRLC–ELSD.

3.3. Comparison of HPLC and RRLC

Initially, the saikosaponins were analyzed by HPLC–ELSD. A commonly used RP–HPLC method was established that allowed a separation of the extract of roots of *Bupleurum* in 80 min (including an equilibration time of 15 min). Though an optimized linear gradient elution and a column filled with sorbents of particle size 3 μm were applied, it is impossible to reduce the retention times in less than 65 min (including an equilibration time of 15 min, Fig. 3A). For a complex mixture, reliable methods using HPLC for the separation of saponins in most plant extracts were relatively time-consuming, about 60 min per analysis [30]. The advantage of long methods was the good resolution of adjacent peaks. But those methods were hardly suitable for rapid determination. Moreover they resulted in inefficiency, as they required the use of large amounts of organic solvents and instrument time.

Methods for rapid, highly resolving and efficient determination of extracts of *Bupleurum* are of great interest. RRLC methods have been introduced to offer greater resolution, good sensitivity and high speed of analysis for the separation of a complex mixture [21]. Comparing with the above-mentioned HPLC method, a newly developed RRLC method only needs 15 min for analysis (including an equilibration time of 3 min), which is only 1/4 of analysis time of HPLC (Fig. 3B). The serious reduction in retention time was made possible by performing the separation at very high pressure using a shorter analytical column packed with sorbents of particle size 1.8 μm. With the much shorter analysis time, lower solvent consumption and satisfactory resolution, the RRLC method was effective for the comprehensive analysis for large numbers of samples of *Bupleurum*.

3.4. Method validation

The linearity of each analyte was determined by using a series of working standard solutions, and each standard solution was measured in triplicate. Calibration curves were plotted logarithm using peak area versus concentration of each analyte, because of the non-linearity of the detector response versus concentration [18]. Good linear relationships were gained, and the correlation coefficients of all the calibration curves were found to be higher than 0.9954 (Table 2). Limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined on the basis of response and slope of each regression equation at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ ranged in 8.38–25.00 μg/mL and 25.13–45.00 μg/mL, respectively (Table 2).

The precision of the developed assay was determined for intra- and inter-day variations. The intra-day variation was determined by analyzing in triplicate the same mixed standard solution for 3 times within 1 day. While for inter-day variability test, solution was examined in triplicate for consecutive 3 days. The R.S.D.s of retention times and peak areas were taken as a measure tool, and were less than 6.58% for all six compounds (Table 3).

Table 2
Statistical analysis of the linear regression equation employed in the determination of the six saikosaponins.

Compound	Calibration curve[a]	r^2	Test range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
SSc	$y = 1.5642x + 3.2933$	0.9982	30.0–2000	10.0	30.0
SSa	$y = 1.3892x + 3.0992$	0.9967	33.5–3350	8.38	25.2
6″-Acetyl-SSa	$y = 1.8218x + 3.1273$	0.9955	37.5–600	25.0	37.5
SSd	$y = 1.4256x + 3.0996$	0.9954	29.0–2900	14.5	29.0
3″-Acetyl-SSd	$y = 1.3251x + 2.8098$	0.9976	30.0–2400	18.0	30.0
6″-Acetyl-SSd	$y = 1.6341x + 3.0564$	0.9988	60.0–1200	22.5	45.0

[a] In the regression equation $y = ax + b$; y refers to the log-transformed peak area; x to the log-transformed concentration of the reference saikosaponins (mg/mL).

Table 3
Precision and recoveries of the analytical method for the six saikosaponins.

Compound[a]	Intra-day precision ($n=6$)		Inter-day precision ($n=3$)		Recovery (%) (mean, $n=6$)
	R.S.D. of t_R [b] (%)	R.S.D. of PA [c] (%)	R.S.D. of t_R (%)	R.S.D. of PA (%)	
SSc	0.25	3.34	0.35	5.02	97.6
SSa	0.18	4.45	0.28	6.58	100.4
6″-Acetyl-SSa	0.07	4.91	0.17	6.12	97.6
SSd	0.06	3.12	0.14	5.26	96.9
3″-Acetyl-SSd	0.05	4.80	0.19	5.56	99.1
6″-Acetyl-SSd	0.05	3.13	0.16	4.34	100.3

[a] The concentration (mg/mL) of each compounds was: SSc (0.10); SSa (0.17); 6″-acetyl-SSa (0.03); SSd (0.15); 3″-acetyl-SSd (0.12); 6″-acetyl-SSd (0.06).
[b] Retention time.
[c] Peak area.

