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ORIGINAL PAPER

A new strategy for quality control and qualitative analysis of Yinhuang preparations by HPLC-DAD-MS/MS

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Abstract A combinative method using fingerprint analysis (FA) and multi-ingredients quantification (MIQ) was developed and validated for the quality control of Yinhuang (YH) preparations including granule, capsule, and lozenge by high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). Common peaks with or without standard references in FA were confirmed or identified by electrospray ionization tandem mass spectrometry (ESI-MS/MS). The chromatographic separations were achieved on a Sepax GP-C_{18} column (250 mm × 4.6 mm i.d., 5 μm) with a gradient elution using a mixture of 0.1 % formic acid methanol solution and 0.1 % formic acid water solution. In quantitative analysis, nine bioactive constituents (chlorogenic acid, caffeic acid, luteoloside, baicalin, luteolin, wogonoside, baicalein, wogonin, and oroxylin A) were simultaneously determined. The detection wavelength was set at 275 nm, 320 nm, and 350 nm according to the absorption properties of the nine quantified compounds. The linearity, recovery, intraday and interday precision, accuracy, limit of detection (LOD) and quantification (LOQ), repeatability and stability were all tested and good results were obtained. In the FA, 320 nm was selected. The correlation coefficients of similarity were determined on the basis of the relative retention time (RRT) and relative peak area (RPA) of 20 common peaks in chromatographic fingerprints. Results indicated that both the RRT and RPA of 20 common peaks shared a close similarity. From the 20 common peaks, 18 compounds, including the nine quantified compounds, were identified or tentatively characterized by comparing their retention times, UV spectra, and MS spectra with those of standard compounds or literature data. The study not only presents a powerful and reliable analytical tool for the quality control of YH preparations, but also provides the chemical evidence for revealing the material basis of their therapeutic effects.

Keywords Yinhuang preparations · Multi-ingredients quantification · Fingerprint analysis · Qualitative analysis · HPLC-DAD · ESI-MS/MS

Introduction

Traditional Chinese medicine (TCM) has been used in China for centuries and has become increasingly popular around the world in recent decades owing to its low toxicity, high effectiveness, and less side effects [1, 2]. Compared with synthetic drugs that usually focus on a single chemical entity, the curative effects of TCM are based on the synergic effects of their multi-ingredients and multi-targets [3, 4]. However, because of the extreme complexity of TCM, the relationship between the compounds and the pharmacological effects of TCM is still unclear [5, 6]. Thus, it is necessary to establish a method that can not only offer a holistic analytical approach for quality control (QC), but also provide the chemical evidence for revealing the material basis of their therapeutic effects.

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In general, a few chemical markers or bioactive constituents are selected to assess the quality of TCM [7]. Although this method can quantify multiple active compounds from different herbs in prescriptions and has been proved effective for the QC of TCM to some extent, those markers or bioactive constituents are less applicable in the case of more complex herbal products. Furthermore, many reference standards cannot be obtained easily and chromatographic conditions are difficult to optimize.

The fingerprint analysis (FA) has the character of entirety and fuzziness, evaluating the quality consistency and stability of herbal products. It can be used as a tool to control the quality of TCM systematically and comprehensively [8]. Both the Food and Drug Administration (FDA) [9] and European Medicines Agency (EMEA) [10] have clearly denoted that appropriate fingerprint chromatograms should be applied to assess the consistency of botanical drugs. The State Food and Drug Administration of China (SFDA) suggested that all of herbal fingerprint chromatograms should be evaluated according to their similarities [11]. Thus, the chromatographic fingerprint, especially the high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) fingerprint, has recently been widely used for QC of TCM [12]. Peaks existing in all chromatograms of the samples were assigned as "common peaks", and a similarity evaluation was carried out according to these peaks. However, this strategy can only show results of similarity calculated on the basis of the relative value using a preselected marker compound as a reference [13], and the real content of markers cannot be analyzed.

Considering these drawbacks of multi-ingredients quantification (MIQ) and FA, many scholars have combined FA with MIQ to control the quality of TCM [14, 15]. Although this method has proved more effective than FA or MIQ, it should not be neglected that there are still some "common peaks" without standard references that cannot be identified, which is also critically important for the QC of TCM and elucidation of the material basis on their therapeutic effects.

High-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) is now widely accepted to be the predominant tool for the analysis of multi-component herbal medicines [16–19]. This technology facilitates the identification of unknown components in the herbal system remarkably with high sensitivity and accuracy from abundant MS and MS/MS information, especially for those components whose standards are unavailable. Obviously, this tool is powerful in the analysis of TCM. However, hundreds and thousands of complex active components exist in TCM; thus, it is impossible to identify all the components. The "common peaks" in FA were chosen as those peaks existing in almost all of the sample spectra, which can reflect the total characteristics of TCM from different origins; thus, qualitative analysis of these "common peaks" will be helpful for the QC and interpretation of TCM.

In this work, a new strategy for the QC and qualitative analysis of TCM that combines MIQ, FA, and identification of common peaks in the fingerprint by HPLC-DAD-MS/MS was proposed, which not only can be used to control the quality of TCM effectively, but also provides the chemical evidence for revealing the material basis of their therapeutic effects.

Yinhuang (YH), a classical TCM prescription comprising two medicinal herbs, Flos *Lonicerae* and Radix *Scutellariae*, has been proved to possess useful properties including anti-inflammatory, antipyretic, and anti-influenza virus activities [20]. Three main dosage forms of preparations, namely granule, capsule, and lozenge, are widely used in clinical practice in China. Chemical and pharmacological investigations revealed that three types of secondary metabolites, namely phenolic acids, iridoids, and flavonoids, are responsible for the overall therapeutic effects of Flos *Lonicerae* [21], whereas flavonoids such as baicalein, baicalin, wogonin, and wogonoside are the major active components in Radix *Scutellariae* [22].

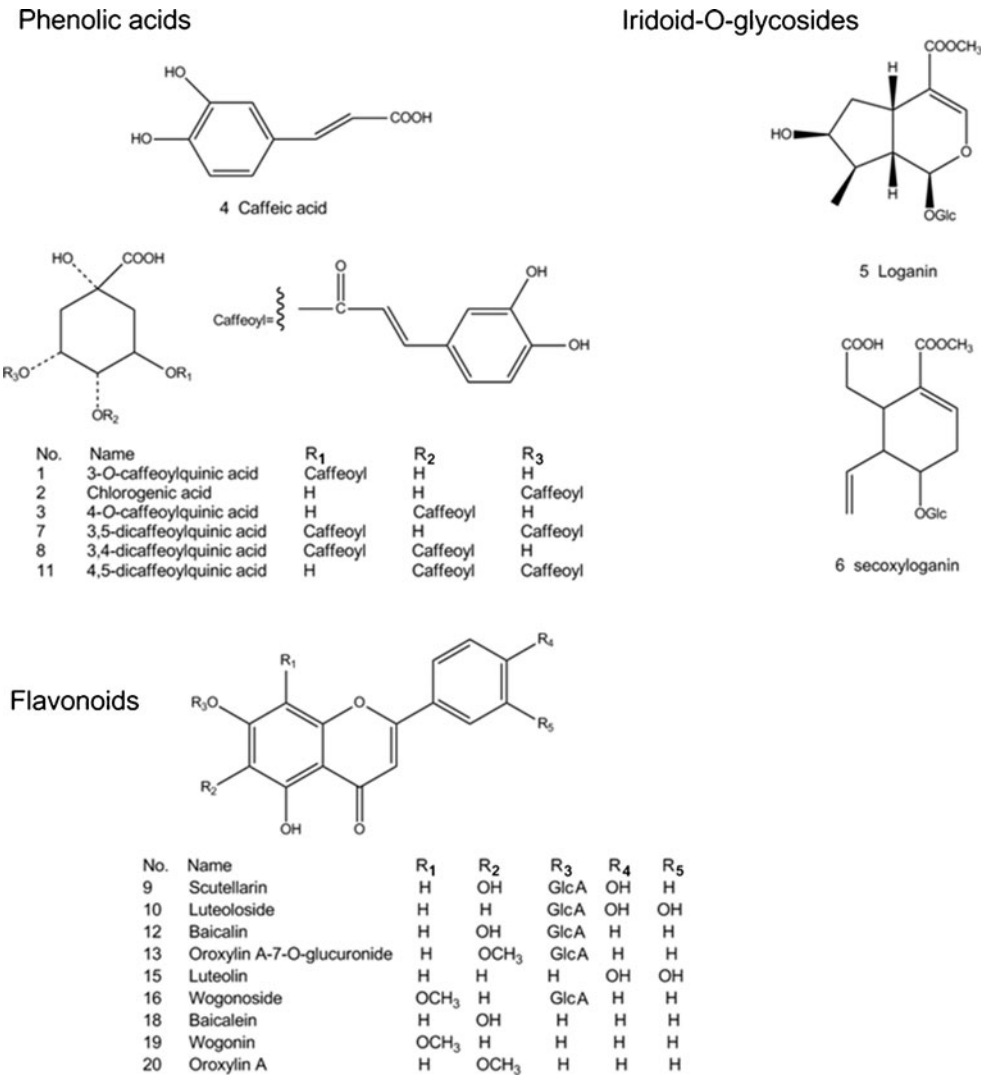
YH granule has been officially listed in the Chinese Pharmacopoeia (Ch. P.) [23]. According to the Ch. P., chlorogenic acid and baicalin in YH granule are required to be qualified by two different HPLC systems for the purpose of QC, which is laborious and time-consuming. Li et al.'s [24] reported the qualitative analysis of YH granules using LC-MS, but multiple bioactive components quantification and FA were not carried out in their work. Several published papers have described the quantitative determination of one or two major compounds such as chlorogenic acid or baicalin for the QC of YH preparations [25, 26]; however, no reports have been concerned with combining chromatographic fingerprint with quantification of multi-ingredients to evaluate the quality of YH preparations.

