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Identification and Determination of the Major Constituents in the Traditional Uighur Medicinal Plant *Saussurea involucrata* by LC-DAD-MS

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Abstract

A high-performance liquid chromatography (LC) coupled with diode array detector (DAD) and electrospray ionization mass spectrometry (ESI-MS) method was developed for the simultaneous analysis of the major constituents in *Saussurea involucrata* (SI). A comprehensive validation of the developed method was conducted, and the unique properties of the present method were confirmed by analyzing eleven SI samples. Seventeen compounds including phenolic acids, flavonoids, and lignanoids were identified by online ESI-MS and by comparison with known data in the literature and standard compounds; of these, eight were simultaneously quantified by LC-DAD. All linear regressions were acquired with $R^2 > 0.99$, and the limits of detection ranged from 0.85 to 3.03 ng. Repeatability was evaluated by intra- and inter-day assays; the relative standard deviation (RSD) value was within 4.95%. Recovery studies for the quantified compounds were found to be within the range 95.20–102.32% with RSD less than 2.18%. Overall, the present hyphenation procedure is highly efficient and reliable, and hence suitable for qualitative and quantitative analysis of a large number of samples.

Keywords

LC-DAD-MS

Saussurea involucrata

Pharmaceutical analysis

Ethnopharmacy

Introduction

Saussurea involucrata Kar. et Kir. (SI), a rare but well-known medicinal plant, grows in the mountains at heights of 4000-4300 m in the Tianshan and A'er Tai areas in China [1]. With a reputation for expelling dampness, diminishing inflammation and facilitating blood circulation, the dried aerial parts of SI have long been used for the treatment of rheumatoid arthritis, cough, stomachache, dysmenorrhea, and altitude sickness in Uighur folk medicine [2]. Various chemical compounds have been isolated from the herb, including flavonoids [3, 4], sesquiterpenes [5], phenolic acids [6] and lignins [6]. In recent years, pharmacologic studies have demonstrated that SI and its constituents have anti-inflammatory [7], analgesic [8], anti-cancer [9] and free radical scavenging [10] effects.

Currently, the quality assessment standard for SI is based on content of the marker compounds chlorogenic acid and/or rutin, which are the primary bioactive compounds identified so far in SI [2]. It is widely accepted that multiple constituents might be involved in any herb's therapeutic functions, and that the content of a single or a few marker compounds cannot accurately reflect the quality of herbal products [11, 12]. However, accurate means for qualitative and quantitative analysis of the different type compounds simultaneously is not reported, even if the determination of flavonoids, phenolic acids and guaianolides in SI has been carried out by thin layer chromatography scan (TLCS) [13], spectrophotometry [14] and high-performance liquid chromatography (LC) [2, 15], respectively. Therefore, to comprehensively assess the quality of SI, the necessity for a precise and accurate method based on rapid separation, identification and quantification of the major constituents in a single run is crucial.

In the present paper, a validated LC coupled with diode array detector (DAD) and electrospray ionization mass spectrometry (ESI-MS) method was developed for the qualitative and quantitative analysis of the major chemical constituents in SI. The results revealed that flavonoids, phenolic acids, and lignanoids are the major constituents of SI; therefore it was feasible to choose these characteristic

compounds for the quality evaluation of the herb. The results demonstrate that the hyphenation of LC-DAD and MS techniques is a powerful approach for the rapid separation, identification and quantification of the constituents in herbal products, and therefore would have wide application in the medicinal herb industry where correct identification and quality control are critical.

Experimental

Plant Materials

The sources of the tested samples are listed in Table 1. Identity of the herbs was confirmed by Dr. Hubiao Chen (School of Chinese Medicine, Hong Kong Baptist University, Kowloon, Hong Kong) by means of geographical origin identification and macroscopic identification. Voucher specimens were deposited in the Chinese medicines center, Hong Kong Baptist University.

Insert Table 1 here

Reagents and Chemicals

Acetonitrile of LC grade and methanol of analytical grade were purchased from Lab-scan (Bangkok, Thailand). Formic acid of LC grade was purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water system (Millipore; Bedford, MA, USA).