In order to examine the recovery of the developed method, the standards of six saikosaponins were added to samples at three concentration levels (approximately equivalent to 0.8, 1.0 and 1.2 times of the concentration of the matrix) with three parallels at each level. The solutions were prepared in accordance with the sample preparation procedure and the mean recovery was calculated for three assays of the standard. The results showed the recovery of these spiked standards ranged from 98.2% to 103.4% and was satisfactory (Table 3).

3.5. Analysis of saikosaponins in samples

The newly developed RRLC–ELSD method was applied to quantify six saikosaponins (compounds **1**–**6**) in 23 samples of roots of *Bupleurum*. Representative chromatograms of the extracts from different *Bupleurum* species are shown in Fig. 4. All the contents were calculated with external standard method, and the mean values from three parallel determinations are summarized in Table 4.

It was found that the well-known bioactive compounds SSc (**1**), SSa (**2**) and SSd (**4**) were detected in almost all samples of *Bupleurum* species. Additionally, some acetyl saikosaponins, including 6″-acetyl-SSa (**3**), 3″-acetyl-SSd (**5**) and 6″-acetyl-SSd (**6**), were shown to be major constituents since their contents (especially for 3″-acetyl-SSd (**5**)) were even higher than those of their corresponding saponins in some *Bupleurum* plants (Table 4). Moreover, the varied contents of acetylated derivatives might have affected the contents of their corresponding saponins, due to a possible transformation of acetyl saikosaponins to their saponins by hydrolysis [26]. Similar transformations could also be found in *R. astragali* [31]. Therefore, the simultaneous analysis of both saikosaponins and acetyl saikosaponins is much more effective than the detection of SSc, SSa and/or SSd alone, and was more capable and comprehensive compared with the reported analytical methods of *R. bupleuri* [12].

Table 4
Contents of the six saikosaponins in roots of different *Bupleurum*.

Sample	Content (mg/g)[a]					
	SSc	SSa	6″-*O*-Ac SSa	SSd	3″-*O*-Ac SSd	6″-*O*-Ac SSd
CH-1	0.72	0.78	–[b]	+	1.06	–
CH-2	1.59	5.86	–	4.13	2.52	1.41
CH-3	0.85	2.82	–	1.99	–	–
CH-4	1.06	3.16	–	2.12	3.63	+[c]
CH-5	0.99	1.92	–	1.61	–	–
CH-6	0.60	1.37	–	0.83	4.49	–
SC-1	2.24	5.35	+	3.40	4.19	1.24
SC-2	1.18	2.85	1.04	1.73	2.53	+
SC-3	1.24	2.66	–	1.66	–	–
YI-1	1.56	6.10	0.86	4.63	4.29	1.72
YI-2	1.69	3.24	–	2.40	4.32	–
YI-3	2.05	6.40	0.92	5.35	4.41	1.82
YI-4	1.15	2.69	–	1.89	4.09	–
ST-1	1.08	5.20	+	3.85	5.94	1.11
AN-1	1.40	3.90	–	3.60	0.69	–
AN-2	1.02	4.88	–	2.82	4.14	+
WE-1	2.12	5.03	–	3.99	8.52	0.95
RO-1	2.27	4.51	–	2.21	0.82	+
KO-1	0.82	1.05	–	0.62	–	–
SM-1	+	0.85	–	5.50	+	3.49
SM-2	0.66	1.23	–	7.91	0.55	5.77
PA-1	+	0.77	–	5.16	+	3.70
BI-1	0.77	1.06	–	6.99	0.93	6.70

[a] Average of triplicates.
[b] Under detection limit.
[c] Under quantification limit.

Due to the similarity in physical appearance and their widely distribution in China, *R. bupleuri* have been complicated with respect to their plant origins; and more than 10 species of *Bupleurum* were utilized as substitutes with the same name for *R. bupleuri* [2]. However, it was found in our study that the distribution and the contents of major saikosaponins in different species vary significantly. The content of SSa (**2**), which is considered as the major active compounds, is not always the highest in the samples. As shown in Fig. 4I–K, unusual low level of SSa (**2**) was found with high level of SSd (**4**) and 6″-acetyl-SSd (**6**) in the chromatograms of *B. smithii* (**SM-1** and **SM-2**), *B. bicaule* (**BI-1**) and *B. smithii* var. *parvifolium* (**PA-1**).

