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Journal of Chromatography A, 1052 (2004) 223–227
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Preparative isolation and purification of coumarins from *Angelica dahurica* (*Fisch. ex Hoffm*) *Benth, et Hook. f* (Chinese traditional medicinal herb) by high-speed counter-current chromatography

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Received 4 June 2004; received in revised form 8 July 2004; accepted 27 August 2004

Abstract

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of coumarins from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f* (Baizhi in Chinese) was successfully established by using *n*-hexane–methanol–water as the two-phase solvent system in gradient elution mode. The upper phase of *n*-hexane–methanol–water (5:5:5, v/v) was used as the stationary phase of HSCCC. The mobile phase used in HSCCC was the lower phase of *n*-hexane–methanol–water (5:5:5, v/v) and *n*-hexane–methanol–water (5:7:3, v/v) that was changed in gradient. Three major components including imperatorin, isoimperatorin and oxypeucedanine were isolated, each at over 98% purity as determined by high-performance liquid chromatography (HPLC). The peak fractions of HSCCC were identified by ^{1}H-NMR and ^{13}C-NMR.

Keywords: High-speed counter-current chromatography; *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f*; Imperatorin; Isoimperatorin; Oxypeucedanine

1. Introduction

Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f (Baizhi in Chinese) has been widely used in traditional Chinese medicine as a common acesodyne. It has strong effects on headache and toothache [1]. In addition, it can be used to treat cough, asthma, coryza, hypertension, vitiligo, psoriasis, acne, herpes zoster, freckle, etc. [2]. The major effective components of this herb are coumarins including imperatorin, isoimperatorin and oxypeucedanine. Pharmacological studies and clinical practice demonstrated that they have remarkable anticancer, antibacterial, and codein effects [3]. They are often used as reference standards in the quality control of Baizhi and its products. So high-purity preparation of them is of great interest. The chemical structures of these compounds are given in Fig. 1.

High-speed counter-current chromatography (HSCCC), which was first invented by Y. Ito [4], is a kind of liquid–liquid partition chromatography. The stationary phase of this method is also a liquid. It is retained in the separation column by centrifugal force. Because no solid support is used in the separation column, HSCCC successfully eliminates irreversible adsorption loss of samples onto the solid support used in conventional chromatographic column [5]. As an advanced separation technique, it offers various advantages including high sample recovery, high-purity of fractions, and high-loading capacity [6]. So it is suitable for separation of active components from traditional Chinese herbs and other natural products. In the past 30 years, HSCCC has gained great progress in preparation of various reference standards for pharmacological studies and good manufacturing practice, such as coumarins [7–9], alkaloids [10–12], flavonoids [13–15], hydroxyanthraquiones [16–18] and dyes [19,20].

The present paper describes HSCCC separation of imperatorin, isoimperatorin and oxypeucedanine from aether

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doi:10.1016/j.chroma.2004.08.127

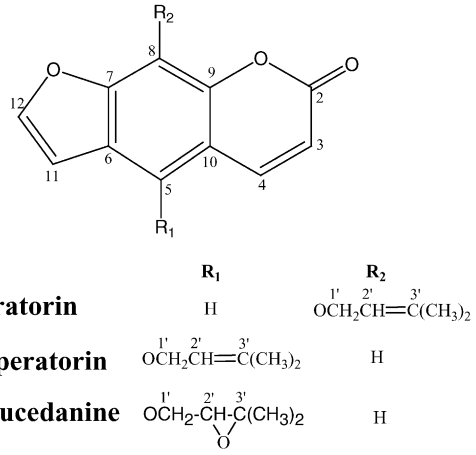

Fig. 1. Chemical structures of imperatorin, isoimperatorin and oxypeucedanine.

crude extract of *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f*. High-performance liquid chromatography (HPLC) was used for selecting two-phase solvent system. The upper phase of *n*-hexane–methanol–water (5:5:5, v/v) was used as the stationary phase of HSCCC. The mobile phase used in HSCCC was the lower phase of *n*-hexane–methanol–water (5:5:5, v/v) and *n*-hexane–methanol–water (5:7:3, v/v) that was changed in gradient. Three major components including imperatorin, isoimperatorin and oxypeucedanine were isolated, each at over 98% purity as determined by HPLC.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 260 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the β values of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co., Ltd., Beijing, China) was used to control the separation temperature. An ÄKTAprime (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co., Ltd., Hangzhou, China).

