Research article

Biomedical Chromatography

Received 5 August 2010, Revised 7 October 2010, Accepted 8 October 2010 Published online in Wiley Online Library: 10 February 2011

(wileyonlinelibrary.com) DOI 10.1002/bmc.1570

Comparative study on enantiomeric excess of main akannin/shikonin derivatives isolated from the roots of three endemic Boraginaceae plants in China

Wen Zhou, Hu Da Gu La Jiang, Ying Peng and Shao Shun Li*

ABSTRACT: This work systematically investigated the enantiomeric excess (e.e.) of main components isolated from the roots of three endemic Boraginaceae plants distributed extensively in China, named *Arnebia euchroma* (Royle) Johnst (*A.e.*), *Lithospermum erythrorhizon* Sieb. et Zucc. (*L.e.*) and *Onosma confertum* W. W. Smith (*O.c.*), and the optical purity of their hydrolysis products separately, by means of three different approaches. The influence of HCl on the e.e. values of the major constituents was also studied. Analysis of the absolute configurations and e.e. values of all the derivatives acquired was performed by CD and chiral-HPLC respectively. The results of the main constituents demonstrated that *A.e.* mainly yields *S*-form naphthoquinone derivatives, while the *R*-form is predominant in the derivatives of *L.e.* and *O.c.* The optical purity of alkannin and shikonin and their derivatives was not influenced by acid treatment in the course of separation and hydrolysis. Additionally, it was found that 100% e.e. of shikinon could be acquired from a specific shikinon ester derivative, *β,β*-dimethylacrylshikonin occurring in the roots of *O.c.*, as did 100% e.e. of alkannin from *β,β*-dimethylacrylalkannin contained in the roots of *A.e.*

Keywords: alkannin; shikonin; Boraginaceae; acid-treatment; chiral-HPLC

Introduction

Alkannins (A) and shikonins (S) (Fig. 1), which were first identified as enantiomers and correctly assigned their structures by Brockmann (1936), are mainly found in the roots of the Boraginaceae family. Biological investigations over the past 30 years have demonstrated that the chiral pair A/S and theirs derivatives were endowed with a wide spectrum of functions such as wound healing, antimicrobial, anti-inflammatory, antioxidant and antithrombotic biological activity, which were shown to have no relation to the chirality (Papageorgiou *et al*., 1999). Recently, significant and extensive scientific researches have been focused on A/S effectiveness on several tumors and on their mechanism of anticancer action (Liu *et al*., 2008; Xuan and Hu, 2009; Wang *et al*., 2009), but no comparative evidence was provided to support the relationship of the antitumor activity with the enantiomeric excess (e.e.) of A/S.

Since health and regulatory authorities stipulate full documentation of methods of analysis, and determination of the individual enantiomers and the pharmacological and pharmacokinetic profiles for approved enantiomeric drugs (Assimopoulou *et al*., 2004a), further researches on the analysis of A/S and on the parameters that affect the enantiomeric ratio of A/S are required (Papageorgiou *et al*., 2006), and the assurance of highly optical purity A/S is crucial for their research and development in pharmaceuticals.

Much effort has been made to identify the enantiomeric ratio of A/S extracted from the Boraginaceae family, which is a major source of the chiral pair. The results of the identification, qualitative and quantitative determination of A/S and their derivatives in raw materials showed that the ratio of A/S was directly bound up with breeds and sources. Generally speaking, alkannin derivatives occurred primarily in the roots of the Boraginaceae family of Europe, especially *Alkannatinctoria Tausch*, while shikonin derivatives were mainly found in the roots of the plant *Lithospermm erythrorhizon Sieb et Zucc* of East Asia (Papageorgiou *et al*., 1999). Nevertheless, Jain and coworkers reported that the racemic mixture of alkannin and shikonin existed in the roots of the Indian plant *Arnebia hispidissima* (Jain and Mathur, 1965). The formation of stereoisomeric mixtures of naphthoquinone derivatives in *Echium lycopsis callus* cultures was also investigated, and the examination of the absolute configurations of these compounds revealed that the *Echium lycopsis callus* cultures yielded both the *R*-form (shikonin) and the *S*-form (alkannin) in various ratios with the range of the esterified derivatives (Fukui *et al*., 1983). It is also noticeable that all *Alkanna species* grown in Greece contained 100% e.e. of alkaninin derivatives (Assimopoulou *et al*., 2004a). Therefore, high optical purity of shikonin or

* Correspondence to: Shao Shun Li, School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China. E-mail: ssli@sjtu.edu.cn

School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

Abbreviations used: A, alkannin; S, shikonin; CD, circular dichroism; e.e., enantiomeric excess.

