Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1140 (2007) 107–111
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

One-step separation and purification of 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography

Ming Gu *, Xiaolei Wang, Zhiguo Su, Fan Ouyang

National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China

Received 26 September 2006; received in revised form 15 November 2006; accepted 20 November 2006
Available online 4 December 2006

Abstract

Three kinds of polyphenols of *Salvia miltiorrhiza* Bunge, 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde, were separated and purified in one step with solvent system *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1.5:8) by high-speed counter-current chromatography. Acetic acid was successfully used to increase the partition of high polar target compounds in organic phase to modify partition coefficient value. 3,4-Dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde were purified from 100 mg water extracted crude sample of *Salvia miltiorrhiza* Bunge at purity of 97.6%, 94.2% and 98.2% and at yield of 98.6%, 73.6% and 90.2%. High-speed counter-current chromatography together with organic/aqueous solvent system supplied an efficient method to purify water-soluble compounds directly from crude samples of traditional Chinese medicines.

Keywords: High-speed counter-current chromatography; Polyphenol; 3,4-Dihydroxyphenyllactic acid; Salvianolic acid B; Protocatechualdehyde

1. Introduction

Salvia miltiorrhiza Bunge, a popular traditional Chinese medicinal plant, has been used extensively for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea and neuroasthenic insomnia and cytotoxicity against human tumor cell lines [1–3]. Studies on the chemical constituents of *Salvia* were mainly confined to the diterpenoids and the tanshinones at the beginning of research. In recent years, much attention has been directed to the biologically active water-soluble components, polyphenols, in the dried root decoction. These studies, particularly in China, have led to the isolation and identification of a host of caffeic acid-derived metabolites, many of which possess a variety of biological activities including antioxidant, antiplatelet, antitumor and antiviral activity [4,5]. 3,4-Dihydroxyphenyllactic acid, also known as danshensu (a Chinese word which literally mean the element of Danshen), and salvianolic acid B (lithospermic acid B) are major active components of polyphenols in *Salvia miltiorrhiza* Bunge. 3,4-Dihydroxyphenyllactic acid was first isolated from *Salvia miltiorrhiza* Bunge and found to be a coronary vasodilator and to scavenge the free oxygen radicals [6]. Salvianolic acid B has significant scavenging effects on oxygen free radicals and protective effects on heart and brain injuries induced by ischemia-reperfusion [7]. Protocatechualdehyde (protocatechuic aldehyde) is normally used as a symbolic component in the analysis of related preparation of *Salvia miltiorrhiza* Bunge.

Decoction has been the most popular preparation of traditional Chinese medicine (TCM) for more than 2000 years. It is implied that water-soluble components are very important active parts in TCM. However, the current separation and purification technologies suitable for total water-soluble compounds with high polarity are very limited. The origin method for purification of 3,4-dihydroxyphenyllactic acid is decocting the powdered dried root of *Salvia miltiorrhiza* Bunge with water and precipitating the extract with ethanol, which is still popular nowadays for large-scale preparation of injection of 3,4-dihydroxyphenyllactic acid. Macro pore resin chromatography is another separation method [8]. However, the purity of 3,4-dihydroxyphenyllactic acid by these two methods was not satisfactory. Preparative high-performance

* Corresponding author. Tel.: +86 10 82627061.
E-mail address: rainbow_gm@yahoo.com (M. Gu).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.11.057

liquid chromatography is efficient to yield this high purity compound, although the cost was very high and the loaded sample has to be pretreated strictly, which is not suitable for scale-up in TCM area [9]. More methods need to be developed for purification of 3,4-dihydroxyphenyllactic acid at high purity. It was reported that high-speed counter-current chromatography (HSCCC) was an active chromatography method for purification of tanshinones [10–13] and some kinds of polyphenols [14–16] of *Salvia miltiorrhiza* Bunge, such as salvianolic acid B, salvianolic acid E, rosmarinic acid and lithospermic acid. Because 3,4-dihydroxyphenyllactic acid is of low molecular weight and of three hydroxyl groups, it is of high polarity and is difficult to be separated and purified by normal organic/aqueous solvent systems of HSCCC. By now, purification of 3,4-dihydroxyphenyllactic acid, a very important active polyphenol, by HSCCC has not been reported.