Therefore, the aim of the present paper was to establish a reliable HPLC-DAD-ESI-MS/MS method for the qualitative analysis and QC of YH preparations. FA and simultaneous quantification of nine bioactive components, namely chlorogenic acid, caffeic acid, luteoloside, baicalin, luteolin, wogonoside, baicalein, wogonin, and oroxylin A (Fig. 1) were carried out to assess the quality of YH preparations from different Chinese manufacturers by HPLC-DAD. The qualitative analysis was performed by ESI-MS/MS in negative ionization mode to acquire plentiful mass data in full-scan mode and MS/MS in a data-dependent product ion spectrum.

Materials and methods

Reagents and materials

The reference compounds including chlorogenic acid, caffeic acid, luteoloside, baicalin, luteolin, wogonoside, baicalein,



No.	Name	R_1	R_2	R_3
1	3-O-caffeoylquinic acid	Caffeoyl	H	H
2	Chlorogenic acid	H	H	Caffeoyl
3	4-O-caffeoylquinic acid	H	Caffeoyl	H
7	3,5-dicaffeoylquinic acid	Caffeoyl	H	Caffeoyl
8	3,4-dicaffeoylquinic acid	Caffeoyl	Caffeoyl	H
11	4,5-dicaffeoylquinic acid	H	Caffeoyl	Caffeoyl

No.	Name	R_1	R_2	R_3	R_4	R_5
9	Scutellarin	H	OH	GlcA	OH	H
10	Luteoloside	H	H	GlcA	OH	OH
12	Baicalin	H	OH	GlcA	H	H
13	Oroxylin A-7-O-glucuronide	H	OCH_3	GlcA	H	H
15	Luteolin	H	H	H	OH	OH
16	Wogonoside	OCH_3	H	GlcA	H	H
18	Baicalein	H	OH	H	H	H
19	Wogonin	OCH_3	H	H	H	H
20	Oroxylin A	H	OCH_3	H	H	H

Fig. 1 Chemical structures of the identified compounds in YH preparations

wogonin, and oroxylin A (≥98.0 %) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPP, Beijing, China). HPLC-grade methanol was obtained from Fisher Scientific (USA). Purified water was used from a Milli-Q system (Millipore, Bedford, MA, USA). Formic acid and other reagents were of analytical grade. The deionized water was used for all solutions and dilutions.

Twelve batches of YH preparations were collected from different pharmaceutical companies in China (Table 1). The reference standard stock solutions of the nine compounds were prepared in methanol or water and stored in brown vials at 4 °C. YH granule (sample A, lot no. 20060725) was selected as the sample for method validation and optimization of HPLC-DAD-ESI-MS/MS conditions.

Preparation of sample solutions and negative control (NC) solutions

YH preparations were pulverized into fine powder. The powder (one-twentieth of the max dose per day) was accurately weighed and extracted with 25 mL of 80 % methanol in an ultrasonic water bath (Ningbo Xinzhi Biotechnology Instrument Co., Ningbo, China) for 30 min. Additional solvent was added to adjust the weight to the pre-extraction weight, and then the extracts were centrifuged at 15,000 rpm for 10 min. The supernatants were filtered through a 0.45-μm membrane filter and transferred to an autosampler vial for HPLC-DAD-ESI-MS/MS analysis.

According to the prescription and preparation protocol of YH formula recorded by the Ch. P., two NC samples comprising the formula without Flos *Lonicerae* or Radix *Scutellariae* were prepared to validate the specificity of the method. The medicinal herbs were ground into powder with a particle size of 40–60 mesh and the NC samples were prepared according to the method for analysis described above.

Preparation of standard solutions

Standard stock solutions of the nine reference standards (chlorogenic acid, caffeic acid, luteoloside, baicalin, luteolin,

Table 1 Summary of the tested samples

Sample no.	Preparation form	Origins	Batch no.
A	Granule	Jiahe Medicine Co., Ltd, Guangzhou, China	100113
B	Granule	Beiwei Medicine Co., Ltd, Beijing, China	100513
C	Granule	Zhongzhi Medicine Co., Ltd, Zhongshan, China	20100306
D	Granule	Yadong Biological Medicine Co., Ltd, Zhongshan, China	1004012
E	Granule	Yongan Medicine Co., Ltd, Yunnan, China	100903
F	Granule	Yiheng Medicine Co., Ltd, Nanjing, China	1101210
G	Granule	Jiminkexin Medicine Co., Ltd, Jiangxi, China	101202
H	Capsule	Tianyang Medicine Co., Ltd, Shanxi, China	20101209
I	Capsule	Zhongzhi Medicine Co., Ltd, Zhongshan, China	20110107
J	Capsule	Taihuatang Medicine Co., Ltd, Sichuan, China	101103
K	Lozenge	Xinyijiahua Medicine Co., Ltd, Shanghai, China	100616
L	Lozenge	Diao Medicine Co., Ltd, Chengdu, China	1011029

wogonoside, baicalein, wogonin, and oroxylin A) were prepared by dissolving them in methanol or water. They were then diluted to six concentrations for construction of calibration plots in the ranges of 0.21–42.5, 0.35–70, 0.40–80, 8.00–400, 0.44–88.2, 0.50–100, 0.36–72, 0.38–77.5, and 0.36–72 μg mL^{-1}, respectively. All the solutions were stored in a refrigerator at 4 °C.

HPLC-DAD-ESI-MS system

Chromatographic analysis

HPLC analysis was performed on an Agilent 1260 series HPLC system equipped with a binary pump, online degasser, auto plate-sampler, column oven, and diode array detector (DAD). Different HPLC parameters including various columns, mobile phases, and column temperatures were examined and compared. Finally, a Sepax GP-C_{18} column (250 mm×4.6 mm i.d., 5 μm) (Sepax, US) was used at a column temperature of 30 °C. The mobile phase was prepared from 0.1 % formic acid methanol solution (component A) and 0.1 % formic acid water solution (component B). The gradient elution program (% A in B) was optimized and conducted as follows: 0–15 min, 15 %; 15–25 min, 15–32 %; 25–40 min, 32–35 %; 40–52 min, 35 %; 52–70 min, 35–42 %; 70–80 min, 42–45 %; 80–90 min, 45–50 %; 90–120 min, 50–55 %; 120–135 min, 55 %. The flow rate was 1.0 mL min^{-1}. The DAD spectra were recorded between 190 and 400 nm. In the quantitative analysis, wavelengths were set at 275 nm, 320 nm, and 350 nm according to the absorption properties of the analyzed compounds (baicalin, wogonoside, baicalein, wogonin, and oroxylin A at 275 nm; chlorogenic acid and caffeic acid at 320 nm; luteoloside and luteolin at 350 nm). In the FA, the wavelength was set at 320 nm to exhibit the vast majority of chromatography peaks.

Mass spectrometry

The above system, interfaced with an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, MA, USA), was used for carrying out the HPLC-ESI-MS/MS analysis. The conditions of ESI source were as follows: source voltage, 3,500 V; drying gas (N_2) flow rate, 11.0 L min^{-1}; drying gas temperature, 350 °C; nebulizer, 15 psi. The mass spectrometric data was acquired from *m*/*z* 100 to 1,000 in negative ion modes.

Validation of the quantitative analysis

Linearity, limits of detection and quantification (LODs and LOQs), precision (interday and intraday), absolute recovery, accuracy, repeatability, and stability were determined to validate the quantitative method on the basis of the International Conference on Harmonization (ICH) guideline [27].

HPLC fingerprint analysis

Data analysis was performed using the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A) commercial software, which was recommended by the SFDA. The relative retention time (RRT) and relative peak area (RPA) of each common peak related to its reference peak were calculated for quantitative expression of the chemical properties in the chromatographic pattern of the YH formula. On the basis of this, the correlation coefficients of entire chromatographic profiles of samples were calculated and the simulated mean chromatogram was generated.