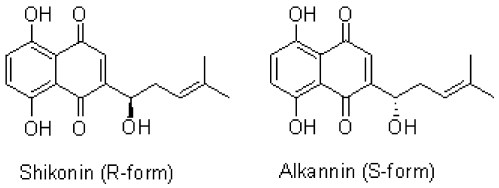

Figure 1. Structures of the chiral pair alkannin and shikonin.

alkannin acquired from the Boraginaceae family, which contains complicated enantiomeric ratios of A/S derivatives, remains largely elusive.

In previous methods acids such as HCl were inevitably used for the preparation of A/S and related products. For instance, diluted HCl (1:1) was applied to recover the copper complex and to neutralize the alkaline layer in the course of the preparation of shikalkin (Jain and Mathur, 1965). Also, the insoluble Cu-chelates of copper acetate with the red pigment extracted from the *Macrotomia cephalotes* D.C. were displaced with 10% HCl to prepare alkannin derivatives (Papageorgiou, 1979). As reported, four naphthoquinone derivatives, isolated from *Onosma heterophylla Griseb*, were converted into Cu-chelates with copper acetate, and after acidic decomposition the mixture of the free pigments was subjected to silica gel columns (Mellidis and Papageorgiou, 1987). During the preparation of A/S and their derivatives, acids were required to displace Cu-chelates and release the red pigments. However, there has been no related research with respect to the influence of acids on chirality.

Taken together, although there are a few articles dealing with the effects of environmental factors and hydrolysis conditions on the enantiomeric ratios of the pharmaceutical substance alkannin and shikonin (Assimopoulou *et al*., 2004a), most of the scientific works concerning Boraginaceae family focus mainly on chemistry, biology, chromatographic conditions and detection techniques of A/S and their derivatives. The objectives of this work were to investigate the e.e. values of the main A/S derivatives in the roots of several wild Chinese Boraginaceous plants, not examined to date, with the aim of exploring the detailed relationship of the e.e. values between main A/S derivatives and their hydrolyzed products, and the influence of acids on the optical purity in the course of the preparation of A/S and their derivatives.

Experimental

Chemicals

The roots of Boraginaceous plants were purchased from various Chinese geographical regions. *Arnebia euchroma* (Royle) Johnst (*A.e.*) roots were purchased from Aksu area (Xinjiang, China). *Lithospermum erythrorhizon* Sieb. et Zucc (*L.e.*) roots were collected from Qingyuan county (Liaoning, China). Additionally, the roots of the medicinal Boraginaceous plant *Onosma confertum* W. W. Smith (*O.c.*) were purchased from Shanghai Ley's Pharmaceutical Limited Company. All materials purchased were identified by associate professor Wang M. Y., Shanghai Jiao Tong university.

Shikonin (National Institute for the Control of Pharmaceutical and Biological products, China), alkannin, acetylalkannin and acetylshikonin were kindly provided by FunSea Beijing Technology Co. Ltd, China, and were used as standards for RP-HPLC and chiral-HPLC. *β,β*-Dimethylacrylalkannin, *β,β*-dimethylacrylshikonin, *β*-hydroxyisovalerylalkannin, *β*-hydroxyisovalerylshikonin, *β*-acetoxyisovalerylalkannin, *β*-acetoxyisovalerylshikonin, *α*-methylbutyrylalkannin and *α*-methylbutyrylshikonin, prepared according to the procedure proposed by Papageorgiou (1978), also used as standards, were confirmed by our groups.

All solvents but isopropanol and hexane used for RP-HPLC and chiral-HPLC were HPLC grade. Deionized water (resistivity >8 MΩ/cm) was obtained using the Milli-Q SP Reagent Water System (Millipore, Bedford, MA). Silica gel (200–300 mesh), applied to isolate the main components from the roots, was purchased from Branch of Qingdao Haiyang Chemical Ltd Co. All solvents and solutions were filtered through 0.45 μM PTFE filters from Rephile Corporation (Shanghai, China) before use.