HSCCC is a continuous liquid–liquid partition chromatography with no solid support matrix, the stationary phase of which is retained in the separation columns by gravity and centrifugal force field. Therefore, HSCCC avoids the disadvantages arising from the interaction of samples with the solid support such as absorption and denaturation of target products. HSCCC has the unique features of high recovery, high efficiency and the ease to scale-up. Industrial HSCCC is in the course of development. It has widely been used in the separation and purification of natural products, antibiotics and rare elements with organic/aqueous systems [17].

To build up two phases of organic/aqueous systems of HSCCC, liquids have to be of much different polarities, which makes purification of total water-soluble components very difficult. Normally, *n*-butanol/water is the most polar basic solvent system for HSCCC separation with organic solvent/water. However, retention of stationary phase of this system is normally lower than 50%, which has a negative effect on resolution [18]. Also, this system is not stable enough for long-term run on HSCCC leading to the loss of stationary phase. It is helpful to use acids and salts in solvent system to increase polarity to separate and purify components with high polarity. It is not easy to remove salts from target fractions, and therefore, acid was under consideration to modify *K* value. Moreover, acid is positive for the stability of polyphenols, especially for 3,4-dihydroxyphenyllactic acid whose most stable pH value is 2.

In this report, organic acid was used as a major member of solvent system to successfully develop polar separation environment more suitable for the purification of 3,4-dihydroxyphenyllactic acid, beneficial to the purification of many other total water-soluble components. Another two polyphenols of *Salvia miltiorrhiza* Bunge, protocatechualdehyde and salvianolic acid B were purified at the same time. The chemical structures of these three components are shown in Fig. 1.

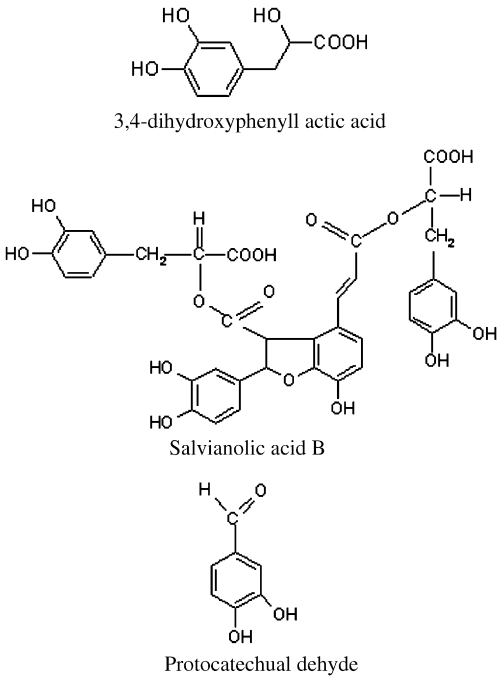

Fig. 1. Structures of 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde.

2. Experimental

2.1. Reagents

Analytical-grade *n*-hexane, ethyl acetate, methanol and acetic acid for HSCCC separation were from Atoz Fine Chemicals, Tianjin, China. Methanol of HPLC grade and acetic acid were obtained from Concord Tech, Tianjin, China. All aqueous solutions were prepared with pure water produced by Milli-Q system (18 MΩ, Millipore, Bedford, MA, USA).

Water extracted crude powder sample of *Salvia miltiorrhiza* Bunge was a gift from Dalian Institute of Physics and Chemistry, Chinese Academy of Sciences. Standard samples of 3,4-dihydroxyphenyllactic acid (110855–200405), salvianolic acid B (111562–200506) and protocatechualdehyde (0810–200004) were supplied by the State Food and Drug Administration of China (SFDA).

2.2. Apparatus

HSCCC (TBE-300A) is from Tauto Biotech, Shanghai, China, with three preparative coils connected in series (diameter of 2.6 mm, total volume 200 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (*R*) was 5 cm, and the β value varied from 0.5 at internal terminal to 0.8 at the external terminal ($\beta = r/R$, where *r* is the distance from the coil to the holder shaft). The HSCCC systems are equipped with a Model S constant-flow pump, a Model 8823A UV monitor operating at 280 nm and a Model 3057 recorder.