Identification of compounds

Identification of constituents in the YH preparations extract was carried out by HPLC-ESI-MS/MS analysis. In the full-scan mass spectra, most of the constituents exhibited their

$[M-H]^-$ peak in negative ion mode under the soft electrospray ionization condition. Precursor ions were subjected to collision-induced dissociation (CID) to generate the fragment ions and the fragmentation patterns were proposed for the structural identification of constituents.

Results and discussion

Optimization of HPLC/DAD/ESI-MS conditions

To obtain an ideal separation containing sufficient information of compounds with good resolution and reasonable analysis time, some HPLC analytical parameters, such as separation column, mobile phase, gradient elution procedure, and column temperature, were investigated. Three kinds of columns, namely Sepax GP- C_{18} (250 mm× 4.6 mm i.d., 5 μm), Agilent Zorbax SB-C_{18} (250 mm× 4.6 mm i.d., 5 μm), and Kromasil C_{18} (250 mm×4.6 mm i.d., 5 μm) , were tested. The best separation was achieved on the Sepax GP-C_{18} column. Different mobile phase compositions including methanol–water or acetonitrile–water containing different concentrations of formic acid or acetic acid were studied. As a result, the optimal mobile phase system consisting of methanol containing 0.1 % formic acid and water containing 0.1 % formic acid was selected as it had greater baseline stability and ionization efficiency.

The quantification of constituents in YH preparations was achieved at 275 nm, 320 nm, and 350 nm (baicalin, wogonoside, baicalein, wogonin and oroxylin A at 275 nm; chlorogenic acid and caffeic acid at 320 nm; luteoloside and luteolin at 350 nm), where the UV spectra of the nine analytes exhibited maximum absorbance, in which better response and less interference could be accomplished (Fig. 2a, b). In the FA, the wavelength was set at 320 nm, where most chromatograph peaks were detected (Fig. 2b).

By comparing positive-ion mode with negative-ion mode, the latter was selected for MS analysis in this study according to the number and abundance of peaks. Furthermore, MS parameters such as source voltage, drying gas (N_2) flow rate, and drying gas temperature were optimized (listed in Mass spectrometry), and the total ion current (TIC) chromatogram was acquired (Fig. 3).

Sample extraction protocol

In order to achieve the optimum extraction efficiency, the main conditions such as solvent, solvent volume, and extraction time were optimized. Because of the different polarity of the tested compounds, pure and aqueous methanol or ethanol solutions were investigated. The best solvent was found to be 80 % methanol, which allowed efficient extraction of the compounds. In

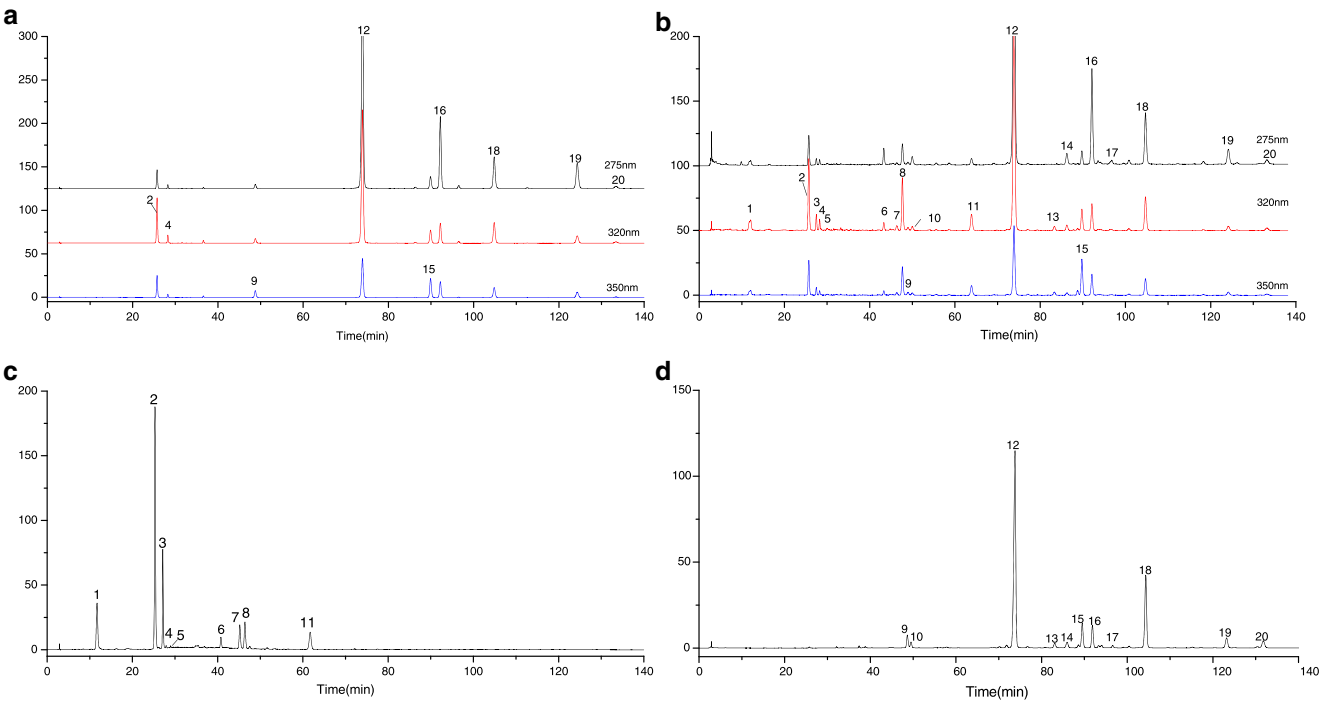


Fig. 2 Representative HPLC-DAD chromatograms of **a** mixed standard solutions at 275 nm, 320 nm, and 350 nm; **b** YH granule at 275 nm, 320 nm, and 350 nm; **c** the NC sample without Radix *Scutellariae* at 320 nm; **d** the NC sample without Flos *Lonicerae* at 320 nm. *2* chlorogenic acid, *4* caffeic acid, *9* luteoloside, *12* baicalin, *15* luteolin, *16* wogonoside, *18* baicalein, *19* wogonin, *20* oroxylin A

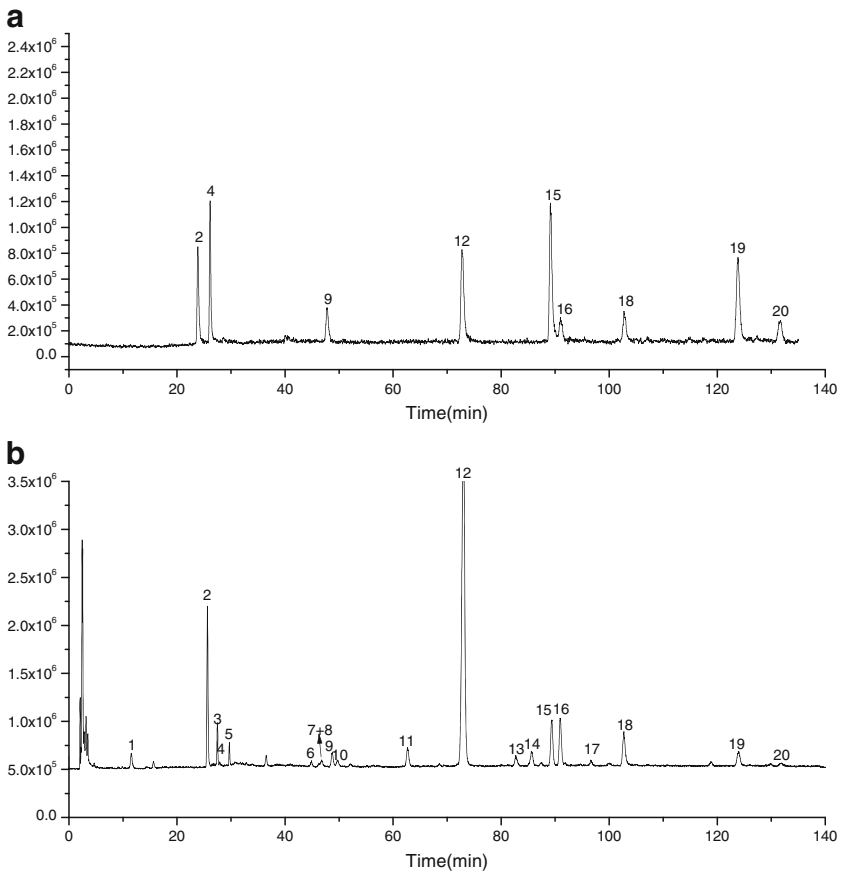


Fig. 3 HPLC-ESI-MS total ion chromatogram (TIC) in negative ion mode of **a** the mixed standard, **b** YH granule

addition, reflux and ultrasonic extraction were compared for their suitability in extracting the targets from matrix. As described in other works, the reflux extraction could cause the loss of the compounds as a result of ionization, hydrolysis, and oxidation during extraction [28–30]; furthermore, it might lead to consumption of a large amount of solvent, low extraction efficiency, and is time-consuming [31–33]. Therefore, the ultrasound-assisted extraction (UAE) method was more suitable for the benefit of saving time and its easier operation. In the present paper, the extraction time and sample-to-solvent ratio were investigated, and the results indicated that an efficient extraction was produced when 0.5 g of sample was extracted with 25 mL of 80 % methanol by UAE for 30 min.

