Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1070 (2005) 43–48
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Quality evaluation of *Flos Lonicerae* through a simultaneous determination of seven saponins by HPLC with ELSD

Xing-Yun Chai [a], Song-Lin Li [b], Ping Li [a,*]

[a] *Key Laboratory of Modern Chinese Medicines and Department of Pharmacognosy, China Pharmaceutical University, No. 24, Tongjia Lane, Nanjing 210009, People's Republic of China*
[b] *Institute of Nanjing Military Command for Drug Control, Nanjing 210002, People's Republic of China*

Received 19 November 2004; received in revised form 29 January 2005; accepted 9 February 2005
Available online 24 February 2005

Abstract

A new HPLC coupled with evaporative light scattering detection (ELSD) method has been developed for the simultaneous quantitative determination of seven major saponins, namely macranthoidin B (**1**), macranthoidin A (**2**), dipsacoside B (**3**), hederagenin-28-*O*-β-D-glucopyranosyl(6→1)-*O*-β-D-glucopyranosyl ester (**4**), macranthoside B (**5**), macranthoside A (**6**), and hederagenin-3-*O*-α-L-arabinopyranosyl(2→1)-*O*-α-L-rhamnopyranoside (**7**) in *Flos Lonicerae*, a commonly used traditional Chinese medicine (TCM) herb. Simultaneous separation of these seven saponins was achieved on a C_{18} analytical column. The mobile phase consisted of (A) acetonitrile–acetic acid (95:0.5) and (B) 0.5% aqueous acetic acid using a gradient elution of 29%A at 0–10 min, 29–46%A at 10–25 min and 46%A at 25–30 min. The drift tube temperature of ELSD was set at 106 °C, and with the nitrogen flow-rate of 2.6 l/min. All calibration curves showed good linear regression ($r^2 > 0.9922$) within test ranges. This method showed good reproducibility for the quantification of these seven saponins in *Flos Lonicerae* with intra- and inter-day variations of less than 3.0% and 6.0%, respectively. The validated method was successfully applied to quantify seven saponins in five sources of *Flos Lonicerae*, which provides a new basis of overall assessment on quality of *Flos Lonicerae*.

Keywords: HPLC-ELSD; *Flos Lonicerae*; Saponins; Quantification

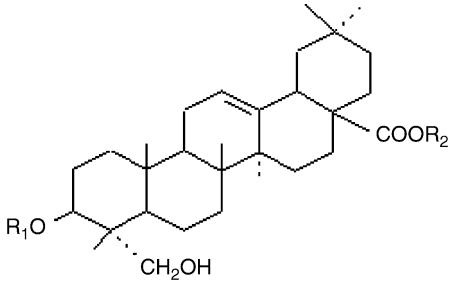
1. Introduction

Flos Lonicerae (Jinyinhua in Chinese), the dried buds of several species of the genus *Lonicera* (*Caprifoliaceae*), is a commonly used traditional Chinese medicine (TCM) herb. It has been used for centuries in TCM practice for the treatment of sores, carbuncles, furuncles, swelling and affections caused by exopathogenic wind-heat or epidemic febrile diseases at the early stage [1]. Though four species of *Lonicera* are documented as the sources of *Flos Lonicerae* in China Pharmacopeia (2000 edition), i.e. *L. japonica*, *L. hypoglauca*, *L. daystyla* and *L. confusa*, other species such as *L. similes* and *L. macranthoides* have also been used on the same purpose in some local areas in China [2]. So it is an important issue to comprehensively evaluate the different sources of *Flos Lonicerae*, so as to ensure the clinical efficacy of this Chinese herbal drug.