2.3. Preparation of solvent system for HSCCC separation

Hydrophilic organic/aqueous solvent system was prepared by thoroughly mixing organic solvents and water in separatory funnel at room temperature. Two phases were separated just before use. The optimized solvent system of water-soluble components of *Salvia miltiorrhiza*

Bunge was *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1.5:8).

2.4. Preparation of sample solution for HSCCC separation

Hundred milligrams of water extracted crude sample of *Salvia miltiorrhiza* Bunge was dissolved in 5 ml lower phase of solvent system assisted by ultrasonic.

2.5. HSCCC operation

The coiled column of HSCCC was filled with the upper phase of solvent system. Then, the apparatus was rotated at 850 rpm and at temperature of 26 °C and at the same time, the lower phase of solvent system was pumped through the column at a flow-rate of 1.5 ml/min. After the mobile phase emerged in the effluent and the hydrodynamic equilibrium was established in the column, 5 ml of the sample solution containing 100 mg of water extracted crude sample of *Salvia miltiorrhiza* Bunge was injected through the valve. Retention of stationary phase was 50%. The effluent was monitored with a UV–vis detector at 280 nm, and the peak fractions were collected.

2.6. Measurement of partition coefficient (K)

Partition coefficient (*K*) was expressed as the absorbency of sample in the upper phase divided by that in the lower phase.

Measurement of *K* value of crude sample is as follows. Solvent systems were prepared and equilibrated. Three milliliters of upper phase and 3 ml of lower phase were put in a 10 ml test tube. Two milligrams of crude powder sample was weighted accurately and put into the tube. The test tube was stoppered and shaken vigorously and then was thoroughly equilibrated. Samples dissolved in the upper phase and lower phase were analyzed by UV spectrophotometer at 280 nm.

Measurement of *K* value of 3,4-dihydroxyphenyllactic acid is as follows. One milliliter of upper phase and 1 ml of lower phase were put in a 5 ml test tube. Crude powder sample (0.5 mg) was weighted accurately and put into the tube. The test tube was stoppered and shaken vigorously and then was thoroughly equilibrated. Samples dissolved in the upper phase and lower phase were analyzed by HPLC and detected at 280 nm.

2.7. HPLC analysis

The water extracted crude sample and fractions separated by HSCCC were analyzed by HPLC system (10 Avp, Shimadzu, Japan) composed of two pumps, UV detector, oven, system controller and 20 μl sample loop. The column used was Ultrasphere C18 column (250 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan). The mobile phase was solvent A (methanol–water–acetic acid = 10:89.2:0.8), and solvent B (methanol–water–acetic acid = 89.2:10:0.8) in the gradient mode was as follows: 0–60 min, 0–60% B. The flow-rate was 1.0 ml/min, and the temperature was 40 °C. The effluent was monitored at 280 nm. Crude sample and three kinds of reference material were dissolved with solvent A.

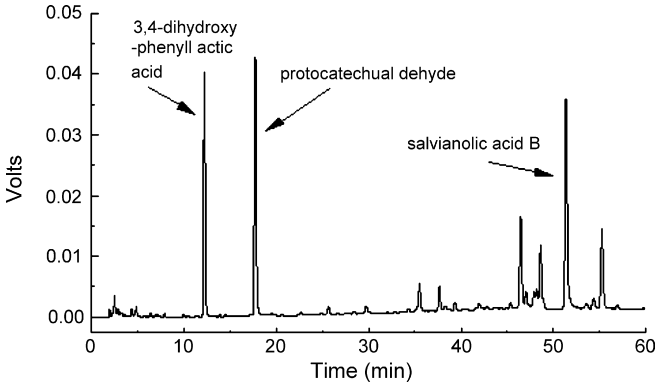

Fig. 2. Analysis of water extracted crude sample of *Salvia miltiorrhiza* Bunge by HPLC. Experimental conditions are as follows: the column used was Ultrasphere C18 column (250 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan). The mobile phase was solvent A (methanol–water–acetic acid = 10:89.2:0.8) and solvent B (methanol–water–acetic acid = 89.2:10:0.8) in the gradient mode was as follows: 0–60 min, 0–60% B. The flow-rate was 1.0 ml/min and temperature was 40 °C. The effluent was monitored at 280 nm. The crude sample and three kinds of reference material were dissolved with solvent A and loaded through 20 μl sample loop.