Method validation of quantitative analysis

Nine peaks in the chromatogram of YH preparations with reasonable heights and good resolution were chosen as marker compounds, namely chlorogenic acid, caffeic acid, luteoloside, baicalin, luteolin, wogonoside, baicalein, wogonin, and oroxylin A, which were generally considered as active components. HPLC profiles of YH and standard substances detected at 275 nm, 320 nm, and 350 nm are displayed in Fig. 2a, b, respectively.

Specificity of the method

In order to investigate the specificity of the method, different NC samples were prepared and analyzed, and the chromatograms are shown in Fig. 2c, d. It was obvious that there were no interferences for determination of the nine compounds by comparing the retention times, UV spectra, and MS spectra with those of the standards.

Calibration curves, the limit of detection (LOD) and quantification (LOQ)

A series of standard solutions of nine compounds were used to determine the linear range of the analyses by the external standard method. Calibration curves were generated by plotting the peak areas versus the corresponding concentrations. The linearity of the calibration process was investigated by means of R^2, the quality coefficient (Q), and the lack-of-fit test. As shown in Table 2, correlation coefficients were better than 0.9998 for all analytes with Q values less than 3 %. For the lack-of fit test, significance values were greater than 0.05 for all analytes at the 95 % confidence level, which indicated that a linear regression model provided a good interpolation of the experimental data.

Table 2 Detection wavelength, linear regression data, LODs, and LOQs for nine active compounds analyzed by HPLC-DAD

Compound	λ (nm)	Regression equation[a]	Linear range (μg mL^{-1})	R^2	Q[b] (%)	p value[c]	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Chlorogenic acid	320	y=62,373x−84.19	0.21–42.50	1.0000	0.83	0.123	0.05	0.17
Caffeic acid	320	y=115,357x+8,488.3	0.35–70.00	0.9999	1.05	0.232	0.05	0.22
Luteoloside	350	y=38,263x+2,734.5	0.40–80.00	0.9999	1.17	0.077	0.10	0.33
Baicalin	275	y=43,099x+15,644	8.00–400.00	1.0000	0.53	0.103	0.12	0.51
Luteolin	350	y=48,389x+6,357.3	0.44–88.20	0.9998	1.55	0.119	0.05	0.22
Wogonoside	275	y=22,014x−2,334	0.50–100.00	0.9998	1.84	0.176	0.06	0.27
Baicalein	275	y=56,408x−2,235.2	0.36–72.00	0.9999	2.73	0.070	0.03	0.12
Wogonin	275	y=37,402x+1,137	0.38–77.50	0.9999	1.45	0.175	0.06	0.25
Oroxylin A	275	y=66,150x−162.11	0.36–72.00	0.9999	1.22	0.084	0.04	0.18

[a] In the regression equation $y = ax + b$, x is the concentration of the compound (μg mL^{-1}), y indicates the peak area, and R^2 is the correlation coefficient of the equation

[b] Quality coefficient of the regression model

[c] p value of lack-of-fit test (confidence level at 95 %)

The diluted solution of the nine reference compounds was further diluted to a series of concentrations with methanol for the purpose of obtaining the LODs and LOQs. They were determined at signal-to-noise (S/N) ratios of 3 and 10, respectively. As shown in Table 2, the range of LODs for all compounds was from 0.04 to 0.12 μg mL^{-1}, and the LOQ was from 0.12 to 0.51 μg mL^{-1}.

Recovery

The recovery was determined by adding an accurately known amount of the corresponding marker compounds at three different levels (high, middle, and low) to a sample of YH (sample A). As shown in Table 3, the recoveries were between 97.3 % and 103.5 % with a relative standard deviation (RSD) value of less than 2.6 % for all the nine compounds, which indicated that the absolute recovery of the nine compounds meets the requirement of quantitative analysis.

Precision and accuracy

Precision and accuracy were evaluated together. The intraday and interday precisions were conducted by six replicate injections of mixed standard solutions with three concentrations during a single day and on three consecutive days. An RSD value was used to evaluate precision. As shown in Table 4, the intraday and interday precision RSD values of the nine compounds were all less than 2.2 %.

Accuracy values were calculated from accuracy (%)= (mean of measured concentration/nominal concentration)× 100. The results are given in Table 4. For all nine compounds, the accuracy values were between 95.4 % and 103.6 %, which indicated that the established method had a good and high accuracy.

Table 3 Recovery of nine analytes

Compound	Original (mg g^{-1})	Spiked (mg g^{-1})	Found (mg g^{-1})	Recovery[a] (%)	RSD (%) (n=3)
Chlorogenic acid	0.937	0.450	1.383	99.1	1.23
		0.900	1.865	103.2	0.85
		1.350	2.323	102.7	1.30
Caffeic acid	0.030	0.016	0.047	104.0	2.51
		0.032	0.063	103.5	1.56
		0.048	0.079	103.0	1.13
Luteoloside	0.044	0.023	0.067	101.5	1.55
		0.046	0.090	100.7	1.60
		0.069	0.114	102.1	0.47
Baicalin	2.919	1.550	4.484	101.0	1.02
		3.100	5.976	98.6	0.72
		4.650	7.436	97.1	0.43
Luteolin	0.528	0.240	0.774	102.5	1.59
		0.480	1.017	101.9	1.85
		0.720	1.248	100.0	0.88
Wogonoside	0.242	0.150	0.394	101.3	1.67
		0.300	0.536	97.9	1.15
		0.450	0.700	101.8	1.05
Baicalein	0.554	0.250	0.796	96.9	2.51
		0.500	1.039	96.9	1.22
		0.750	1.289	98.0	1.05
Wogonin	0.048	0.025	0.074	104.7	0.93
		0.050	0.099	102.3	1.31
		0.075	0.123	99.5	1.21
Oroxylin A	0.046	0.022	0.069	103.1	2.33
		0.044	0.088	96.2	1.80
		0.066	0.114	102.8	1.28

[a] Recovery (%)=[(found amount−original amount)/spiked amount]×100

Table 4 Precision and accuracy of nine analytes

Compound	Nominal concentration (μg mL^{-1})	Intraday (n=6)			Interday (n=3)		
		Found (μg mL^{-1})	RSD (%)	Accuracy (%)	Found (μg mL^{-1})	RSD (%)	Accuracy (%)
Chlorogenic acid	1.70	1.73	1.62	101.8	1.71	1.60	100.6
	17.00	17.20	1.69	101.2	17.25	1.31	101.5
	34.00	34.80	1.17	102.4	34.88	1.41	102.6
Caffeic acid	2.80	2.87	1.89	102.5	2.90	0.52	103.6
	28.00	28.09	1.62	100.3	27.91	1.05	99.7
	56.00	56.01	1.20	100.0	55.89	0.89	99.8
Luteoloside	3.20	3.26	1.40	101.9	3.15	1.01	98.4
	32.00	31.60	1.42	98.8	31.53	0.65	98.5
	64.00	64.22	0.81	100.3	64.12	0.46	100.2
Baicalin	16.00	16.10	0.40	100.6	15.88	0.92	99.2
	160.00	160.55	0.88	100.3	158.15	0.75	98.8
	320.00	321.08	0.30	100.3	318.22	0.55	99.4
Luteolin	3.53	3.65	0.75	103.4	3.55	1.28	100.6
	35.28	35.95	1.18	101.9	35.63	1.94	101.0
	70.56	70.88	1.87	100.4	70.91	0.81	100.5
Wogonoside	4.00	4.08	0.48	102.0	4.10	0.96	102.5
	40.00	40.27	1.96	100.7	39.93	1.47	99.8
	80.00	80.56	1.27	100.7	80.86	1.86	101.1
Baicalein	2.88	2.91	1.61	101.0	2.80	1.33	97.2
	28.80	28.75	2.18	99.8	28.55	2.10	99.1
	57.60	58.17	1.24	101.0	58.25	0.58	101.1
Wogonin	3.10	3.15	0.63	101.6	3.01	1.60	97.1
	31.00	31.56	1.06	101.8	31.11	1.31	100.4
	62.00	62.70	1.24	101.1	62.93	1.41	101.5
Oroxylin A	2.88	2.92	1.01	101.4	2.75	0.52	95.5
	28.80	29.10	1.93	101.0	28.36	1.05	98.5
	57.60	57.22	1.16	99.3	57.96	0.89	100.6

Repeatability and stability

Repeatability was investigated with six independently prepared sample solutions of YH (sample A), one of which was injected into the apparatus at 0, 4, 8, 12, 24, and 48 h, separately, to determine the stability of the solution. As shown in Table 5, repeatability and stability RSD values of the nine compounds were all less than 2.2 %.