The standard compounds of syringoside, chlorogenic acid and rutin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Protocatechuic acid, 1, 5-dicaffeoylquinic acid, quercitroside, diosmetin 3-*O*-glucoside, kaempferol 3-*O*-rhamnoside, acacetin 7-*O*-glucoside, arctiin, quercetin and apigenin were isolated from *Saussurea stella* Maxim., a plant of same genus as *S. involucrata* [16]. The purity of these chemical standards was determined to be more than 98% by normalization of the peak areas detected by LC-DAD. Their chemical structures are shown in Fig. 1.

Insert Fig. 1 here

Preparation of Solutions

Preparation of Standard Solutions

The stock solutions of eight standards (1000 mg L^{-1}) were prepared in 70% methanol and stored in the refrigerator. The working solutions were prepared by appropriate dilution of the stock solutions with 70% methanol, and the resulting concentrations were 1, 50, 100, 150, and 200 mg L^{-1}, respectively. An aliquot of 10 μL for each calibration standard solution was injected for LC analysis.

Preparation of Sample Solutions

Herbal sample powder (0.5 g) was extracted with 10 mL of 70% methanol by means of sonication at room temperature for 0.5 h. The operations were repeated two times, and the residue was washed with 4 mL of fresh extraction solvent. Total extracts were combined in a 25-mL volumetric flask, which was filled up to the calibration mark with extraction solvent. The extracts were then filtered through a syringe filter (0.2 μm, Alltech, Beerfield, IL, USA). An aliquot of 10 μL solution was injected for LC-DAD-MS analysis.

LC–DAD–ESI-MS Conditions

An Agilent 1100 series LC system consisting of a vacuum degasser, binary pump, autosampler, thermostated column compartment and diode array detector (DAD), was used for quantitative analysis and UV acquisition. For mass spectrometric determination, the LC-DAD system was hyphenated to a Bruker MicrOTOFQ system by an electrospray ionization (ESI) interface (Bruker Daltonics, Bremen, Germany).

For chromatographic separation, an Alltima C_{18} column (5 μm, 4.6 × 250 mm, Alltech Associates) with a compatible guard column (C_{18}, 5 μm, 4.6 × 7.5 mm) was used. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using a gradient program of 5% (B) in 0-15 min, and 25-55% in 15-60 min. The solvent flow rate was 1 mL min^{-1} and the column temperature was set to 30 ^{o}C. The effluent from DAD was drained to the MS system with a split ratio

of 10:1. The conditions of MS analysis in the positive ion mode were as follows: drying gas (nitrogen), flow rate, 5 L min^{-1}; gas temperature, 170 ^{o}C; scan range, 50-1000 *m/z*; end plate offset voltage, -500 V; capillary voltage, 4500 V; nebulizer press, 0.7 Bar.

Assay Validation and Sample Determination

Calibration curves were established for each standard compound. Repeatability was evaluated in intra- and inter-day assays. Recovery of all the quantified constituents was determined by sample in different concentration levels using a mixture of standards with 50, 100 and 200% of the quantified levels of constituents in the samples. All herb samples collected from various regions were analyzed using the present method.

Results and Discussion

Optimization of Analysis Conditions

Compared to systems with methanol, systems with acetonitrile had a better resolution and a smoother baseline. Different ratios of water and acetonitrile were further tried, and a satisfactory separation within a suitable period of time was obtained. In order to ensure the stability of phenolic acids constituents and to promote the formation of quasi-molecular ions $[M+H]^+$ in MS analysis [17], 0.1% formic acid was used in the mobile phase.

By comparing the LC chromatograms of the herbs recorded at wavelengths from 203 to 500 nm and the corresponding UV absorption maximum for each chemical standard, it was found that a wavelength of 280 nm could represent the profile of the major constituents in SI. The representative LC chromatogram is shown in Fig. 2(a).