The HPLC equipment used was Agilent1100 system including a G1311A QuatPump, a G1315B DAD, a 7725i injection valve with a 20 μl loop, a G1332A degasser and Agilent HPLC workstation.

Nuclear magnetic resonance (NMR) spectrometer used here was Mercury Plus 400 NMR (Varian Inc., America).

2.2. Reagents

All solvents used for preparation of crude extract and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water used was distilled water.

Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f was purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of crude extract

Preparation of crude extract was carried out according to the literature [21]. The dried roots of *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f* were ground to powder (about 30 mesh). The powder (100 g) was extracted with aether at 40 °C for 2 h four times (200 ml of aether each time). The extracts were combined and evaporated under reduced pressure. 1.0 g of powder was obtained. It was stored in a refrigerator (4 °C) for subsequent HSCCC separation.

2.4. Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficients (K) of the target components. The K values were determined by HPLC analysis. Suitable amount of crude extract was dissolved in the upper phase. The solution was determined by HPLC. The peak area was recorded as A_1. Then equal volume of the lower phase was added to the solution and mixed thoroughly. After partition equilibration, the upper phase solution was determined by HPLC again, and the peak area was recorded as A_2. The partition coefficient (K) was obtained by the following equation: $K = A_2/A_1 - A_2$.

2.5. Preparation of two-phase solvent system and sample solution

In the present study, the two-phase solvent system composed of *n*-hexane–methanol–water at various volume ratios were used for HSCCC separation. The upper phase of *n*-hexane–methanol–water (5:5:5, v/v) was used as the stationary phase. The lower phases of *n*-hexane–methanol–water (5:5:5, v/v) and *n*-hexane–methanol–water (5:7:3, v/v) were used as the mobile phase in gradient elution. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated and degassed by sonication for 30 min shortly before use.

The sample solution was prepared by dissolving the crude extract in the upper phase of *n*-hexane–methanol–water (5:5:5, v/v) at suitable concentration.

2.6. HSCCC separation procedure

HSCCC was performed with a TBE-300A HSCCC instrument as follows: the upper phase (the stationary phase) and the lower phase of *n*-hexane–methanol–water (5:5:5, v/v) were pumped into the column with volume ratio of 60:40. After the column was totally filled, the apparatus was rotated at 900 rpm. At the same time, the lower phase of *n*-hexane–methanol–water (5:5:5, v/v) was pumped into the column at a flow rate of 2.0 ml min^{-1}. About half an hour later, hydrodynamic equilibrium was reached, and 5 ml of the sample solution containing 100 mg of the crude extract was injected into the column through the injection valve. In the first 150 min, the mobile phase was the lower phase of *n*-hexane–methanol–water (5:5:5, v/v), and in the subsequent 150 min, the volume ratio of the lower phase of *n*-hexane–methanol–water (5:7:3, v/v) was changed linearly from 0 to 100%. All through the experiment the separation temperature was controlled at 20 °C. The effluent from the tail end of the column was continuously monitored with a UV absorbance detector at 254 nm. The data were collected immediately after sample injection. Each fraction was collected manually according to the obtained chromatogram. Each collection was evaporated under reduced pressure and dissolved by methanol for subsequent purity analysis by HPLC.

2.7. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis was performed with a SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. The mobile phase was methanol: water (68:32, v/v). The effluent was monitored at 254 nm and the flow rate was kept at 1.0 ml min^{-1} constantly.

The structure identification of HSCCC peak fractions was carried out by ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Mercury Plus 400 NMR with TMS (for ^{1}H-NMR) and $C^{2}HCl_3$ (for ^{13}C-NMR) as internal standards.

3. Results and discussion

3.1. Optimization of HPLC method

Selection of two-phase solvent system and purity analysis of HSCCC peak fractions were performed by HPLC. So in the first place, a good HPLC method should be developed for the subsequent research. Different kinds of solvent systems were used as the mobile phase to analyze crude extract from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f* by HPLC. The results revealed that when methanol and water (68:32, v/v) were used as the mobile phase, three major peaks can be obtained, and each peak got baseline separation. The peak purity was analyzed with Agilent 1100 workstation. The results were satisfactory. HPLC chromatogram of crude extract from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f* was shown in Fig. 2(A).