To further explore the variation of *Bupleurum*, hierarchical cluster analysis was performed based on 14 major selected compounds (peaks **1**–**14**, peaks **7**–**14** were undetermined) from the chromatogram profiles of the tested 23 specimens of 12 *Bupleurum* species (Figs. 4 and 5). Ward's method, a very efficient method for the analysis of variance between clusters, was applied and Squared Euclidean distance was selected as measurement. Fig. 5 shows the resulting dendrogram, which are divided into two main clusters. Three species of *B. smithii* (**SM-1** and **SM-2**), *B. bicaule* (**BI-1**) and *B. smithii* var. *parvifolium* (**PA-1**) are separated into the dendrogram of cluster II, indicating their chemical distinctiveness from other species. Similar result was obtained by high performance thin layer chromatographic analysis of saikosaponins and the specie of *B. bicaule* demonstrated different chemica1 distribution from other *Bupleurum* species, including *B. chinense*, *B. scorzonerifolium* and *B. marginatum* var. *stenophyllum* [32]. From the chemotaxonomical point of view, the presence of most abundant of SSd (**4**), 6″-acetyl-SSd (**6**), peak **13** (t_R = 8.7 min) and other unknown peaks (t_R > 10 min), but trace levels of SSc (**1**) and SSa (**2**), suggested that these compounds might be employed as the most important characteristic for the preliminary identification of *B. smithii* (**SM-1** and **SM-2**), *B. bicaule* (**BI-1**) and *B. smithii* var. *parvifolium* (**PA-1**). Therefore, the information might be viewed as chemotaxonomically relevant to the genus *Bupleurum*.

The remaining 19 samples of nine species, belonged to cluster I, are branched into two subgroups. Though their chemical profiles were similar, the contents of the investigated saikosaponins were different significantly (from low to high). Subgroup I, including samples CH-1 to YI-4, have relative low levels of saikosaponins, in which the contents of SSa and SSd were ranged from 0.78 to