Quantitative determination of YH preparations

The contents (n=3) of nine makers are summarized in Table 6. It was recognized that chlorogenic acid, baicalin, luteolin, and wogonoside were the dominant compounds in all examined samples. However, the dominant compounds or the total content of certain types of constituents varied in different YH preparations. For example, chlorogenic acid was abundant in batches of D, G, K, and L, but lower in batches B and F. Baicalein was not detected in batches E and F. Oroxylin A was not detected in the batch F. The other components also had obvious variations. The differences in

Table 5 Repeatability and stability of nine analytes

Compound	Repeatability (n=6) (mg g^{-1})		Stability (48 h, n=3) (mg g^{-1})	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Chlorogenic acid	1.874±0.026	1.36	1.870±0.024	1.28
Caffeic acid	0.062±0.001	1.66	0.059±0.001	1.62
Luteoloside	0.088±0.001	0.58	0.085±0.001	0.93
Baicalin	5.830±0.015	0.25	5.822±0.023	0.40
Luteolin	1.053±0.013	1.23	1.055±0.018	1.71
Wogonoside	0.484±0.004	0.91	0.480±0.006	1.25
Baicalein	1.108±0.013	1.17	1.100±0.013	1.18
Wogonin	0.093±0.001	1.42	0.091±0.002	2.16
Oroxylin A	0.090±0.001	0.86	0.088±0.001	1.16

Table 6 Contents (mg g^{-1}, mean±SD, n=3) of the nine compounds in YH preparations (n=3)

Sample no.	Chlorogenic acid	Caffeic acid	Luteoloside	Baicalin	Luteolin	Wogonoside	Baicalein	Wogonin	Oroxylin A
A	1.874±0.001	0.060±0.004	0.089±0.002	5.837±0.005	1.055±0.002	0.484±0.001	1.107±0.001	0.096±0.001	0.091±0.001
B	0.292±0.002	0.018±0.000	0.086±0.002	3.942±0.010	0.422±0.002	0.326±0.002	0.255±0.001	0.141±0.000	0.070±0.001
C	1.484±0.001	0.015±0.000	0.481±0.010	25.090±0.020	2.326±0.003	0.109±0.000	1.637±0.003	0.040±0.000	0.052±0.000
D	4.622±0.004	0.165±0.002	0.535±0.008	28.918±0.033	2.384±0.010	0.619±0.003	1.334±0.004	0.068±0.000	0.033±0.000
E	1.827±0.001	0.059±0.000	0.094±0.001	3.683±0.015	0.493±0.004	0.428±0.003	ND	0.106±0.003	0.041±0.000
F	0.974±0.002	0.041±0.000	0.114±0.001	7.415±0.012	0.880±0.002	0.045±0.000	ND	0.021±0.000	ND
G	9.307±0.005	0.077±0.001	1.314±0.015	99.285±0.047	10.05±0.014	1.386±0.010	1.086±0.001	0.258±0.003	0.422±0.005
H	3.222±0.003	0.061±0.001	0.344±0.002	24.702±0.022	2.880±0.011	0.214±0.003	0.025±0.000	0.061±0.000	0.059±0.000
I	2.521±0.003	0.051±0.000	0.147±0.002	6.959±0.020	1.533±0.003	1.039±0.005	0.468±0.003	0.232±0.001	0.123±0.001
J	1.364±0.002	0.010±0.000	0.429±0.001	29.435±0.035	3.186±0.008	0.165±0.002	0.016±0.000	0.090±0.001	0.075±0.000
K	4.824±0.005	0.946±0.006	2.724±0.006	32.812±0.053	3.710±0.014	0.117±0.003	1.483±0.010	0.069±0.003	0.079±0.000
L	10.905±0.003	0.071±0.002	0.749±0.003	51.466±0.066	4.934±0.008	0.803±0.005	3.258±0.009	0.126±0.001	0.132±0.001
Range[a]	0.292–10.905	0.010–0.946	0.094–2.724	3.683–99.285	0.422–2.384	0.045–1.386	0.016–1.637	0.040–0.232	0.033–0.422

ND not detected

[a] Range of contents of each compound in all collected samples

the content of constituents among different samples might be attributed to many factors, such as different raw materials and extraction processes. Therefore, the detection of a single component or only several components could not control the quality of YH preparations effectively. To ensure the stability, safety, and efficacy for clinical use, guidelines and QC for YH preparations should be standardized.

Chromatographic fingerprint analysis of YH preparations

To construct a standard fingerprint, 12 batches of YH preparations (granule, capsule, and lozenge) were collected and analyzed with the established HPLC-DAD procedure. The average chromatogram from the 12 batches was regarded as the standard fingerprint of the YH preparations. Peaks existing in all chromatograms of the samples were assigned as "common peaks". As shown in Fig. 4, the chromatograms of the YH preparations contained 20 distinct common peaks. Peak 12 (baicalin) was the most abundant of all the 20 peaks. Therefore, it was selected as a reference peak to calculate the RRT and RPA according to the following formulas: $RRT=RT_{peak}/RT_{peak12}$ and $RPA=PA_{peak}/PA_{peak12}$. RRT and RPA of the common peaks in 12 samples are shown in Table 7. The RSD values of the RRT were less than 1.2 %, which demonstrated good stability and reproducibility of the FA by HPLC-DAD. The similarity

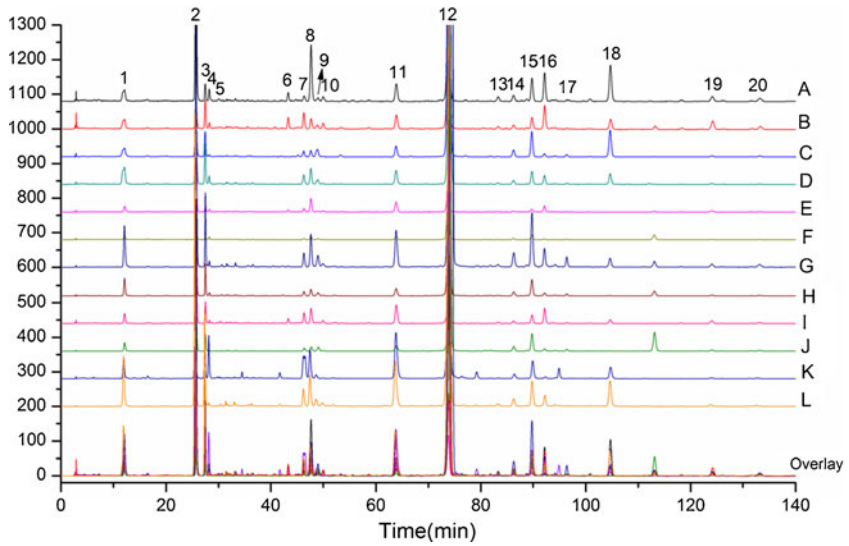


Fig. 4 Chromatogram of the investigated 12 samples of YH preparations

Table 7 RRT and RPA of 20 common peaks with respect to peak 12 in chromatograms of YH preparations

Peak no.	RRT	RSD%	RPA	RSD%
1	0.16	1.21	0.06	97.1
2	0.33	1.12	0.22	75.9
3	0.35	0.91	0.05	54.0
4	0.37	0.59	0.03	122.0
5	0.39	0.52	0.006	86.1
6	0.51	0.71	0.006	86.9
7	0.62	0.32	0.03	96.5
8	0.65	0.29	0.07	111.2
9	0.66	0.45	0.01	26.2
10	0.67	0.32	0.01	117.6
11	0.87	0.41	0.10	99.6
12(s)	1.00	0.00	1.00	0.0
13	1.18	0.34	0.04	47.5
14	1.22	0.31	0.09	39.3
15	1.25	0.31	0.11	133.8
16	1.26	0.40	0.06	58.0
17	1.31	0.32	0.08	57.7
18	1.42	0.31	0.08	112.7
19	1.71	0.31	0.05	123.7
20	1.82	0.28	0.01	94.2

indexes were calculated by the mean fusion vector method. As listed in Table 8, the similarity index of all 12 samples was higher than 0.890, which suggested that the YH preparations from different manufacturers shared similar chromatographic patterns. However, the RSD values of RPA from the 12 samples were very high (approximately 26.2–133.8 %), which might have originated from a number of factors, such as different origin, production process, storage conditions, and alternative environment.

Table 8 Similarities of chromatograms of 12 samples

No.	Similarities[a]
A	0.991
B	0.952
C	0.993
D	0.998
E	0.891
F	0.997
G	0.998
H	0.999
I	0.933
J	0.990
K	0.994
L	0.993

[a]The reference fingerprint was developed with the median of all chromatograms

Qualitative analysis by HPLC-ESI-MS/MS

As mentioned above, the chemical fingerprint of the YH preparations contained 20 common peaks including the nine quantified components, and the peaks exist in almost all spectra from the batches. Therefore, the identification of the common peaks is essential to total QC of YH preparations and reveals the material basis of their therapeutic effects. LC-MS was used to identify the common peaks.