Instrumentation

RP-HPLC was used for qualitative determination of the methanolic extract of the roots of plants, chiral-HPLC was applied to test the optical purity, and CD was performed to determine the absolute configuration by reference to the literature (Ai *et al*., 1993). The HPLC system was equipped with a UV–vis detector (G1315C/D), an Agillent 1200 series G1322A IV pump and software for process control and data handling (Agilent ChemStation for LC 3D Systems). The detector wavelength was set at 516 nm.

The column used for RP-HPLC was a Sepax GP-C_{18} (no. 07290918445, 250 × 4.6 mm), obtained from Sepax Technologies Inc. (Newark, USA), and separations were carried out at ambient temperature. Gradient elution (A is methanol from 35 to 95% in 10 min, where B is 0.5% v/v phosphoric acid in water) was adopted as the mobile phase degassed before use. The flow rate of the mobile phase was adjusted to 1.0 mL/min and the injection volume was 10 μL, so as to control the reasonable resolution of the red pigment.

The chiral HPLC column applied was Sino-Chiral OD, no. 0A02014-C [Packing cellulose-tris(3,5-dimethylphenyl carbamate)], purchased from FunSea Beijing Technology Co. Ltd (Beijing, China), 150 × 4.6 mm, and separations were also conducted at ambient temperature. The mobile phase, hexane–isopropanol, 90:10 (analytically pure), was degassed before application. The mobile phase flow rate was adjusted to 0.45 mL/min and the injection volume of 5 μL was employed, in order to obtain sufficient resolution of alkannin and shikonin and their corresponding derivatives.

CD spectra were run on a Jasco J-815, made in Japan, provided with a 150 W air-cooled xenon lamp, continuous scan mode and wavelength accuracy with the range of ±0.3nm. NMR spectra were recorded on a Varian Mercury 300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C); chemical shifts of ^{1}H and ^{13}C spectra were recorded with tetramethylsilane as internal standard. Mass spectra were recorded on a Shimadzu LCMS-2010EV mass spectrometer.

Preparation of standards and samples

The preparation of the standards, which are not available commercially, is crucial for the reproducibility and accuracy of the analysis for the relationship of the e.e. values between the main A/S derivatives and their hydrolyzed products. The pulverized roots (200 g) of *L.e.* were extracted twice with ethyl acetate (2 × 300 ml) at 50°C, the combined organic layer was evaporated in *vacuo*, and the residue was re-dissolved in methanol to remove insoluble substance, and then concentrated again to dryness. The residue dissolved in suitable amount of ethyl acetate was subjected to the silica gel column chromatography with the solvent system of petroleum ether and ethyl acetate (v/v 10:1 to 5:1) to give *α*-methylbutyrylshikonin and *β*-hydroxyisovaleryl-shikonin.

***α*-Methylbutyrylshikonin.** ^{1}H NMR (300 MHz, $CDCl_3$, *δ*, ppm): *δ* 12.59 (s, 1H), 12.41 (s, 1H), 7.18 (s, 2H, benzene ring H), 7.00 (s, 1H, quinone ring H), 6.03 (dd, 1H, J = 5.4, 7.2 Hz), 5.13 (t, 1H, J = 7.2 Hz), 2.62–2.58 (m, 1H), 2.56–2.48 (m, 1H), 2.44 (m, 1H), 1.69 (s, 3H), 1.57 (s, 3H) 1.49 (m, 2H), 1.17 (d, 3H, J = 6.9 Hz), 0.95 (t, 3H, J = 7.2 Hz). ^{1}H NMR (75 MHz, $CDCl_3$, *δ*, ppm): *δ* 178.4, 176.9, 175.3, 166.9, 167.5, 148.5, 135.7, 132.9, 132.7, 131.4, 118.0, 111.9, 111.5, 69.2, 41.1, 33.3, 26.6, 25.8, 17.9, 16.3, 11.5. ESI-MS: *m/z* 395.4 $(M + Na)^+$. Retention time (chiral HPLC): 8.04 min. CD spectral data: $[\theta] = +3045.3$ at 365 nm, CH_3OH.