Insert Fig. 2 here

Online ESI-MS Identification of the Major Constituents

The mass spectrometric conditions were optimized in both positive and negative ion modes; the positive ion mode was found to be more sensitive. Most constituents exhibited their quasi-molecular ions $[M+H]^+$ and $[M+Na]^+$ in positive ion mode. A typical total ion chromatogram (TIC) obtained from the LC-DAD-ESI-MS analysis of a SI sample is shown in Fig. 2(b).

Based on comparison with chromatograms of standard compounds, thirteen peaks were unambiguously identified as protocatechuic acid (1), syringoside (3), chlorogenic acid (4), rutin (6), 1, 5-dicaffeoylquinic acid (8), quercitroside (9), diosmetin 3-*O*-glucoside (10), kaempferol 3-*O*-rhamnoside (11), arctiin (12), quercetin (14), acacetin 7-*O*-glucoside (15) and apigenin (17). Another five peaks were tentatively identified as 3α-hydroxyl-11β, 13-dihydrodehydro-costuslactone 8-*O*-glucoside (5), isoquercitroside (7), involucratolactone 8-*O*-glucoside (13), hispidulin (18) and jaceosidin (19) by comparing their *m/z* values and UV spectra with literature data [3-6, 18-20]. The structures of the identified compounds are shown in Fig. 1. From the results, it is obvious that the major types of constituents in the herb are flavonoids, phenolic acids, and lignanoids along with a few sesquiterpene lactones. The results demonstrated, generally, that hyphenation of LC-DAD and MS techniques was a powerful tool for the efficient separation and identification of the constituents in an herbal sample.

Validation of the Analysis Method

Linearity of the assay for standards was determined with five data points over the range 1-200 mg L^{-1} in 70% methanol (1, 50, 100, 150, and 200 mg L^{-1}, respectively). The calibration curve was established by plotting the peak area against the concentrations of the standards with linear regression analysis. Calibration curves showed that there was a linear correlation between peak areas and the concentrations of the standards, and a good linearity with $R^2 > 0.99$ was achieved (Table 2). Based on

visual evaluation with a signal-to-noise ratio of about 3:1, the LOD of the quantified constituents was reported to be between 0.85 and 3.03 ng. Therefore, the system was considered to be satisfactory for the analysis.

Insert Table 2 here

The method precision was evaluated by intra- and inter-day (n = 3) assays, and the same batch of SI sample was determined within 1 day and 3 separate days, respectively. The relative standard deviation (RSD) values were found to be within the range of 0.64 to 3.70% for intra-day assays and of 1.10 to 4.95% for inter-day assays. The accuracy of the method was validated by the determination of recovery. Recoveries of all of the quantified constituents were determined using samples of SI, for which the respective chemical contents had been predetermined, spiked at three different concentration levels. In each case a mixture of standards with 50, 100 and 200% of the quantified levels of constituents were spiked into the appropriate samples. Samples were then extracted, processed and quantified in accordance with the established procedures. Triplicate sample analysis was conducted for the determination of recovery at each spiked level. The average recovery of protocatechuic acid, syringoside, chlorogenic acid, rutin, 1, 5-dicaffeoylquinic acid, quercitroside, diosmetin 3-*O*-glucoside and arctiin were 97.42% (RSD 1.58%), 98.05% (RSD 2.02%), 99.30% (RSD 1.03%), 100.51% (RSD 1.69%), 101.17% (RSD 1.32%), 97.31% (RSD 2.18%), 97.80% (RSD 1.47%) and 99.02% (RSD 1.19%), respectively. The overall procedure is reliable and accurate and is therefore suitable for high throughput quantification of a large number of samples.

Sample Analysis

The SI samples collected from various regions were determined with the present hyphenation method, and the results are listed in Table 1. The results demonstrate a variation in the contents of the quantified constituents in SI samples. Such variations may presumably be attributed to differences in

source and year of harvest. Comparison of the average contents of eight analytes shows that the total amounts of chlorogenic acid and rutin represented about 90 % of total analyte content. It is undoubted that such two compounds applicable as markers for quality evaluation of SI [2]. In recent years, actiin was reported to inhibit the lung cancer of mice [21], and syringoside was found to cause a moderate free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) [22]. Therefore, to reflect the roles of multiple constituents for its many pharmacological activities [23], arctiin and syringoside of SI should be chosen as analytical markers besides chlorogenic acid and rutin. Thus, the present hyphenation method could meet this need, which made a comprehensive analysis of the different type compounds in a single run available.