3. Results and discussion

3.1. Analysis of polyphenols in the crude sample by HPLC

Water extracted crude sample was analyzed by HPLC with C18 column. Acetic acid was added to the mobile phase to keep it at about pH 3, which makes polyphenols stable. The mobile phase was solvent A (methanol–water–acetic acid = 10:89.2:0.8), and solvent B (methanol–water–acetic acid = 89.2:10:0.8) running in the gradient mode was as follows: 0–60 min, 0–60% B. Three major components in the crude sample were identified by mixing suitable concentration of reference material with the crude sample. It is shown in Fig. 2 that peaks A, B and C are 3,4-dihydroxyphenyllactic acid, protocatechualdehyde and salvianolic acid B. Four concentrations of each reference material were prepared and ran on HPLC to plot standard curve. Contents of 3,4-dihydroxyphenyllactic acid, protocatechualdehyde and salvianolic acid B in the crude sample were 4.00%, 0.63% and 6.40%, respectively.

3.2. HSCCC separation of polyphenols of Salvia miltiorrhiza Bunge

HSCCC separation system is composed of two-phase liquids. It is essential to select a suitable solvent system by an important parameter, partition coefficient (*K*), whose ideal value is around 1 that gives a high separation ability and reasonable running time. Because there were three major target components in the water extracted crude sample, it was not economical to detect the *K* value of different solvent systems that were supposed to be used for HSCCC separation with three kinds of reference material. Also, it was not convenient, prompt and economical to detect *K* values of three target compounds of crude extract in many different solvent systems by HPLC. Therefore, UV spectrophotometer was used in the fast detection of *K* value, which

gave a universal clue of distributions of compounds. Crude samples were mixed with different solvent systems and distributed in the upper phase and lower phase in test tubes (details shown in section 2.6). *K* value was the absorbency of sample in the upper phase divided by that in the lower phase.

Organic acid, such as acetic acid, is helpful for modifying *K* value of negatively charged analytes. These molecules become more hydrophobic and favor partition to the organic phase. Also, adding the acid to the solvent system often substantially shortens the settling time, improving retention of stationary phase. For polyphenols, organic acid is helpful for their stability. Therefore, acetic acid became a major composite of solvent system in our experiments. Solvent systems composed of *n*-butanol and water with or without acetic acid was of high polarity; however, *K* value was far away from 1. In the solvent systems composed of ethyl acetate, ethanol and water without acetic acid, *K* value of ethyl acetate–ethanol–water (2:1:2) was close to 1. Retention of stationary phase of this system on HSCCC was just 30%, which was not positive to the resolution. Solvent systems composed of *n*-hexane, ethyl acetate, methanol and water were important systems in HSCCC separation [19]. The common high polarity ratio of it was 1:5:1:5; however, it was not polar enough for the separation of 3,4-dihydroxyphenyllactic acid. In our experiments, ratio of solvents was adjusted to 1:6:1.5:5.5 or 1:6:1.5:7.5 to increase the ratio of polar solvents. Acetic acid was added at the same time to increase the partition of polyphenols to the organic phase improving *K* value and to keep the stability of polyphenols. Solvent systems composed of *n*-hexane–ethyl acetate–methanol–acetic acid–water at the ratios of 1:6:1.5:1.5:4 and 1:6:1.5:1.5:6 were of ideal *K* values. Consequently, these two systems were tried on HSCCC to separate the crude sample.

Retention of stationary phase of these two solvent systems was around 50%. Solvent system composed of *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1.5:6) showed better resolution than the system at the ratio of 1:6:1.5:1.5:4 as shown in Fig. 3. It suggested that high ratio of polar solvents in the solvent system was of help for the separation. Then, the ratio of 1:6:1.5:1.5:8 was tried on HSCCC at a flow-rate of 1.5 ml/min and at a speed of 850 rpm (shown in Fig. 4). Eight peak fractions were eluted and analyzed by HPLC with C18 column. Retention times of peak fraction numbers 2, 6 and 7 were similar to those of three kinds of reference material. These three peak fractions were then mixed, respectively, with corresponding reference material. It was confirmed that peak fraction numbers 2, 6 and 7, respectively, were 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde at purity of 97.6%, 94.2% and 98.2%, respectively. Yields of 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde were 98.6%, 73.6% and 90.2%, respectively. Other peak fractions were of much lower purity than the above three target compounds and of low value to do further analysis.