β-Hydroxyisovalerylshikonin. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 7.17 (s, 2H, benzene ring H), 7.02 (s, 1H, quinone ring H), 6.10 (1H, dd, J = 4.5, 4.5 Hz), 5.13 (t, 1H, J = 6.9 Hz), 2.66–2.62 (m, 3H), 2.58–2.45 (m, 1H), 1.68 (s, 3H), 1.58(s, 3H), 1.28 (s, 3H), 1.24 (s, 3H). ^{1}H NMR (75 MHz, $CDCl_3$, δ, ppm): δ 176.7, 175.4, 171.6, 168.4, 167.8, 147.5, 136.4, 133.2, 133.1, 131.4, 117.7, 111.6, 111.4, 69.7, 69.4, 46.3, 32.6, 29.3, 29.1, 25.8, 17.8. ESI-MS: m/z 411.4 $(M + Na)^+$. Retention time (chiral HPLC): 10.96 min. CD spectral data: $[\theta] = +1147.6$ at 365 nm, CH_3OH.

According to the same procedure as the roots of *L.e.*, β,β-dimethylacrylalkannin and β-acetoxyisovalerylalkannin were isolated and purified from the roots of *A.e.*; however, β,β-dimethylacrylshikonin was obtained from the roots of *O.c.*

β,β-Dimethylacrylalkannin. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): δ 12.59 (s, 1H), 12.41 (s, 1H), 7.17 (s, 2H, benzene ring H), 6.97 (d, 1H, J = 3.9 Hz, quinone ring H), 6.02 (m, 1H), 5.75 (s, 1H), 5.12 (t, 1H, J = 7.2 Hz), 2.64–2.59 (m, 1H), 2.50–2.45 (m, 1H), 2.15 (s, 3H), 1.93 (s, 3H), 1.68 (s, 3H), 1.57 (s, 3H). ^{1}H NMR (75 MHz, $CDCl_3$, δ, ppm): δ 178.7, 177.2, 175.5, 167.5, 166.9, 166.7, 148.9, 139.9, 136.1, 132.9, 132.8, 131.7, 118.2, 112.1, 111.8, 69.5, 33.2, 27.5, 25.9, 20.8, 18.1. ESI-MS: m/z 393.4 $(M + Na)^+$. Retention time (chiral HPLC): 6.06 min. CD spectral data: $[\theta] = -2214.6$ at 365 nm, CH_3OH.