3.2. Optimization of HSCCC conditions

Appropriate solvent system plays an important role in separation by HSCCC. Selecting solvent system means choosing the stationary phase and the mobile phase simultaneously. In order to achieve efficient resolution of target compounds, many different solvent systems were examined and the *K* values were shown in Table 1. The results indicated that when *n*-hexane–methanol–water (5:5:5, v/v) was used as two-phase solvent system, imperatorin and oxypeucedanine can be separated, but the elution time of isoimperatorin was too long. When *n*-hexane–methanol–water (5:7:3, v/v) was used, isoimperatorin can be well separated. But imperatorin and oxypeucedanine were eluted together in a poor resolution. When the lower phase of *n*-hexane–methanol–water (5:5:5, v/v) and *n*-hexane–methanol–water (5:7:3, v/v) were used in gradient elution (0–150 min, only the lower phase of *n*-hexane–methanol–water (5:5:5, v/v); 150–300 min, the volume ratio of the lower phase of *n*-hexane–methanol–water (5:7:3, v/v) changed from 0 to 100%), three pure fractions can be obtained and the separation time was acceptable.

The influence of revolution speed, flow rate of the mobile phase, and temperature on the HSCCC separation were also investigated. The results indicated that when the flow rate was 2 ml min^{-1}, revolution speed was 900 rpm, separation temperature was 20 °C, retention percentage of the stationary phase could reach 60% and good separation results could be obtained. Under the optimum conditions, three major peaks were obtained and yielded 29 mg of Peak I (collected during 173–210 min), 35 mg of Peak II (collected during 228–255 min), and 28 mg of Peak III (collected during 330–360 min) from 100 mg of crude extract. The purity of these compounds was 99.1%, 98.3%, and 99.8%, respectively, as determined by HPLC. The HSCCC chromatogram of crude extract from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f* was given in Fig. 3.

3.3. Results of HPLC analysis and identification of HSCCC peak fraction

Each HSCCC peak fraction was analyzed by HPLC. The purity of peak I, peak II and peak III fraction in Fig. 3 was 99.2%, 98.1% and 99.7%, respectively. HPLC chromatograms and UV spectra of the HSCCC peak fractions were shown in Fig. 2(B)–(D).

Identification of each HSCCC fraction was carried out by ^{1}H-NMR and ^{13}C-NMR.

HSCCC peak I in Fig. 3: ^{1}H-NMR (400 MHz, $C^{2}HCl_3$): δ ppm: 7.764 (1H, d, J = 9.6 Hz, C_4–H), 7.691 (1H, d, J