These three polyphenols of *Salvia miltiorrhiza* Bunge were separated and purified effectively in one step by HSCCC at high purity and high yield, which showed the great advantage of this technology. Organic acid played an important role in purification of high polar compounds by increasing partition in organic phase. HSCCC supplied an effective method to purify

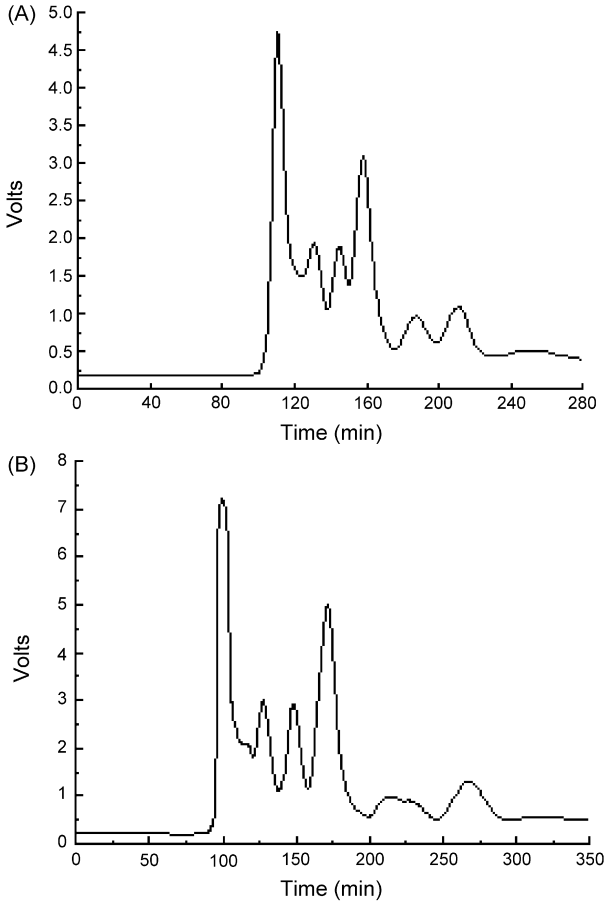

Fig. 3. Optimization of solvent system of HSCCC separation. Experimental conditions for HSCCC are as follows—apparatus: TBE-300A with 200 ml column; retention of stationary phase: 50%; solvent system of A: *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1.5:4); solvent system of B: *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1.5:6); mobile phase: lower phase; elution mode: head to tail; flow-rate: 1.5 ml/min; revolution: 850 rpm; temperature: 26 °C; detection wavelength: 280 nm.

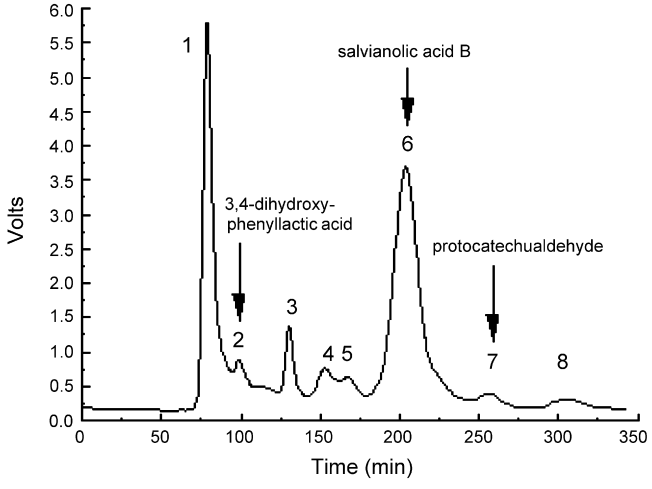

Fig. 4. HSCCC separation of *Salvia miltiorrhiza* Bunge with optimized condition. Experimental conditions for HSCCC are as follows—apparatus: TBE-300A with 200 ml column; retention of stationary phase: 50%; solvent system: *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1.5:8); mobile phase: lower phase; elution mode: head to tail; flow-rate: 1.5 ml/min; revolution: 850 rpm; temperature: 26 °C; detection wavelength: 280 nm; sample loading: 100 mg/5 ml.