Chemical and pharmacological investigations on *Flos Lonicerae* resulted in discovering several kinds of bioactive components, i.e. chlorogenic acid and its analogues, flavonoids, iridoid glucosides and triterpenoid saponins [3]. Previously, chlorogenic acid has been used as the chemical marker for the quality evaluation of *Flos Lonicerae*, owing to its antipyretic and antibiotic property as well as its high content in the herb. But this compound is not a characteristic component of *Flos Lonicerae*, as it has also been used as the chemical marker for other Chinese herbal drugs such as *Flos Chrysanthemi* and so on [4,5]. Moreover, chlorogenic acid alone could not be responsible for the overall pharmacological activities of *Flos Lonicerae* [6]. On the other hand, many studies revealed that triterpenoidal saponins of *Flos Lonicerae* possess protection effects on hepatic injury caused

* Corresponding author. Tel.: +86 25 8324 2299/8539 1244/13505142632; fax: +86 25 8532 2747.
E-mail address: lipingli@publicl.ptt.js.cn (P. Li).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.02.031



Compound	R_1	R_2
1	Ara(2-1)rha(3-1)glc(4-1)glc	Glc(6-1)glc
2	Ara(2-1)rha(3-1)glc	Glc(6-1)glc
3	Ara(2-1)rha	Glc(6-1)glc
4	H	Glc(6-1)glc
5	Ara(2-1)rha(3-1)glc(4-1)glc	H
6	Ara(2-1)rha(3-1)glc	H
7	Ara(2-1)rha	H

Fig. 1. Chemical structures of seven saponins from *Lonicera confusa* macranthoidin B (**1**), macranthoidin A (**2**), dipsacoside B (**3**), hederagenin-28-*O*-β-D-glucopyranosyl(6→1)-*O*-β-D-glucopyranosyl ester (**4**), macranthoside B (**5**), macranthoside A (**6**), and hederagenin-3-*O*-α-L-arabinopyranosyl(2→1)-*O*-α-L-rhamnopyranoside (**7**).

by Acetaminophen, Cd, and CCl_4, and conspicuous depressant effects on swelling of ear croton oil [7–11]. Therefore, saponins should also be considered as one of the markers for quality control of *Flos Lonicerae*. Consequently, determinations of all types of components such as chlorogenic acid, flavonoids, iridoid glucosides and triterpenoidal saponins in *Flos Lonicerae* could be a better strategy for the comprehensive quality evaluation of *Flos Lonicerae*.

Recently an HPLC-ELSD method has been established in our laboratory for qualitative and quantitative determination of iridoid glucosides in *Flos Lonicerae* [12]. But no method was reported for the determination of triterpenoidal saponins in *Flos Lonicerae*. As a series studies on the comprehensive evaluation of *Flos Lonicerae*, we report here, for the first time, the development of an HPLC-ELSD method for simultaneous determination of seven triterpenoidal saponins in the Chinese herbal drug *Flos Lonicerae*, i.e. macranthoidin B (**1**), macranthoidin A (**2**), dipsacoside B (**3**), hederagenin-28-*O*-β-D-glucopyranosyl(6→1)-*O*-β-D-glucopyranosyl ester (**4**), macranthoside B (**5**), macranthoside A (**6**), and hederagenin-3-*O*-α-L-arabinopyranosyl(2→1)-*O*-α-L-rhamnopyranoside (**7**) (Fig. 1).

2. Experimental

2.1. Samples, chemicals and reagents

Five samples of *Lonicera* species, *L. japonica* from Mi county, HeNan province (LJ1999-07), *L. hypoglauca* from Jiujang county, JiangXi province (LH2001-06), *L. similes* from Fei county, ShanDong province (LS2001-07), *L. confusa* from Xupu county, HuNan province (LC2001-07), and *L. macranthoides* from Longhu county, HuNan province (LM2000-06), respectively, were collected in China. All samples were authenticated by Dr. Ping Li, professor of department of Pharmacognosy, China Pharmaceutical University, Nanjing, China. The voucher specimens were deposited in the department of Pharmacognosy, China Pharmaceutical University, Nanjing, China.

Seven saponin reference compounds: macranthoidin B (**1**), macranthoidin A (**2**), dipsacoside B (**3**), hederagenin-28-*O*-β-D-glucopyranosyl(6→1)-*O*-β-D-glucopyranosyl ester (**4**), macranthoside B (**5**), macranthoside A (**6**), and hederagenin-3-*O*-α-L-arabinopyranosyl(2→1)-*O*-α-L-rhamnopyranoside (**7**) were isolated previously from the dried buds of *L. confusa* by repeated silica gel, sephadex LH-20 and Rp-18 silica gel column chromatography, their structures were elucidated by comparison of their spectral data (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) with references [13–15]. The purity of these saponins were determined to be more than 98% by normalization of the peak areas detected by HPLC with ELSD, and showed very stable in methanol solution.