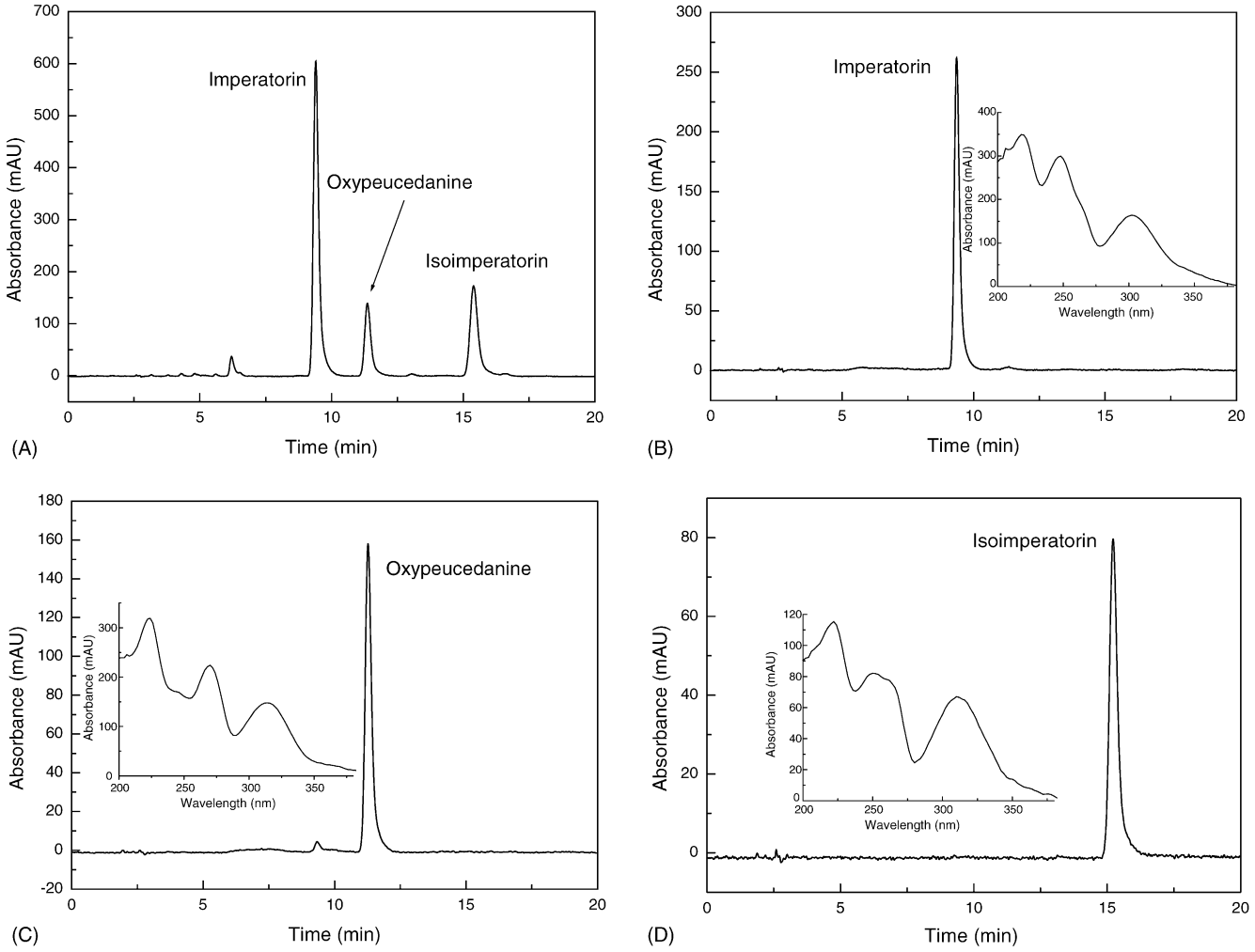

Fig. 2. HPLC chromatograms. (A) crude extract from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f*; (B) HSCCC fraction of peak I; (C) HSCCC fraction of peak II; (D) HSCCC fraction of peak III. Column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D, 5 μm); mobile phase: methanol: water (68:32, v/v); flow rate: 1.0 ml min^{-1}; detection wavelength: 254 nm.

= 2.0 Hz, C_{12}–H), 7.359 (1H, s, C_5–H), 6.814 (1H, d, J = 2.0 Hz, C_{11}–H), 6.370 (1H, d, J = 9.6 Hz, C_3–H), 5.613 (1H, m, C_2'–H), 5.007 (2H, d, J = 7.2 Hz, C_1'–H), 1.744, 1.722 (3H each, s, 2× CH_3); ^{13}C-NMR (400 MHz, C^2HCl_3): δ ppm: 160.507 (C-2), 148.580 (C-7), 146.581 (C-12), 144.308 (C-4), 143.805 (C-9), 139.739 (C-3′), 131.016 (C-8), 125.813 (C-6), 119.716 (C-2′), 116.447 (C-10), 114.668 (C-3), 113.095 (C-5), 106.663 (C-11), 70.121 (C-1′), 25.772 (–CH_3), 18.079 (–CH_3). These data were identical with the literature [22], and peak I was identified as imperatorin.