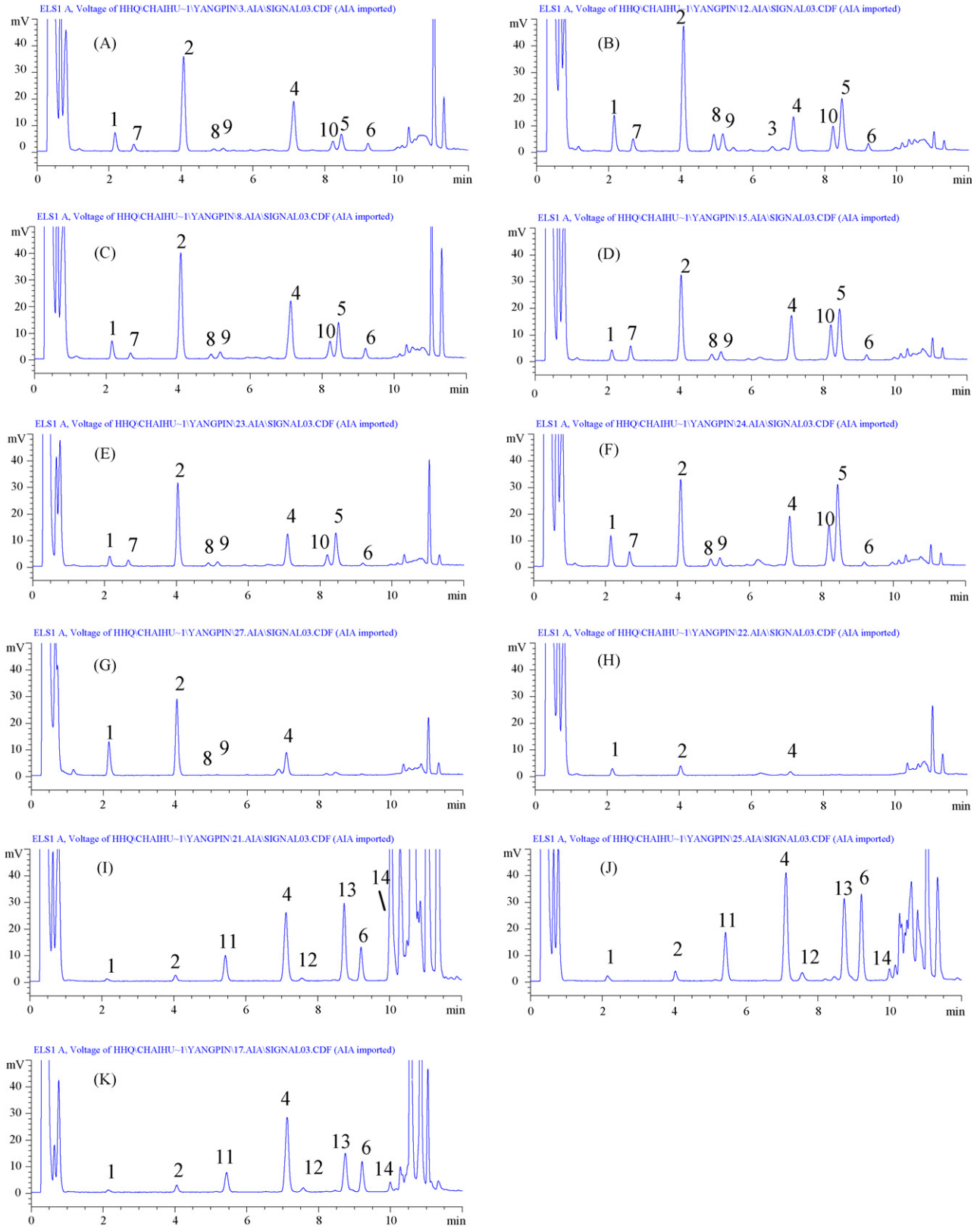

Fig. 4. Typical RRLC–ELSD chromatograms of roots of *Bupleurum* from different species. (A) *B. chinense*; (B) *B. scorzonerifolium*; (C) *B. yinchowense*; (D) *B. marginatum* var. stenophyllum; (E) *B. angustissimum*; (F) *B. wenchuanense*; (G) *B. rockii*; (H) *B. komarovianum*; (I) *B. smithii*; (J) *B. smithii* var. *parvifolium*; (K) *B. bicaule*.

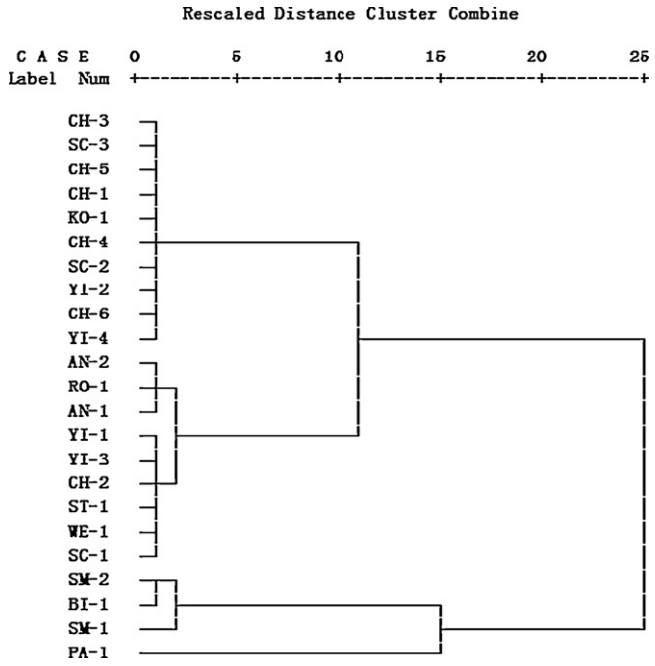

Fig. 5. Dendrograms of hierarchical cluster analysis for the 23 tested samples of roots of *Bupleurum*. The hierarchical clustering was done by SPSS software. Ward's method was applied, and Squared Euclidean distance was selected as measurement.

3.24 mg/g and 0.62–2.40 mg/g, respectively (Fig. 5, Table 4). Subgroup II, including samples AN-2 to SC-1, contained high abundant of saikosaponins, in which the contents of SSa and SSd were ranged from 3.90 to 6.40 mg/g and 2.21–5.35 mg/g, respectively (Fig. 5, Table 4). Among these samples, different populations within a single species of *Bupleurum* or different species collected from the same place vary in the amount of bioactive components due in difference in their origins, genetic variation, growth circumstance, harvest time, storage conditions, and so on. However, definite differentiation of these species may need further investigation of the analysis based on detailed chemical information and a large sample size.

4. Conclusion

In this work, a fast RRLC–ELSD method has been developed for simultaneous determination of six major saikosaponins in *Bupleurum*. Furthermore, the established method was applied for the quality evaluation of different species or locations of *Bupleurum*. The distribution of saikosaponins in 23 samples of roots of this genus was analyzed and the chemical profiles provide evidence for chemotaxonomic investigation. Thus, the proposed method may be suitable for quality control of the species of *Bupleurum* used as *R. bupleuri*.

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