HPLC-grade acetonitrile from Merck (Darmstadt, Germany), the deionized water from Robust (Guangzhou, China), were purchased. The other solvents, purchased from Nanjing Chemical Factory (Nanjing, China) were of analytical grade.

2.2. Apparatus and chromatographic conditions

Aglient 1100 series HPLC apparatus was used. Chromatography was carried out on an Aglient Zorbax SB-C_{18} column (250 × 4.6 mm, 5.0 μm) at a column temperature of 25 °C. A Rheodyne 7125i sampling valve (Cotati, USA) equipped with a sample loop of 20 μl was used for sample injection. The analog signal from Alltech ELSD 2000 (Alltech, Deerfield, IL, USA) was transmitted to a HP Chemstation for processing through an Agilent 35900E (Agilent Technologies, USA).

The optimum resolution was obtained by using a linear gradient elution. The mobile phase consisted of (A) acetonitrile–acetic acid (95:0.5) and (B) 0.5% aqueous acetic acid using a gradient elution of 29%A at 0–10 min, 29–46%A at 10–25 min and 46%A at 25–30 min. The drift tube temperature for ELSD was set at 106 °C and the nitrogen flow-rate was of 2.6 l/min. The chromatographic peaks were identified by comparing their retention time with that of each reference compound which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference compounds further confirmed the identities of the peaks.

2.3. Calibration curves

Methanol stock solutions containing seven analytes were prepared and diluted to appropriate concentration for the con-

Table 1
Calibration curves for seven saponins

Analytes	Calibration curve[a]	r^2	Test range (μg)	LOD (μg)	LOQ (μg)
1	$y = 6711.9x - 377.6$	0.9940	0.56–22.01	0.26	0.88
2	$y = 7812.6x - 411.9$	0.9922	0.54–21.63	0.26	0.84
3	$y = 6798.5x - 299.0$	0.9958	0.46–18.42	0.22	0.72
4	$y = 12805x - 487.9$	0.9961	0.38–15.66	0.10	0.34
5	$y = 4143.8x - 88.62$	0.9989	0.42–16.82	0.18	0.24
6	$y = 3946.8x - 94.4$	0.9977	0.40–16.02	0.16	0.20
7	$y = 4287.8x - 95.2$	0.9982	0.42–16.46	0.12	0.22

[a] y: Peak area; x: concentration (mg/ml).

struction of calibration curves. Six concentration of the seven analytes' solution were injected in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The results were demonstrated in Table 1.

2.4. Limits of detection and quantification

Methanol stock solution containing seven reference compounds were diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions were injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. LOD and LOQ for each compound were shown in Table 1.

2.5. Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. Approximately 2.0 g of the pulverized samples of *L. macranthoides* were weighed, extracted and analyzed as described in Section 2.6. For intra-day variability test, the samples were analyzed in triplicate for three times within one day, while for inter-day variability test, the samples were examined in triplicate for consecutive three days. Variations were expressed by the relative standard deviations. The results were given in Table 2.

Recovery test was used to evaluate the accuracy of this method. Accurate amounts of seven saponins were added to approximate 1.0 g of *L. macranthoides*, and then extracted and analyzed as described in Section 2.6. The average recoveries were counted by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%, and RSD (%) = (SD/mean) × 100%. The results were given in Table 3.

2.6. Sample preparation

Samples of *Flos Lonicerae* were dried at 50 °C until constant weight. Approximately 2.0 g of the pulverized samples, accurately weighed, was added to a round-bottomed flask containing 25 ml of 60% aqueous ethanol and the mixture

Table 2
Precision of the assay

Analyte	Intra-day variability			Inter-day variability		
	Content (mg/g)	Mean	RSD (%)	Content (mg/g)	Mean	RSD (%)
1	46.16 46.28 46.22	46.22	0.13	46.22 45.36 47.42	26.33	2.23
2	5.38 5.38 5.16	5.31	2.40	5.28 5.31 5.04	5.22	3.04
3	4.37 4.30 4.18	4.28	2.24	4.28 4.46 4.02	4.25	5.20
4	nd[a]	–	–	nd	–	–
5	1.76 1.80 1.82	1.79	1.70	1.79 1.68 1.84	1.77	4.70
6	1.28 1.24 1.22	1.25	2.45	1.25 1.34 1.20	1.26	5.72
7	tr[b]	–	–	tr	–	–

RSD (%) = (SD/mean) × 100%.
[a] Not detected.
[b] Trace.