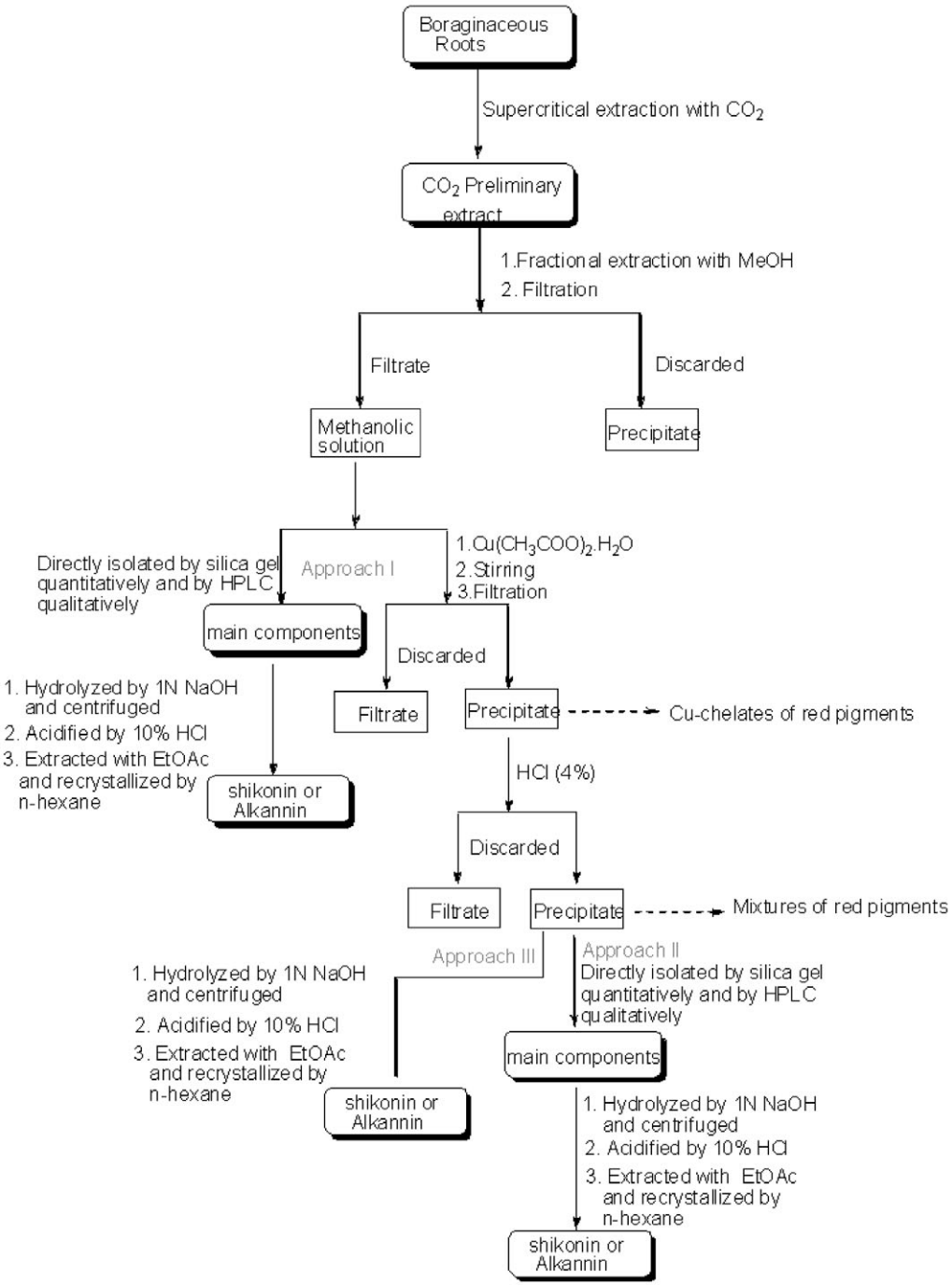

Figure 2. Isolation and preparation procedures of alkannin or shikonin with various enantiomeric ratios.

***β*-Acetoxyisovalerylalkannin.** ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): δ 12.58 (s, 1H), 12.41 (s, 1H), 7.18 (s, 2H, benzene ring H), 7.00 (s, 1H, quinone ring H), 6.03 (m, 1H), 5.11 (t, 1H, J = 7.2 Hz), 3.04–2.90 (m, 2H), 2.64–2.57 (m, 1H), 2.51–2.41 (m, 1H), 1.99 (s, 3H), 1.67 (s, 3H), 1.55(s, 3H), 1.52 (s, 6H). ^{1}H NMR (75 MHz, $CDCl_3$, δ, ppm): δ 177.6, 176.1, 170.5, 169.3, 168.2, 167.7, 148.2, 136.2, 133.1, 132.8, 131.4, 117.7, 111.9, 111.6, 79.4, 69.8, 44.5, 32.9, 26.8, 26.6, 25.7, 22.5, 18.0. ESI-MS: *m/z* 453.3 $(M + Na)^+$. Retention time (chiral HPLC): 8.84 min. CD spectral data: $[\theta] = -2256.8$ at 365 nm, CH_3OH.

***β,β*-Dimethylacrylshikonin.** The spectroscopic data of ^{1}HNMR, ^{13}CNMR and MS were identical to those of *β,β*-dimethylacrylalkannin. Retention time (chiral HPLC): 7.25 min. CD spectral data: $[\theta] = +2407.7$ at 365 nm, CH_3OH.

β-Hydroxyisovalerylalkannin and *α*-methylbutyrylalkannin were prepared using alkannin (1 mmol) as starting material, which came from hydrolysis products of *β,β*-dimethylacrylalkannin, with the corresponding 3-hydroxy-3-methylbutanoic acid (1.05 mmol) and 2-methylbutanoic acid (1.05 mmol) in the presence of dicyclohexylcarbodiimide (1.05 mmol) and 4-(dimethylamino) pyridine (0.25 mmol) under the nitrogen atmosphere. When alkannin was replaced by shikonin, which originated from hydrolysate of *β,β*-dimethylacrylshikonin, *β*-acetoxyisovalerylshikonin was synthesized.

***β*-Hydroxyisovalerylalkannin.** The spectroscopic data of ^{1}HNMR, ^{13}CNMR and MS were identical to that of *β*-hydroxyisovalerylshikonin. Retention time (chiral HPLC) was 9.73 min. CD spectral data: $[\theta] = -1231.5$ at 365 nm, CH_3OH.