HSCCC peak II in Fig. 3: ^{1}H-NMR (400 MHz, C^2HCl_3): δ ppm: 8.125 (1H, d, J = 9.6 Hz, C_4–H), 7.625 (1H, d, J = 2.0 Hz, C_{12}–H), 7.10 (1H, broad, C_8–H), 6.997 (1H, d, J = 2.0 Hz, C_{11}–H), 6.286 (1H, d, J = 9.6 Hz, C_3–H), 4.852 (2H, d, J = 7.2 Hz, C_1'–H), 3.661 (1H, t, J = 7.2 Hz, C_2'–H), 1.741, 1.705 (3H each, s, 2× CH_3); ^{13}C-NMR

Table 1
The K (partition coefficient) values of imperatorin, oxypeucedanine and isoimperatorin in *n*-hexane–methanol–water solvent system

n-Hexane–methanol–water (v/v)	K		
	Imperatorin	Oxypeucedanine	Isoimperatorin
5:4:6	5.54	6.62	23.83
5:4.5:5.5	2.92	3.59	11.35
5:5:5	1.64	2.13	6.44
5:6:4	0.57	0.73	2.51
5:7:3	0.31	0.32	0.86

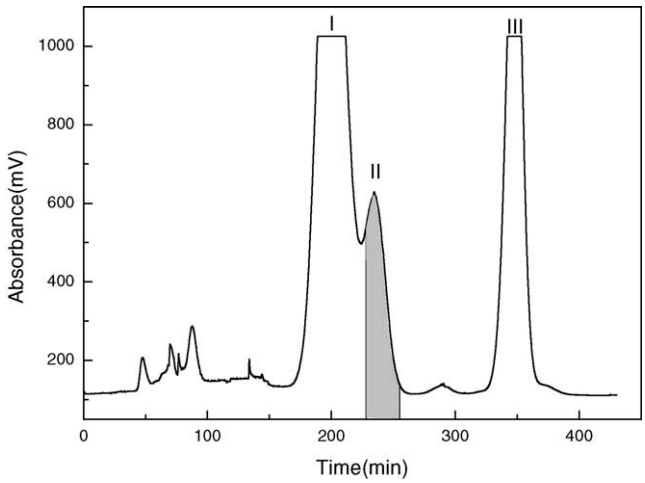

Fig. 3. HSCCC chromatogram of crude extract from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f*. Column: multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 260 ml; stationary phase: the upper phase of *n*-hexane–methanol–water (5:5:5, v/v); mobile phase: the lower phase of *n*-hexane–methanol–water (5:5:5, v/v) and *n*-hexane–methanol–water (5:7:3, v/v) in gradient elution mode (0–150 min, the lower phase of *n*-hexane–methanol–water (5:5:5, v/v); 150–300 min, the volume ratio of the lower phase of *n*-hexane–methanol–water (5:7:3, v/v) from 0 to 100%); flow rate: 2.0 ml min^{-1}; revolution speed: 900 rpm; detection wavelength: 254 nm; separation temperature: 20 °C; sample size: 100 mg of crude sample dissolved in 5 ml of the upper phase of *n*-hexane–methanol–water (5:5:5, v/v); retention percentage of the stationary phase: 60%. I: imperatorin (collected during 173–210 min); II: oxypeucedanine (collected during 228–255 min), III: isoimperatorin (collected during 330–360 min).

(400 MHz, C^2HCl_3): δ ppm: 160.517 (C-2), 158.090 (C-7), 150.761 (C-9), 148.915 (C-5), 145.045 (C-12), 139.357 (C-4), 114.167 (C-6), 112.791 (C-3), 107.492 (C-11), 105.006 (C-10), 94.212 (C-8), 70.340 (C-1′), 60.756 (C-2′), 58.310 (C-3′), 25.560 (–CH_3), 18.037 (–CH_3). Comparing these data with the literature [23], peak II was identified as oxypeucedanine.

HSCCC peak III in Fig. 3: ^{1}H-NMR (400 MHz, C^2HCl_3): δ ppm: 8.164 (1H, d, J = 9.6 Hz, C_4–H), 7.612 (1H, d, J = 2.3 Hz, C_{12}–H), 7.163 (1H, s, C_8–H), 6.958 (1H, d, J = 2.3 Hz, C_{11}–H), 6.275 (1H, d, J = 9.6Hz, C_3–H), 5.541 (1H, t, C_2'–H), 4.922 (2H, d, J = 6.8 Hz, C_1′–H), 1.803, 1.701 (3H each, s, 2× CH_3); ^{13}C-NMR (400 MHz, C^2HCl_3): δ ppm: 161.306 (C-2), 158.090 (C-7), 152.624 (C-9), 148.915 (C-5), 144.855 (C-12), 139.702 (C-11), 139.572 (C-4), 119.039 (C-2′), 114.167 (C-6), 112.532 (C-3), 107.543 (C-10), 105.006 (C-3′), 94.212 (C-8), 69.696 (C-1′), 25.787 (–CH_3), 18.185 (–CH_3). According to the literature [22], peak III was identified as isoimperatorin.