Conclusion

A LC-DAD-ESI-MS method was developed for the simultaneous analysis of the major constituents in SI. Seventeen compounds were identified by online ESI-MS and eight of them were simultaneously quantified by LC-DAD in eleven SI samples. With respect to already existing methods, the present method, hyphenating LC to both DAD and MS techniques, has the advantages of faster, more accurate, and simultaneous qualitative and quantitative analysis of SI herbal samples. The method contributes to the practical applications of hyphenation science in the field of chromatographia as well as herbal medicine.

Acknowledgements

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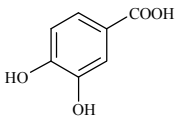
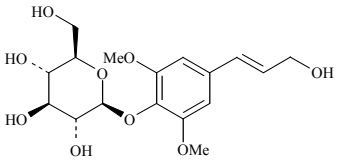
Legends for Figures

Fig. 1. Chemical structures of the compounds identified in the HPLC chromatograms

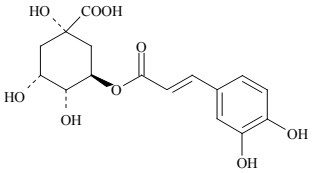
Fig. 2. LC chromatogram of a sample of *Saussurea involucrata* with (a) detection at 280 nm, (b) the total ion chromatogram of a sample in positive ion mode. Key to peak identity as in Fig. 1. (For chromatographic protocol see Experimental section.)



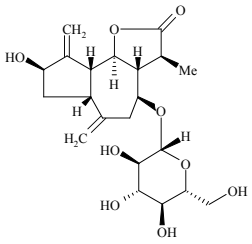
Protocatechuic acid (1)



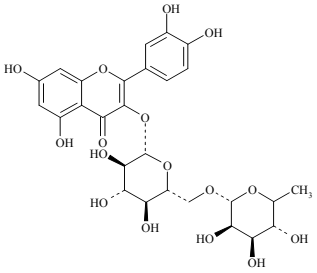
Syringoside (3)



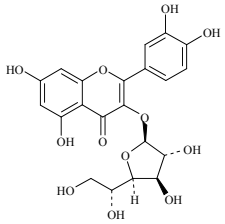
Chlorogenic acid (4)



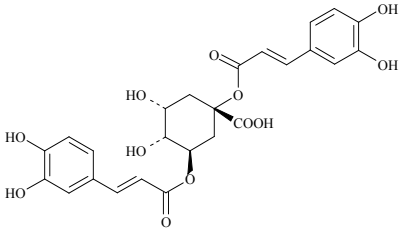
3α-Hydroxyl-11β, 13-dihydrodehydro-costuslactone 8-*O*-glucoside (5)



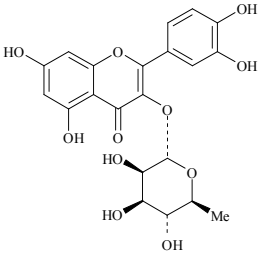
Rutin (6)



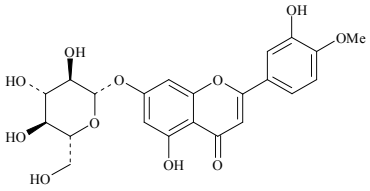
Isoquercitroside (7)



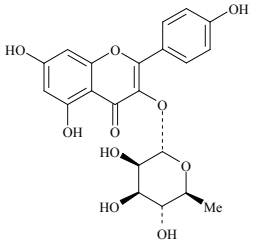
1,5-Dicaffeoylquinic acid (8)