The ESI-MS/MS data and fragmentations of the nine standard compounds are listed in Table 9. YH granule (sample A) was selected for this study; a total of 18 "common peaks" described in the fingerprint chromatogram including the nine quantified compounds were identified or tentatively characterized (Table 10). Their chemical structures are provided in Fig. 1.

Compounds **2**, **4**, **10**, **12**, **15**, **16**, **18**, **19**, and **20** were unambiguously identified by comparing their HPLC retention time, UV spectra, and MS data with those of the reference standards. For other constituents, the structures were tentatively characterized on the basis of their retention times and MS/MS fragmentation behavior. Among the compounds, there were seven phenolic acids and two iridoid-*O*-glycosides from Flos *Lonicerae*, and nine flavonoids mainly from Radix *Scutellariae*.

Identification of phenolic acids

All the phenolic acids identified in YH were from Flos *Lonicerae*. In general, the structures of these compounds have one or more caffeic acid substituents bound to a quinic acid moiety, usually located at position 3 and/or 4 and/or 5.

It was reported that the linkage position of acyl groups on quinic acid could be determined on the basis of the MS^2 fragmentation [34]. Generally, when the acyl group was linked to 3-OH or 5-OH, the [quinic acid−H]$^-$ ion at *m*/*z* 191 was the base peak, and the [caffeic acid]$^-$ ion at *m*/*z* 179 was more significant for 3-*O*-caffeoylquinic acid. The [quinic acid−H_2O−H]$^-$ ion at *m*/*z* 173 was the prominent peak when the acyl group was linked to 4-OH.

On the basis of this study, three monocaffeoylquinic acid isomers (compounds **1**–**3**) and three dicaffeoylquinic acid isomers (compounds **7**–**11**) were identified. Compound **2** was unambiguously identified as chlorogenic acid (5-*O*-caffeoylquinic acid) by comparison of retention time and mass spectra with those of the standard (see Fig. 5b). Compounds **1** and **3** both displayed [M−H]$^-$ ions at *m*/*z* 353, the same as chlorogenic acid. However, these two isomers exhibited different MS^2 spectra.

Table 9 MS/MS product ions and fragmentations obtained from standard compounds in this study

Compounds	$[M-H]^-$ (*m/z*)	Fragmentor	Collision energy	Fragment ions (*m/z*)
Chlorogenic acid	353.0	80	10	191.3 [quinic acid−H]$^-$, 172.9 [quinic acid−H−H_2O]$^-$, 126.9 [quinic acid−H−CO−$2H_2O$]$^-$
Caffeic acid	178.9	90	10	134.9 [M−H−CO_2]$^-$
Luteoloside	447.1	140	20	285.1 [M−H−Glc]$^-$, 174.9 [M−H−Glc−catechol]$^-$, 151.1 [$^{1,3}A^-$], 132.9 [$^{1,3}B^-$]
Baicalin	445.0	110	5	268.9 [M−H−GluA]$^-$, 174.7 [GluA−H]$^-$, 112.8 [GluA−H−CO_2−H_2O]$^-$
Luteolin	284.9	120	20	174.7 [M−H−catechol], 150.8 [$^{1,3}A^-$], 132.9 [$^{1,3}B^-$]
Wogonoside	459.0	100	10	283.0 [M−H−GluA]$^-$, 268.0 [M−H−GluA−CH_3]$^-$, 162.8 [$^{0,2}A^-$]
Baicalein	268.9	140	15	241.0 [M−H−CO]$^-$, 222.8 [M−H−H_2O−CO]$^-$
Wogonin	283.1	110	10	267.9 [M−H−CH_3]$^-$, 238.8 [M−H−CO−H]$^-$, 163.0 [$^{0,2}A^-$]
Oroxylin A	283.0	80	10	267.7 [M−H−CH_3]$^-$, 239.1 [M−H−CH_3−COH]$^-$, 211.1 [H−CH_3−COH−CO]$^-$, 195 [M−H−CH_3−COH−CO_2]$^-$

In the MS^2 spectrum of compound **1**, the *m/z* 191 was the base peak, whereas the ion at *m/z* 173 produced the base peak for compound **3**. By referring to literature data [35], compounds **1** and **3** were identified as 3-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid, respectively (see Fig. 5a, c). Compounds **7**, **8**, and **11** all gave $[M-H]^-$ ions at *m/z* 515 and the [M−H −162]$^-$ ions at *m/z* 353. Compound **8** produced a base peak ion at *m/z* 191, as reviewed above, which was identified as 3,5-dicaffeoylquinic acid (see Fig. 5f), which was consistent with the previous report [35]. Two other compounds produced base peaks at *m/z* 179 or 173, hence they were identified as 4-substituted quinic acids. According to the literature [36], 3,5-dicaffeoylquinic acid was more easily eluted from the reversed-phase column when compared with 4,5-dicaffeoylquinic acid (see Fig. 5e). Thus, compound **11** was identified as 4,5-

Table 10 Characterization of compounds in YH preparations by HPLC-DAD-ESI-MS/MS

Peak no.	Retention time (t_R, min)	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)	Identification	Source
1	11.8	352.9	191.3, 179.0, 135.4	3-*O*-Caffeoylquinic	FL
2[a]	23.8	353.0	191.1, 172.9, 126.9	Chlorogenic acid	FL
3	25.6	353.1	191.3, 179.0, 173.1	4-*O*-Caffeoylquinic acid	FL
4[a]	26.1	178.9	134.9	Caffeic acid	FL
5	27.9	389.3	345.5, 208.9, 183.4, 165.4	Loganin	FL
6	37.2	403.0	371.0, 223.2, 190.9, 179.0	Secoxyloganin	FL
7	44.7	515.1	353.4, 179.1, 172.9,	3,4-Dicaffeoylquinic acid	FL
8	46.7	515.0	353.2, 190.9,178.9, 134.9	3,5-Dicaffeoylquinic acid	FL
9	47.6	461.3	285.0	Scutellarin	RS
10[a]	48.2	447.1	285.1,174.9, 151.1, 132.9	Luteoloside	RS
11	62.7	515.1	353.2, 190.9, 178.9, 173.1	4,5-Dicaffeoylquinic acid	FL
12[a]	72.3	445.0	268.9, 174.7, 112.8	Baicalin	RS
13	88.2	474.9	298.4, 112.4	Unknown	RS
14	85.6	459.0	283.0, 268.0, 175.0, 113.0	Oroxylin A-7-*O*-glucuronide	RS
15[a]	90.2	284.9	174.7, 150.8, 132.9	Luteolin	RS
16[a]	91.2	459.0	283.0, 268.0, 162.8	Wogonoside	RS
17	94.7	298.9	283.8, 181.8	Unknown	RS
18[a]	102.6	268.9	241.0, 222.8, 206.9	Baicalein	RS
19[a]	123.6	283.1	267.9, 238.8, 163.0	Wogonin	RS
20[a]	131.3	283.0	267.7, 239.1, 211.1, 194.5	Oroxylin A	RS