Table 3
Recovery of the seven analytes

Analyte	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	Mean (%)	RSD (%)
1	23.08	19.71	42.73	99.7	99.8	0.7
	23.14	22.86	46.13	100.6		
	23.11	28.10	51.01	99.3		
2	2.69	2.08	4.73	98.1	98.8	1.6
	2.67	2.91	5.51	97.6		
	2.58	3.16	5.76	100.6		
3	2.17	1.73	3.88	98.8	99.9	2.9
	2.15	2.18	4.40	103.2		
	2.09	2.62	4.65	97.7		
4	nd[a]	1.01	0.98	97.0	102.0	4.2
		1.05	1.10	104.8		
		0.98	1.02	104.1		
5	0.88	0.70	1.56	97.1	98.9	2.6
	0.90	0.87	1.75	97.7		
	0.91	1.08	2.01	101.8		
6	0.64	0.45	1.08	97.7	96.8	0.9
	0.62	0.61	1.21	96.7		
	0.61	0.75	1.33	96.0		
7	tr[b]	1.02	1.03	100.9	100.9	1.8
		1.10	1.11	102.7		
		1.08	1.07	99.1		

Recovery (%) = (amount found − original amount)/amount spiked × 100%, RSD (%) = (SD/mean) × 100%.
[a] Not detected.
[b] Trace.

was heated under reflux for 4 h. The ethanol was evaporated to dryness with a rotary evaporator. Residue was dissolved in water, followed by defatting with 60 ml of petroleum ether for two times, and then the water solution was evaporated, residue was dissolved with methanol into a 25 ml flask. One ml of the methanol solution was drawn and transferred to a 5 ml flask, diluted to the mark with methanol. The resultant solution was at last filtrated through a 0.45 μm syringe filter (Type Millex-HA, Millipore, USA) and 20 μl of the filtrate was injected to HPLC system. The contents of the analytes were determined from the corresponding calibration curves.

3. Results and discussions

The temperature of drift tube and the gas flow-rate are two most important adjustable parameters for ELSD, they play a prominent role to an analyte response. In our previous work [12], the temperature of drift tube was optimized at 90 °C for the determination of iridoids. As the polarity of saponins are higher than that of iridoids, more water was used in the mobile phase for the separation of saponins, therefore the temperature for saponins determination was optimized systematically from 95 °C to 110 °C, the flow-rate from 2.2 l/min to 3.0 l/min. Dipsacoside B was selected as the testing saponin for optimizing ELSD conditions, as it was contained in all samples. Eventually, the drift tube temperature of 106 °C and a gas flow of 2.6 l/min were optimized to detect the analytes. And these two exact experimental parameters should be strictly controlled in the analytical procedure [16].

All calibration curves showed good linear regression ($r^2 > 0.9922$) within test ranges. Validation studies of this method proved that this assay has good reproducibility. As shown in Table 2, the overall intra- and inter-day variations are less than 6% for all seven analytes. As demonstrated in Table 3, the developed analytical method has good accuracy with the overall recovery of high than 96% for the analytes concerned. The limit of detection (S/N = 3) and the limit of quantification (S/N = 10) are less than 0.26 μg and 0.88 μg on column, respectively (Table 1), indicating that this HPLC-ELSD method is precise, accurate and sensitive enough for the quantitative evaluation of major non-chromaphoric saponins in *Flos Lonicerae*.

It has been reported that there are two major types of saponins in *Flos Lonicerae*, i.e. saponins with hederagenin as aglycone and saponins with oleanolic acid as the aglycone [17]. But hederagenin type saponins of the herb were reported to have distinct activities of liver protection and anti-inflammatory [7–11]. So we adopted seven hederagenin type saponins as representative markers to establish a quality control method.

The newly established HPLC-ELSD method was applied to analyze seven analytes in five plant sources of *Flos Lonicerae*, i.e. *L. japonica*, *L. hypoglauca*, *L. confusa*, *L. sim-*

Table 4
Contents of seven saponins in *Lonicera* spp.