***α*-Methylbutyrylalkannin.** The spectroscopic data of ^{1}HNMR, ^{13}CNMR and MS were identical to that of *α*-methylbutyrylshikonin. Retention time (chiral HPLC) was 7.47 min. CD spectral data: $[\theta] = -2981.6$ at 365 nm, CH_3OH.

***β*-Acetoxyisovalerylshikonin.** The spectroscopic data of ^{1}HNMR, ^{13}CNMR and MS were well consistent with that of *β*-acetoxyisovalerylalkannin. Retention time (chiral HPLC) was 9.58 min. CD spectral data: $[\theta] = +2168.4$ at 365 nm, CH_3OH.

Standards of A/S and their derivatives in powdered form or crystalloid were dissolved in the HPLC-grade methanol for RP-HPLC or in the hexane–propanol (90:10, v/v) for chiral-HPLC before injection into the liquid chromatographer. After filtering through a 0.45 μM PTFE filter, the oily extracts of three kinds of Boraginaceae plants dissolved in HPLC-grade methanol were subjected to liquid chromatography to determine their main chemical constituents qualitatively. All derivatives used for CD were dissolved with HPLC-grade methanol and the concentrations were controlled within the range of 1–2 $\times 10^{-4}$ g/mL.

In order to determine the main components of A/S in the three kinds of Boraginaceous roots tested and investigate the influence of acids on the preparation for A/S and their derivatives, three approaches, as shown in Fig. 2, were designed as follows: approach I designated that the extraction from pulverized Boraginaceous roots was performed by supercritical carbon dioxide, and then methanol was selected to dissolve the extract obtained and get rid of the insoluble substance, and afterwards the methanolic solution concentrated was directly isolated by silica gel to obtain A/S esters; subsequently the A/S derivatives were hydrolyzed by 1 mol/L NaOH and acidified by 10% of HCl to obtain shikonin or alkannin. Approach II was similar to Approach I except that copper acetate was used to chelate naphthoquinone derivatives, then acid was applied to displace Cu-complexes of A/S esters derivatives and the chelate was partitioned by ethyl acetate and isolated by silica gel. Approach III, performed according to our improved procedure on the basis of the methods proposed by Assimopoulou and Papageorgiou (2004a), was that all A/S derivatives, in which the Cu-complexes of A/S esters

Table 1. Comparative studies on the e.e. values of main constituents of three Chinese Boraginaceae plants before and after hydrolysis according to Approach I

Boraginaceae plants in China	Main constituents and their contents[a]	Before hydrolysis[b]		After hydrolysis[b]	
		e.e. values (%)	Absolute configuration by CD	e.e. values (%)	Absolute configuration by CD
Arnebia euchroma (Royle) Johnst (*A.e.*)	*β,β*-dimethylacryl alkannin (85.2%)	100	S	100	S
	Acetylalkannin (0.6%)	30.8	S	29.5	S
	β-Acetoxyisovaleryl alkannin (0.25%)	100	S	100	S
Lithospermum erythrorhizon Sieb. et Zucc (*L.e.*)	*α*-Methylbutyryl shikonin (17.9%)	98.8	R	97.7	R
	Acetylshikonin (34.9%)	49.6	R	49.2	R
	β-Hydroxyisovaleryl shikonin (21.4%)	98.5	R	97.7	R
	β,β-Dimethylacryl shikonin (9.7%)	99.3	R	98.6	R
Onosma confertum W. W.Smith (*O.c.*)	*β,β*-Dimethylacryl shikonin (60.6%)	100	R	100	R
	Acetylshikonin (14.6%)	24.5	R	23.7	R

[a] The content is defined as the ratio of the constituent isolated to the total mass of pure red pigments isolated.
[b] Values represent mean values (n = 2); in all cases relative error was lower than 8%.