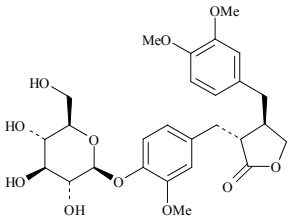
Quercitroside (9)



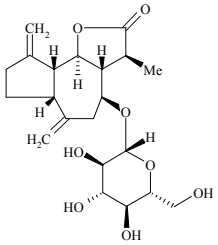
Diosmetin 3-*O*-glucoside (10)



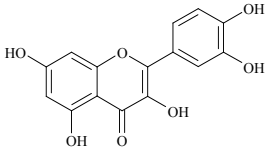
Kaempferol 3-*O*-rhamnoside (11)



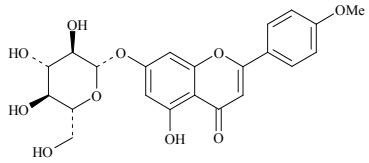
Arctiin (12)



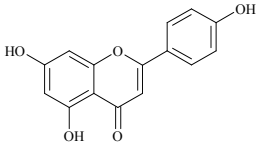
Involucratolactone 8-*O*-glucoside (13)



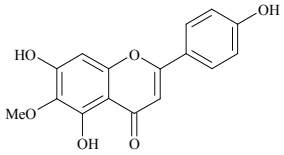
Quercetin (14)



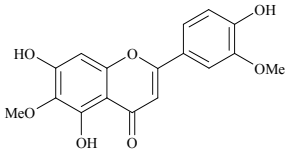
Acacetin 7-*O*-glucoside (15)



Apigenin (17)



Hispidulin (18)



Jaceosidin (19)