	Content (mg/g)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
L. confusa	45.65 ± 0.32	5.13 ± 0.08	4.45 ± 0.11	tr[a]	2.04 ± 0.04	tr	1.81 ± 0.03
L. japonica	nd[b]	nd	3.44 ± 0.09	nd	nd	nd	nd
L. macranthoides	46.22 ± 0.06	5.31 ± 0.13	4.28 ± 0.10	tr	1.79 ± 0.03	1.25 ± 0.03	tr
L. hypoglauca	11.17 ± 0.07	nq[c]	53.78 ± 1.18	nd	1.72 ± 0.02	2.23 ± 0.06	2.52 ± 0.04
L. similes	41.22 ± 0.25	4.57 ± 0.07	3.79 ± 0.09	nd	1.75 ± 0.02	tr	nd

[a] Trace.
[b] Not detected.
[c] Not quantified owing to the suspicious purity of the peak.

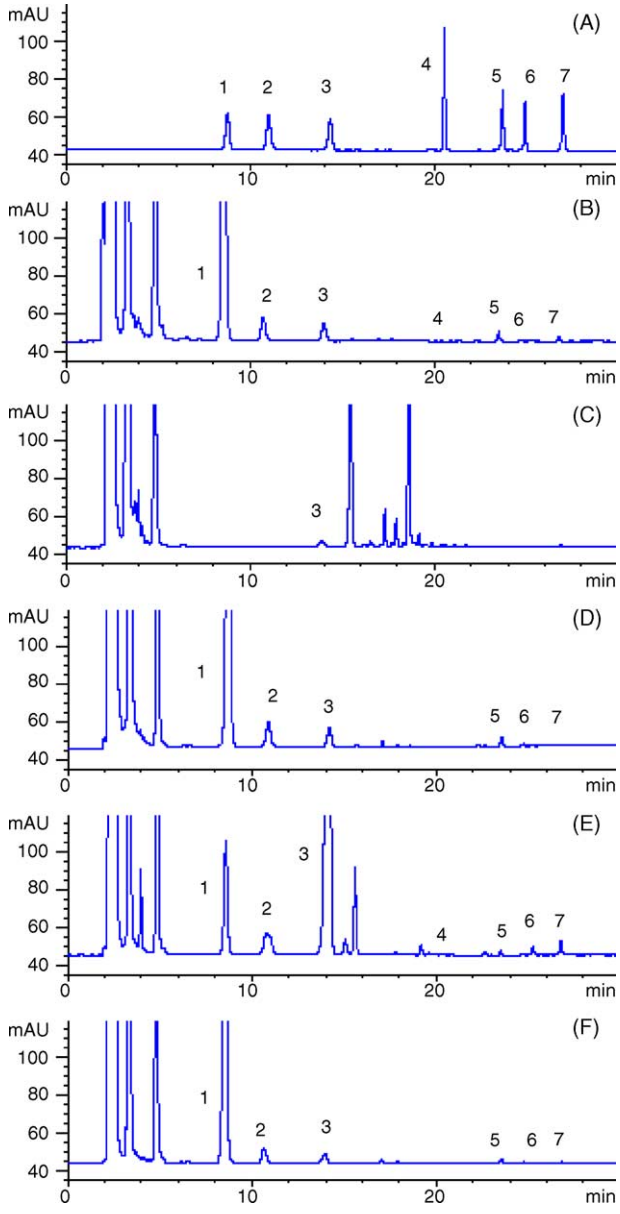


Fig. 2. Representative HPLC chromatograms of mixed standards and methanol extracts of *Flos Lonicerae*. Column: Agilent Zorbax SB-C_{18} column (250 × 4.6 mm, 5.0 μm), temperature of 25 °C; detector: ELSD, drift tube temperature 106 °C, nitrogen flow-rate 2.6 l/min. (A) Mixed standards, (B) *L. confusa*, (C) *L. japonica*, (D) *L. macranthoides*, (E) *L. hypoglauca*, (F) *L. similes*.

iles and *L. macranthoides* (Table 4). It was found that there were remarkable differences of seven saponins contents between different plant sources of *Flos Lonicerae*. All seven saponins analyzed could be detected in *L. confusa* and *L. hypoglauca*, while only dipsacoside B was detected in *L. japonica*. Among all seven saponins interested, only dipsacoside B was found in all five plant species of *Flos Lonicerae* analyzed, and this compound was determined as the major saponin with content of 53.7 mg/g in *L. hypoglauca*. On the other hand, macranthoidin B was found to be the major saponin with the content higher than 41.0 mg/g in *L. macranthoides*, *L. confusa*, and *L. similis*, while the contents of other analytes were much lower.