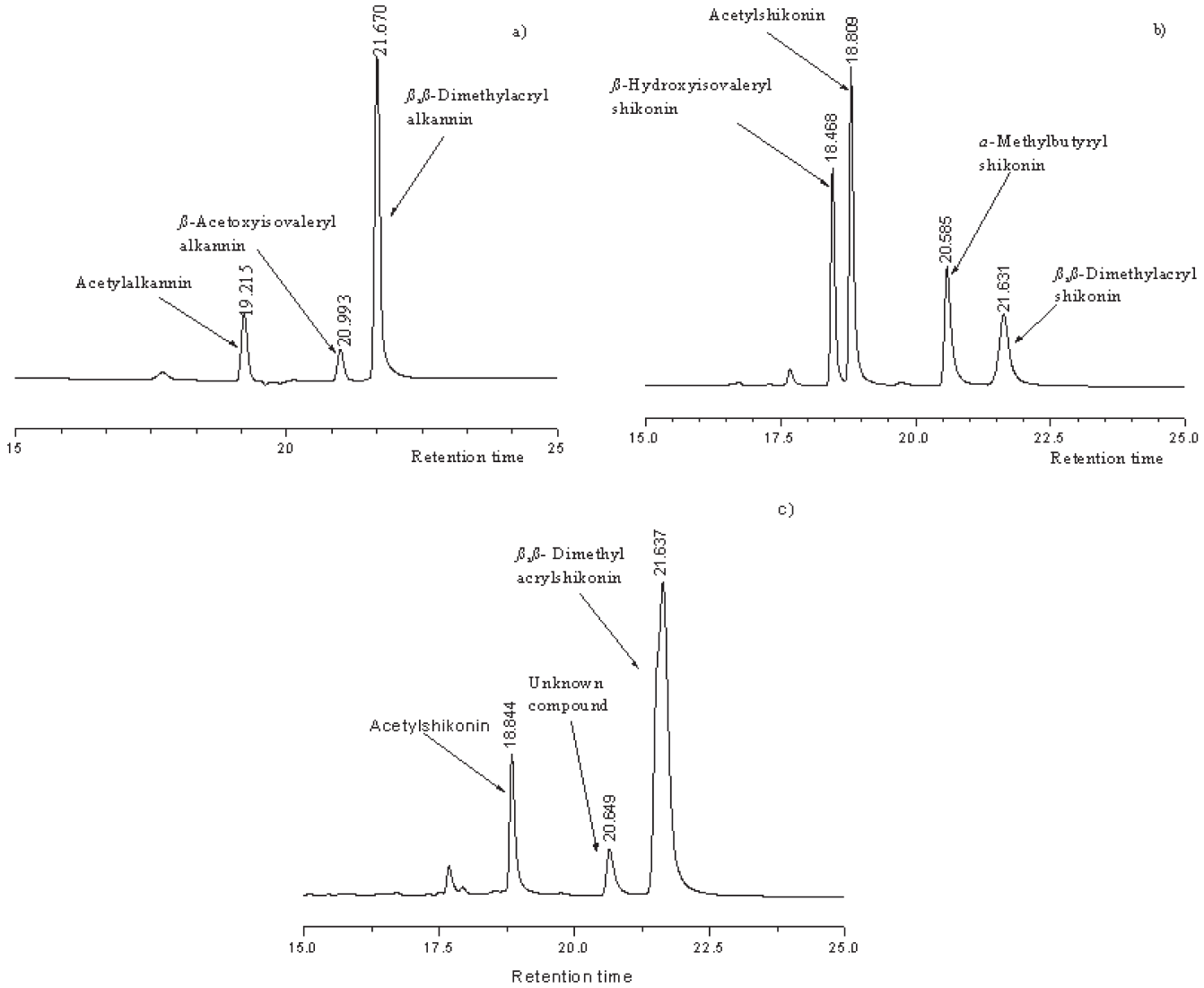

Figure 3. Chromatogram from RP-HPLC analysis of three Chinese Boraginaceae plants at retention time from 15 to 25 min: (a) main constituents of *A.e.*; (b) main constituents of *L.e.*; and (c) main constituents of *O.c.*

derivatives were displaced by acid, were transformed by hydrolysis into alkannin and shikonin.

Hydrolysis was performed as follows: 2.5 g of a pure A/S acyl derivative isolated from Boraginaceous roots obtained according to Approach I or Approach II, or 5 g of the extract from the Boraginaceae plants prepared in accordance with Approach III, was dissolved in 0.5 L of 1 mol/L NaOH and hydrolyzed for several hours while stirring at room temperature. The aqueous phase was then adjusted to pH 4 with 10% of HCl and extracted with ethyl acetate twice. The combined organic layer was washed with brine, then dried by anhydrous Na_2SO_4 and evaporated to dryness in *vacuo*, and finally re-crystallized with *n*-hexane. A suitable amount from the above-refined products was dissolved in a mixture of hexane and isopropanol (hexane–isopropanol 90:10 v/v) and subjected to the chiral-HPLC analysis of its optical purity.