Fig. 1

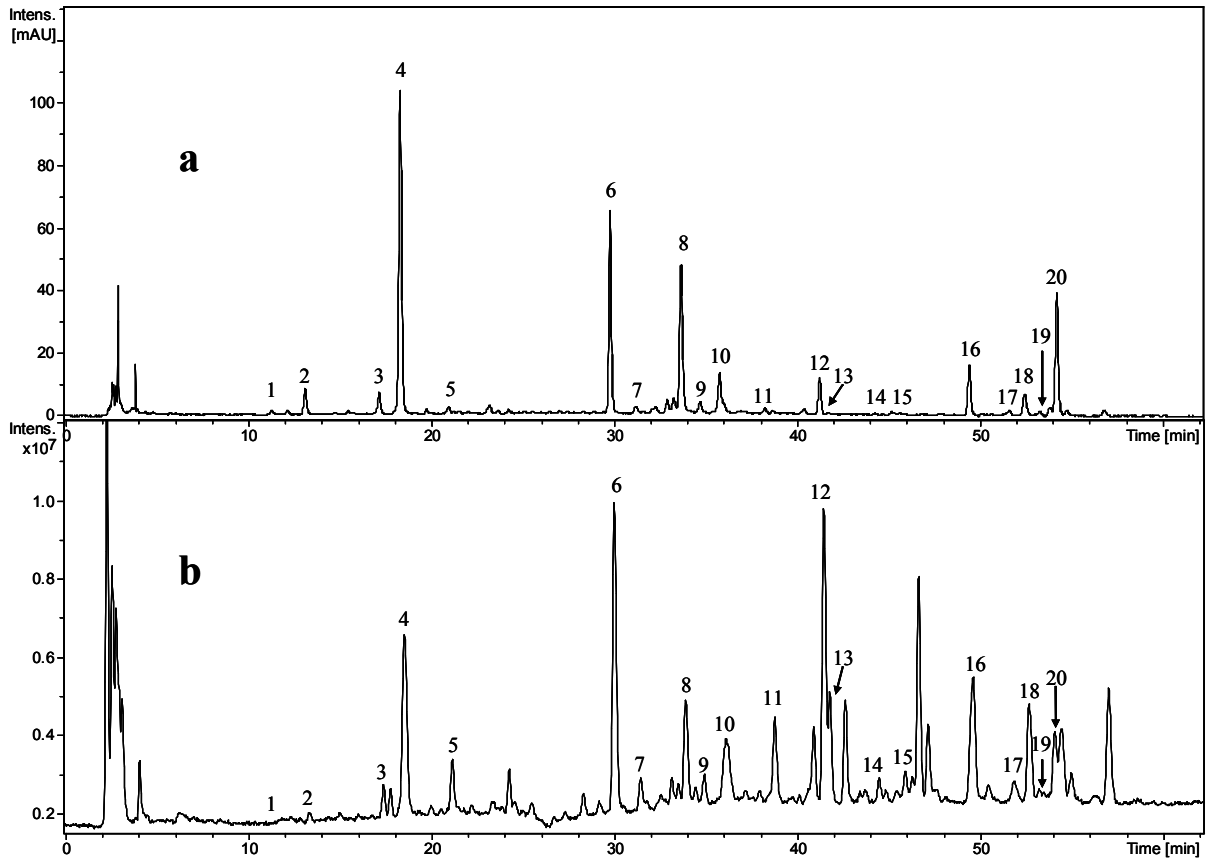


Fig. 2

Legends for Tables

Table 1 Abundance of eight constituents in the herbal samples

Table 2 Linearity calibration curve factors and limits of detection (LOD) for eight constituents

Table 1

Materials [a]	Source and year of harvest	Abundance of eight compounds (mg g^{-1}) [b]							
		Protocatechuic acid (1)	Syringoside (3)	Chlorogenic acid (4)	Rutin (6)	1,5-Dicaffeoyl-quinic acid (8)	Quercitroside (9)	Diosmetin 3-*O*-glucoside (10)	Arctiin (12)
SI-01	Tianshan, Xinjiang, China (2007)	0.15	0.20	7.56	9.25	0.91	0.12	0.23	1.47
SI-02	Tianshan, Xinjiang, China (2007)	0.07	0.04	3.75	11.38	0.72	0.08	0.25	0.38
SI-03	Tianshan, Xinjiang, China (2007)	0.17	0.13	8.98	8.97	0.71	0.12	0.36	0.56
SI-04	Bozhou, Anhui, China (2003)	0.18	0.54	7.86	16.02	0.97	0.11	0.34	0.54
SI-05	Tianshan, Xinjiang, China (2003)	0.20	0.09	13.18	9.06	1.11	0.29	0.86	0.10
SI-06	Lhasa, Tibet, China (2005)	0.17	0.13	3.23	3.55	0.50	0.06	0.17	1.31
SI-07	Yili, Xinjiang, China (2003)	0.18	0.72	6.68	6.71	0.84	0.09	0.28	1.47
SI-08	Kunming, Yunnan, China (2005)	0.16	0.06	10.29	9.39	1.03	0.17	0.51	0.27
SI-09	Tianshan, Xinjiang, China (2003)	0.22	0.36	6.60	15.21	0.72	0.09	0.57	0.18
SI-10	Urumqi, Xinjiang, China (2003)	0.20	0.30	14.43	12.91	1.66	0.26	0.79	0.13
SI-11	Xinyuan, Xinjiang, China (2006)	0.23	0.28	7.75	12.89	0.80	0.07	0.20	0.17

[a] SI-01 to SI-11 are samples of *Saussurea involucrata*

[b] Values shown are mean ± SD (n = 3).

Table 2

Peak	Compounds	Slope (*A*)	Intercept (*B*)	R^2	LOD (ng)
1	Protocatechuic acid	12907.2	- 4.0	0.9998	1.8
3	Syringoside	15533.0	+ 3.4	0.9999	1.7
4	Chlorogenic acid	7275.5	-2.5	0.9998	1.8
6	Rutin	3813.5	-1.2	0.9999	2.9
8	1,5-Dicaffeoylquinic acid	40031.3	- 3.0	0.9999	0.9
9	Quercitroside	8850.3	- 2.8	0.9998	2.6
10	Diosmetin 3-*O*-glucoside	6732.5	- 1.9	0.9997	1.9
12	Arctiin	3800.2	+ 1.1	0.9999	3.0