In our previous study [12], overall HPLC profiles of iridoid glucosides was used to qualitatively and quantitatively distinguish different origins of *Flos Lonicerae*. As shown in Fig. 2, the chromatogram profiles of *L. confusa*, *L. japonica* and *L. similes* seem to be similar, resulting in the difficulty of clarifying the origins of *Flos Lonicerae* solely by HPLC profiles of saponins, in addition to the clear difference of the HPLC profiles of saponins from *L. macranthoides* and *L. hypoglauca*. Therefore, in addition to the conventional morphological and histological identification methods, the contents and the HPLC profiles of saponins and iridoids could also be used as accessory chemical evidence to clarify the botanical origin and comprehensive quality evaluation of *Flos Lonicerae*.

4. Conclusions

This is the first report on validation of an analytical method for qualification and quantification of saponins in *Flos Lonicerae*. This newly established HPLC-ELSD method can be used to simultaneously quantify seven saponins, i.e. macranthoidin B, macranthoidin A, dipsacoside B, hederagenin-28-*O*-β-D-glucopyranosyl(6→1)-*O*-β-D-glucopyranosyl ester, macranthoside B, macranthoside A, and hederagenin-3-*O*-α-L-arabinopyranosyl(2→1)-*O*-α-L-rhamnopyranoside in *Flos Lonicerae*. As the saponin profile alone does not allow the clear distinction of the botanical origin, the results of other conventional methods or the iridoid profile have to be evaluated as well.

Acknowledgements

This project is financially supported by Fund for Distinguished Chinese Young Scholars of the National Science Foundation of China (30325046) and the National High Tech Program (2003AA2Z2010).

References

[1] Ministry of Public Health of the People's Republic of China, Pharmacopoeia of the People's Republic of China, vol. 1, 2000, p. 177.
[2] W. Shi, R.B. Shi, Y.R. Lu, Chin. Pharm. J. 34 (1999) 724.
[3] J.B. Xing, P. Li, D.L. Wen, Chin. Med. Mater. 26 (2001) 457.
[4] Y.Q. Zhang, L.C. Xu, L.P. Wang, J. Chin. Med. Mater. 21 (1996) 204.
[5] D. Zhang, Z.W. Li, Y. Jiang, J. Pharm. Anal. 16 (1996) 83.
[6] T.Z. Wang, Y.M. Li, Huaxiyaoxue Zazhi 15 (2000) 292.
[7] J.Z.H. Shi, G.T. Liu, Acta Pharm. Sin. 30 (1995) 311.
[8] Y.P. Liu, J. Liu, X.S.H. Jia, et al., Acta Pharmacol. Sin. 13 (1992) 209.
[9] Y.P. Liu, J. Liu, X.S.H. Jia, et al., Acta Pharmacol. Sin. 13 (1992) 213.
[10] J.Z.H. Shi, L. Wan, X.F. Chen, ZhongYao YaoLi Yu LinChuang 6 (1990) 33.
[11] J. Liu, L. Xia, X.F. Chen, Acta Pharmacol. Sin. 9 (1988) 395.
[12] H.J. Li, P. Li, W.C. Ye, J. Chromatogr. A 1008 (2003) 167.
[13] Q. Mao, D. Cao, X.S.H. Jia, Acta Pharm. Sin. 28 (1993) 273.
[14] H. Kizu, S. Hirabayashi, M. Suzuki, et al., Chem. Pharm. Bull. 33 (1985) 3473.
[15] S. Saito, S. Sumita, N. Tamura, et al., Chem. Pharm. Bull. 38 (1990) 411.
[16] Alltech ELSD 2000 Operating Manual, Alltech, 2001, p. 16 (in Chinese).
[17] J.B. Xing, P. Li, Chin. Med. Mater. 22 (1999) 366.