In conclusion, HSCCC was successfully used for the isolation and purification of imperatorin, oxypeucedanine and isoimperatorin from *Angelica dahurica (Fisch. ex Hoffm) Benth, et Hook. f*, which yielded 29 mg of imperatorin, 35 mg of oxypeucedanine, and 28 mg of isoimperatorin from 100 mg of the crude extract in one-step separation.

Acknowledgements

Jichun Cui was grateful for his assistance in structure identification.

References

[1] L.P. Zhang, J. Emerg. Tradit. Chin. Med. 6 (1997) 76.
[2] Q.Zh. Zhang, X.Y. Xu, X.L. Zhang, Chin. J. Ethnomed. Ethnopharm. 2 (2002) 98.
[3] Y.B. Ji, Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine, Heilongjiang Science &Technology Press, Haerbin, 1995, pp. 269, 331 (the first division of 1995 edition).
[4] Y. Ito, J. Chromatogr. 214 (1981) 122.
[5] Y. Ito, Anal. Chem. 17 (1986) 65.
[6] Y. Ma, Y. Ito, E. Sokolosky, H.M. Fales, J. Chromatogr. A 685 (1994) 259.
[7] D.E. Schaufelberger, J. Liq. Chromatogr. 12 (1989) 2263.
[8] D.E. Schaufelberger, Planta. Med. 55 (1989) 584.
[9] F.Q. Yang, Q.Y. Du, W.L. Yu, J. Liq. Chromatogr. 18 (1995) 395.
[10] F.Q. Yang, Y. Ito, J. Chromatogr. A 943 (2002) 219.
[11] F.Q. Yang, Y. Ito, J. Chromatogr. A 923 (2001) 281.
[12] L.M. Yuan, M. Zi, P. Ai, X.X. Chen, Z.Y. Li, R.N. Fu, T.Y. Zhang, J. Chromatogr. A 927 (2001) 91.
[13] X.F. Ma, T.Y. Zhang, Y. Wei, P.F. Tu, Y.J. Chen, Y. Ito, J. Chromatogr. A 962 (2002) 243.
[14] Q.Zh. Du, M. Xia, Y. Ito, J. Chromatogr. A 962 (2002) 239.
[15] X.F. Ma, P.F. Tu, Y.J. Chen, T.Y. Zhang, Y. Wei, Y. Ito, J. Chromatogr. A 992 (2003) 193.
[16] F.Q. Yang, T.Y. Zhang, Y. Ito, J. Chromatogr. A 919 (2001) 443.
[17] F.Q. Yang, T.Y. Zhang, G.L. Tian, H.F. Cao, Q.H. Liu, Y. Ito, J. Chromatogr. A 858 (1999) 103.
[18] F.Q. Yang, T.Y. Zhang, B.X. Mo, L.J. Yang, Y.Q. Gao, Y. Ito, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 209.
[19] A. Weisz, E.P. Mazzola, J.E. Matusik, Y. Ito, J. Chromatogr. A 923 (2001) 87.
[20] H. Oka, K.I. Harada, M. Suzuki, K. Fugii, M. Iwaya, Y. Ito, T. Goto, H. Matsumoto, Y. Ito, J. Chromatogr. A 989 (2003) 249.
[21] M.R. Jia, M.Y. Wang, Q.M. Jin, Y.Y. Ma, Sh.W. Tang, Y. Xiong, G.H. Jiang, West China J. Pharm. Sci. 18 (2003) 361.
[22] D.Ch. Chen, Handbook of Reference Substance for Traditional Chinese Herbs, China Pharmaceutical Technology Publishing House, Beijing, 2000, pp. 92, 113.
[23] Y.S. Li, Zh.W. Lin, J. Yunnan Coll. Tradit. Chin. Med. 19 (1996) 1.