FL Flos *Lonicerae*, *RS* Radix *Scutellariae*

[a]Compared with standards

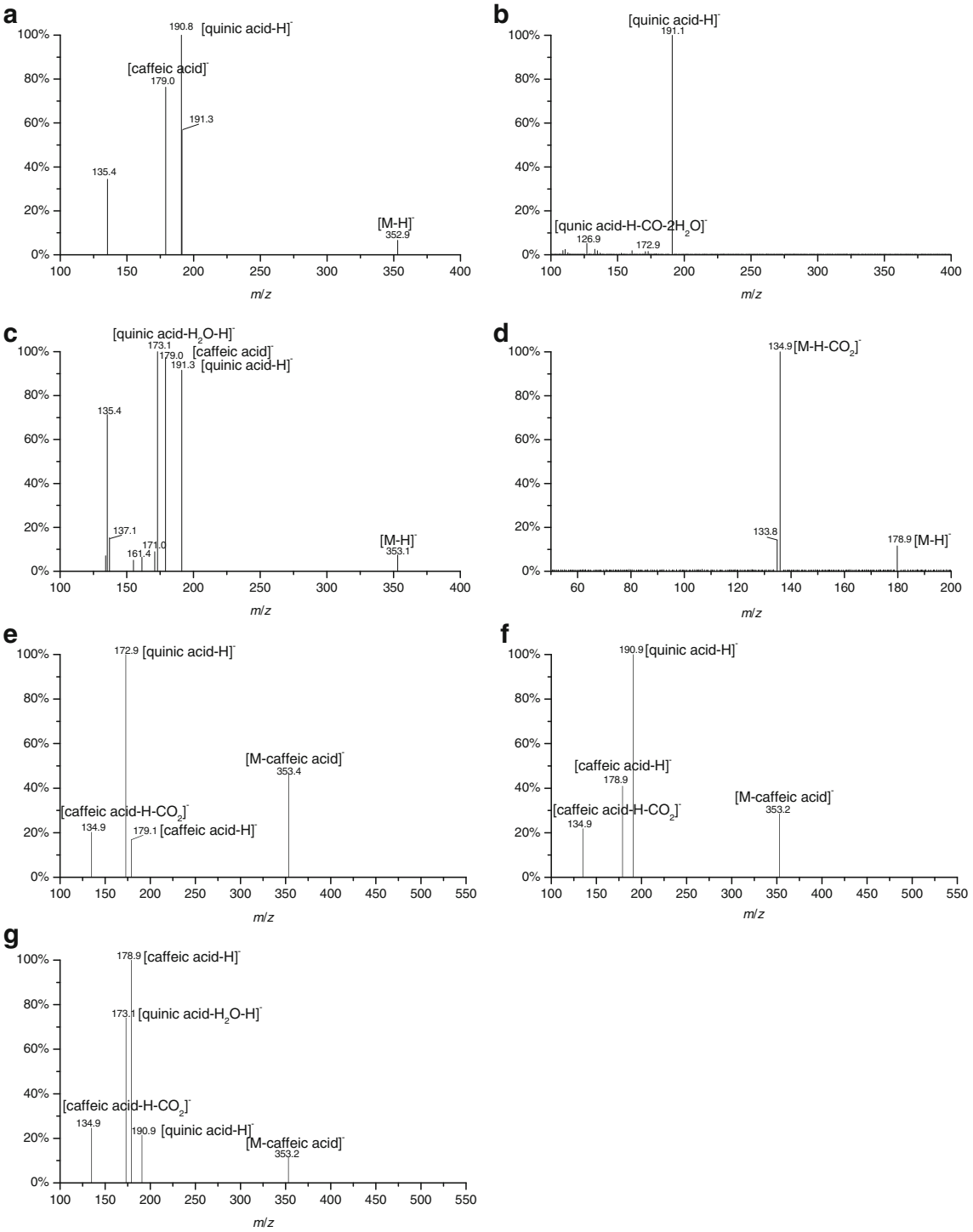


Fig. 5 Negative MS/MS spectra of seven phenolic acids identified in YH preparations. **a** 3-*O*-caffeoylquinic, **b** chlorogenic acid, **c** 4-O-caffeoylquinic acid, **d** caffeic acid, **e** 3,4-dicaffeoylquinic acid, **f** 3,5-dicaffeoylquinic acid, **g** 4,5-dicaffeoylquinic acid

dicaffeoylquinic acid, and compound **7** was identified as 3,4-dicaffeoylquinic acid (see Fig. 5g). Caffeic acid (**4**) was also identified by comparison of its retention time and MS/MS spectra with those of standard (see Fig. 5d).

Identification of iridoid-O-glycosides

Iridoid-*O*-glycosides from Flos *Lonicerae* were reported to possess various biological activities, such as cardiovascular, antiviral, cancer chemopreventive, and

immunomodulator activities [37]. Neutral elimination of a glucose unit (162 Da) is a typical fragmentation for all iridoid glycosides, and subsequent losses of H_2O, CH_3OH, and CO from the aglycone ion could be also observed. In this work, two iridoid-*O*-glycosides (**5** and **6**) were identified in YH by their MS spectra.

Compound **5** displayed an $[M-H]^-$ ion at *m/z* 389, the $[M-H-Glc]^-$ fragment ion at *m/z* 227 was not observed in the ESI-MS/MS spectrum, and the fragment ion $[M-H-Glc-CO_2]^-$ at *m/z* 183 and $[M-H-Glc-H_2O]^-$ at *m/z* 209 were observed predominantly. The fragment ion at *m/z* 121 was generated by successive or simultaneous losses of H_2O, CH_3OH, and CO from the aglycone ion. Referring to the literature, compound **5** could be tentatively identified as loganin [38] (see Fig. 6a).

Compound **6** exhibited a minor $[M-H]^-$ ion at *m/z* 403. The fragment ion $[M-H-Glc-H_2O]^-$ at *m/z* 223 and $[M-H-Glc-H_2O-CO_2]^-$ at *m/z* 179 were predominant. The fragment ion at *m/z* 371 ($[M-H-O_2]^-$) was generated by losses of O_2 from the $[M-H]^-$ ion. In accordance to the literature data [38], compound **6** could be tentatively identified as secoxyloganin (see Fig. 6b).

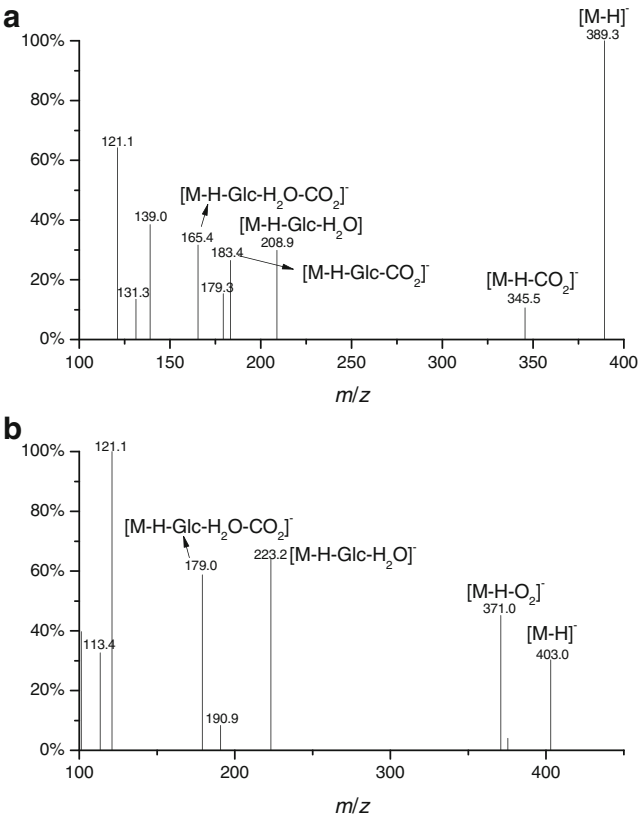


Fig. 6 Negative MS/MS spectra of two iridoid-*O*-glycosides identified in YH preparations: **a** loganin, **b** secoxyloganin

Identification of flavonoids

It has been demonstrated that the flavonoids in Radix *Scutellariae* protect against various inflammatory diseases, hepatitis, tumors, and diarrhea [22].

Compound **9** displayed an $[M-H]^-$ ion at *m/z* 461. The fragment ion at *m/z* 285 ($[M-H-176]^-$) indicated the loss of a glucuronic acid. By referring to the literature, compound **9** was tentatively identified as scutellarin [39] (see Fig. 7a).

Compounds **14** and **16** are a pair of isomers. Both of them gave an $[M-H]^-$ ion at *m/z* 459. Their MS^2 spectrum gave the ion at *m/z* 283 (−176), involving the loss of a glucuronic acid; then the ion at *m/z* 283 yielded an ion at *m/z* 268 (−15), suggesting the presence of a $-CH_3$ group. Compound **16** also had a low signal intensity ion at *m/z* 163 ($^{0,2}A^-$, according to the nomenclature proposed by Fabre and Rustan [40]). Compound **16** was unequivocally identified as wogonoside by comparing with the standard (see Fig. 7f). By examining the known flavonoids in Radix *Scutellariae*, there was another flavonoid named oroxylin A-7-*O*-glucuronide. According to the content difference and the chromatographic retention behavior in HPLC reported before [18], compound **14** was plausibly identified as oroxylin A-7-*O*-glucuronide (see Fig. 7d).

Compared with the standards, compounds **10** (Fig. 7b), **12** (Fig. 7c), **16** (Fig. 7e), **18** (Fig. 7g), **19** (Fig. 7h), and **20** (Fig. 7i) were identified as luteoloside, baicalin, luteolin, baicalein, wogonin, and oroxylin A, respectively (see Fig. 7).

Conclusion

A simple and efficient HPLC-DAD-ESI-MS/MS method was developed to evaluate the quality of YH preparations by combining fingerprint analysis, quantification of nine compounds, and identification of 18 compounds. A total of 12 batches of YH preparations, including granule, capsule, and lozenge, from different sources were identified and distinguished by the chromatographic fingerprint in combination with similarity analysis. The method offered good linearity, precision, repeatability, stability, and recovery. Data analysis on the samples suggested that the concentration of the nine compounds varied significantly from different locations of China. Furthermore, a total of 18 compounds were identified or tentatively characterized by comparing their retention times and MS spectra with those of authentic compounds or literature data. The present study could provide comprehensive information for pharmacological research, clinical applications, and quality evaluation of YH preparations.