Table 1
The *K* values (partition coefficient) of crude extract of *Salvia miltiorrhiza* Bunge in different solvent systems

	Solvent system	*K* value
1	*n*-Butanol–water = 1:1	0.05
2	*n*-Butanol–acetic acid–water = 4:1:5	2.17
3	*n*-Butanol–acetic acid–water = 4:0.5:5	2.02
4	Ethyl acetate–ethanol–water = 2:1:2	1.11
5	Ethyl acetate–ethanol–water = 5:1:5	2.43
6	Ethyl acetate–ethanol–acetic acid–water = 5:1:1:4	3.25
7	*n*-Hexane–ethyl acetate–methanol–acetic acid–water = 1:6:1.5:0:7.5	0.55
8	*n*-Hexane–ethyl acetate–methanol–acetic acid–water = 1.5:5:1.5:0:5	0.37
9	*n*-Hexane–ethyl acetate–methanol–acetic acid–water = 1:6:1.5:1.5:6	1.00
10	*n*-Hexane–ethyl acetate–methanol–acetic acid–water = 1:6:1.5:1.5:4	0.93

high polarity water-soluble compound of TCM directly from crude sample with simple organic/aqueous two-phase system by adding organic acid (Table 1).

Acknowledgements

This work was supported by the Chinese Academy of Sciences (KGCX2-SW-213) and the National Natural Science Foundation Committee of China (20606032).

References

[1] Y.R. Lu, L.Y. Foo, Phytochemistry 59 (2002) 117.
[2] G.C. Lin, W.L. Chang, Phytochemistry 53 (2000) 951.
[3] C.T. Wu, V. Mulabagal, S.M. Nalawade, C.L. Chen, T.F. Yang, H.S. Tsay, Biol. Pharm. Bull. 26 (2003) 845.
[4] J.P. Yuan, H. Chen, F. Chen, J. Agric. Food Chem. 46 (1998) 2651.
[5] M. Petersen, M.S.J. Simmonds, Phytochemistry 62 (2003) 121.
[6] Y. Lu, L.Y. Foo, Phytochemistry 59 (2002) 117.
[7] Y.H. Lin, A.H. Liu, H.L. Wu, C. Westernbroek, Q.L. Song, H.M. Yu, G.J.T. Horst, X.J. Li, Biochem. Biophys. Res. Commun. 348 (2006) 593.
[8] Z.P. Wang, J.Y. Fan, Y.Q. Liu, Y.J. Yuan, Ion Exch. Adsorpt. 19 (2003) 554.
[9] J. Pan, T. Xu, Chin. Trad. Herb. Drugs 34 (2003) 125.
[10] M. Gu, F. Ouyang, Z.G. Su, J. Chromatogr. A 1022 (2004) 139.
[11] G.L. Tian, Y.B. Zhang, T.Y. Zhang, F.Q. Yang, Y. Ito, J. Chromatogr. A 904 (2000) 107.
[12] G.L. Tian, T.Y. Zhang, Y.B. Zhang, Y. Ito, J. Chromatogr. A 945 (2002) 281.
[13] H.B. Li, F. Chen, J. Chromatogr. A 925 (2001) 109.
[14] H.B. Li, J.P. Lai, Y. Jiang, F. Chen, J. Chromatogr. A 943 (2002) 235.
[15] J.H. Chen, F.M. Wang, F.S.C. Lee, X.R. Wang, M.Y. Xie, Tanlanta 69 (2006) 172.
[16] W.B. Zhi, Q.Y. Deng, J. Chromatogr. A 1116 (2006) 149.
[17] Y. Ito, J. Chromatogr. 538 (1991) 3.
[18] L.J. Chen, I.A. Sutherland, J. Chromatogr. A 1114 (2006) 29.
[19] Y. Ito, J. Chromatogr. A 1065 (2005) 145.