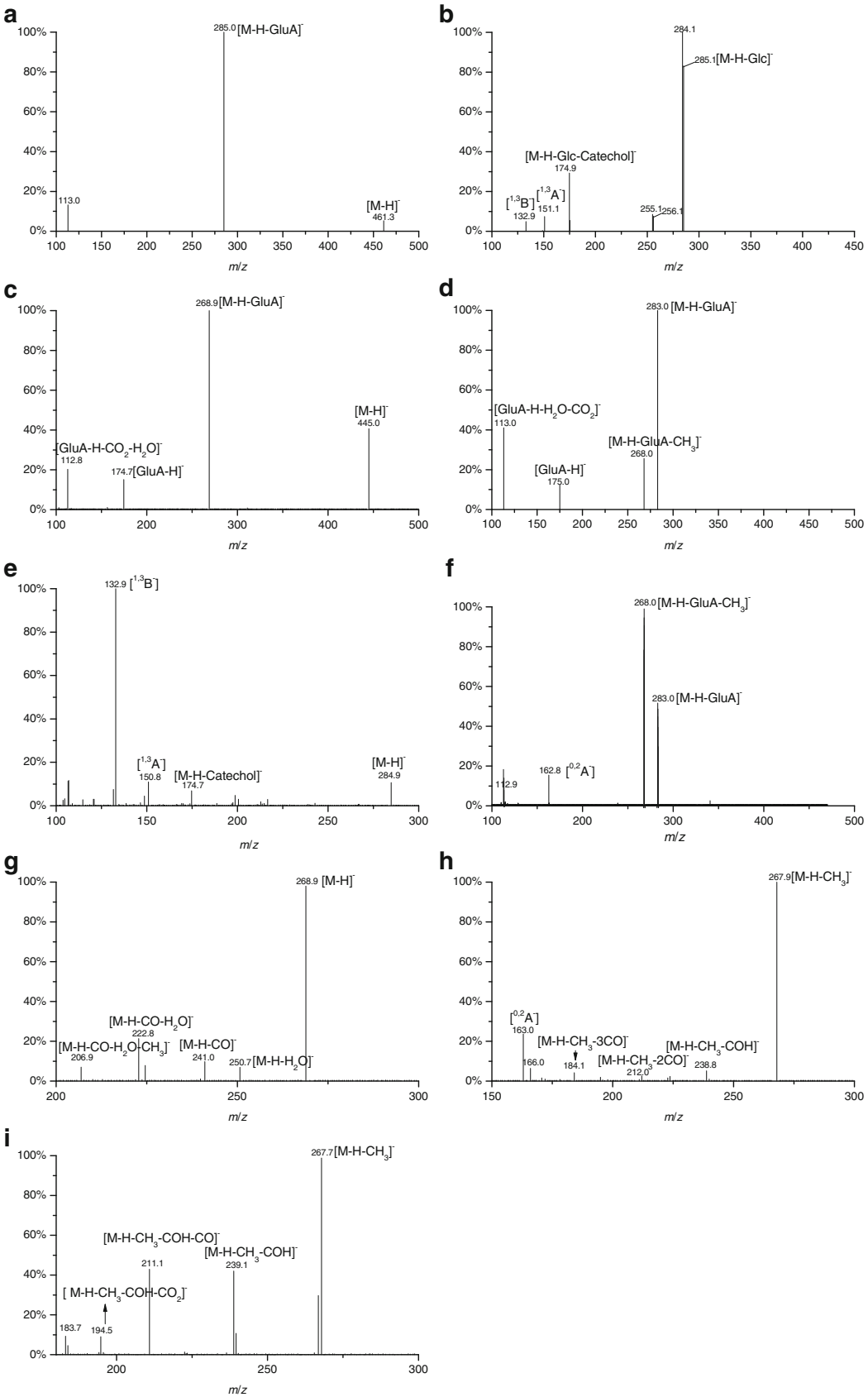


Fig. 7 Negative MS/MS spectra of nine flavonoids identified in YH preparations: **a** scutellarin, **b** luteoloside, **c** baicalin, **d** luteolin, **e** oroxylin A-7-*O*-glucuronide, **f** wogonoside, **g** baicalein, **h** wogonin, **i** oroxylin A

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References

1. Huang ZL, Huang XY, Zheng Q (2010) Medical Recapitulate 16:386–388
2. Ma HD, Shi GB (2009) Pharmaceut Care Res 9:374–376
3. Li P, Qi LW, Wen XD, Sheng LH (2007) Chin J Nat Med 5:1–9
4. Su W, Xu ZY (2007) Shanghai J Tradit Chin Med 41(9):87–88
5. Li YF, Liu L, Cheng YY (2007) Chromatographia 65:749–755
6. He Q, Hu XJ, Cheng YY (2006) J Pharm Biomed Anal 41:485–492
7. Xie J, Wang W, Zhang Y, Bai Y, Yang Q (2007) J Pharm Biomed Anal 45:450–455
8. Liu AH, Lin YH, Yang M, Guo H, Guan SH, Sun JH, Guo DA (2007) J Chromatogr B 846:32–41
9. FDA (2000) Guidance for Industry. Botanical drug products (draft guidance). FDA, Rockville
10. EMEA (2006) Guidance on quality of herbal medicinal products. European Medicines Agency, London
11. State Food and Drug Administration of China (2002) Requirements for studying fingerprints of traditional Chinese medicine injection. SFDA, Beijing
12. Xie B, Gong T, Tang M, Mi D, Zhang X, Liu J, Zhang Z (2008) J Pharm Biomed Anal 48:1261–1266
13. Xu CJ, Liang YZ, Chau FT, Heyden YV (2006) J Chromatogr A 1134(1–2):253–259
14. Xu L, Han X, Qi Y, Xu Y, Yin L, Peng J, Liu K, Sun C (2009) Anal Chim Acta 633:136–148
15. Li Y, Wu T, Zhu J, Wan L, Yu Q, Li X, Cheng Z, Guo C (2010) J Pharm Biomed Anal 52:597–602
16. Duckstein SM, Stintzing FC (2011) Anal Bioanal Chem 401:677–688
17. Wei H, Sun L, Tai Z, Gao S, Xu W, Chen W (2010) Anal Chim Acta 662(1):97–104
18. Han J, Ye M, Guo H, Yang M, Wang BR, Guo DA (2007) J Pharm Biomed Anal 44:430–438
19. Zhang W, Saif MW, Dutschman GE, Li X, Lam W, Bussom S, Jiang Z, Ye M, Chu E, Cheng YC (2010) J Chromatogr A 1217 (37):5785–5793
20. He WY, Gao RM, Li XQ, Jiang JD, Li YH (2010) Yao Xue Xue Bao 45:395–398
21. Li XQ, Sun XH, Cai S, Ying XX, Li FM (2009) Yao Xue Xue Bao 44(8):895–904
22. Han J, Ye M, Xu M, Sun J, Wang B, Guo D (2007) J Chromatogr B 848:355–362
23. National Pharmacopoeia Committee (2010) Chinese pharmacopoeia, vol 1. China Medical Science Press, Beijing
24. Liu EH, Liu Q, Chu C, Li P (2011) J Sep Sci 34:2566–2575
25. Bai Y, Zhang W, Wang X (2010) Zhongguo Zhong Yao Za Zhi 35:166–168
26. Yang X, Han FM, Cheng ZY, Chen Y (1999) Se Pu 17:573–575
27. ICH (1996) Guidance for Industry. Q2B validation of analytical procedures: methodology. ICH, Rockville
28. Ohmishi M, Morishita H, Iwahashi H (1994) Phytochemistry 36:579–583
29. Sakuma H, Matsushima S, Munakata S, Sugawara S (1982) Agric Biol Chem 46:1311–1317
30. Paganga G, Miller N, Rice-Evans CA (1999) Free Radic Res 30:153–162
31. Yan X, Suzuki M, Ohnishi-Kameyama M, Sada Y, Nakanishi T, Nagata T (1999) J Agric Food Chem 47:4711–4713
32. Yu J, Vasanthan T, Temelli F (2001) J Agric Food Chem 49:4352–4358
33. Deyama T, Ikawa T, Kigagawa S, Nishibe S (1987) Chem Pharm Bull 35:1785–1789
34. Schram K, Miketova P, Slanina J, Humpa O, Taborska E (2004) J Mass Spectrom 39:384–395
35. Ye M, Yan Y, Guo DA (2005) Rapid Commun Mass Spectrom 19:1469–1484
36. Löffler C, Sahm A, Wray V, Czygan FC, Proksch P (1995) Biochem Syst Ecol 23:121–128
37. Song Y, Li SL, Wu MH, Li HJ, Li P (2006) Anal Chim Acta 564:211–218
38. Li XQ, Sun XH, Cai S, Ying XX, Li FM (2009) Yao Xue Xue Bao 44:895–904
39. Horvath CR, Martos PA, Saxena PK (2005) J Chromatogr A 1062:199–207
40. Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J (2001) J Am Soc Mass Spectrom 12:707–715