Results and discussion

The isolation and preparation procedures of A/S and their ester derivatives from three Boraginaceae plants with the various enantiomeric ratios are depicted in Fig. 2. Supercritical carbon dioxide extraction was introduced to effectively extract the dry and pulverized roots of Boraginaceae plants with the use of methanolic entrainer, not with other solvents, primarily because of the application of ethanol or water reduced the extraction's efficiency (Manabe *et al.*, 1987). It was reported that the super critical CO_2 extraction had a great advantage over organic solvents regarding the efficiency of extraction and less destruction of A/S derivatives (Liang *et al.*, 2004). Subsequently the purposes of the three different treatments, which were designated as Approaches I–III, on the methanolic solution, shed light on the authentic relationship between the main A/S esters and A/S obtained from hydrolysis as for the absolute configurations and e.e. values, as well as the influence of acids on the chiral centers of A/S and their esters.

The contents of main components in the roots of *Arnebia euchroma* (Royle) Johnst (*A.e.*), *Lithospermum erythrorhizon* Sieb. et Zucc(*L.e.*) and *Onosma confertum* W. W. Smith (*O.c.*) analyzed are described in Table 1 together with their typical RP-HPLC profiles shown in Fig. 3. The results showed

that β,β-dimethylacrylalkannin, acetylalkannin and β-acetoxyisovalerylalkannin were mainly found in the roots of *A.e.* with the contents of 85.2, 0.6 and 0.25% respectively, while the roots of *L.e.* primarily contained acetylshikonin, β-hydroxyisovaleryl-shikonin, α-methylbutyrylshikonin and β,β-dimethylacrylshikonin with the contents of 34.9, 21.4, 17.9 and 9.7%, respectively. In addition, the roots of *O.c.* consisted principally of β,β-dimethylacrylshikonin and acetylshikonin with the contents of 60.6 and 14.6% respectively. This observation was well in agreement with the literature described by Ai *et al*. (1989).

Seven main A/S derivatives, isolated from the roots of three Chinese Boraginaceae plants, with the chemical purity of more than 99%, were analyzed for their absolute configurations and e.e. values using CD and chiral-HPLC respectively. As proved in Table 2, the positive cotton effect (CE) appeared at 405–330, 300–245 and 225–205 nm, respectively, while 330–300 and 245–225 nm gave the negative CE in the CD spectra of the main constituents from *L.e.* and *O.c.*, which was consistent with the *R*-form of shikonin as reported by Ai *et al*. (1993). However, the opposite CE at the correspondingly range was found in the CD of the main constituents from *A.e.*, and confirmed as *S*-form. The results of quantitative determinations for e.e. values in Table 1 further indicated that *A.e.* roots mainly produced the *S*-alkannin derivatives with 100% e.e., like β,β-dimethylacrylalkannin and β-acetoxyisovaleryl-alkannin, depicted in Fig. 4. Acetylalkannin was 30.8% e.e., whereas the roots of *L.e.* and *O.c.* predominantly biosynthesized *R*-shikonin derivatives with e.e. values of more than 98.5%, except for acetylshikonin with 49.6% e.e. and 24.5% e.e. respectively. Therefore, we may conclude that absolute configurations of naphthoquinone derivatives are usually associated with a specific Boraginaceae family, but their e.e. values directly relate to a definite derivative.

Since commercial alkannin and shikonin isolated from the roots of several Boraginaceous species are mainly in the form of their ester, hydrolysis of A/S derivatives is a key step for the yield from the roots. As shown in Table 1, the e.e. values and absolute configurations showed no significant change after or before hydrolysis, except for a very slight decrease which might be attributed to the formation of polymeric A/S during the transformation of monomeic (chiral) to polymeric A/S (Assimopoulou and Papageorgiou, 2004b). In fact, according to a previous report, the experimental result that hydrolysis of the extract from *Alkanna* species grown in Greece could obtain 100% e.e. alkannin proved no influence of the hydrolysis conditions on the e.e. values (Assimopoulou and Papageorgiou, 2004a).

On the other hand, another important conclusion could be also reached from Table 1, that a new approach, through hydrolysis of a specific derivative, to prepare the 100% e.e. of alkannin or shikonin was provided. For example, 100% e.e. of shikonin (Fig. 5b) could be easily obtained from β,β-dimethylacrylshikonin (Fig. 5a) from the roots of *O.c.* Similarly, 100% e.e. alkannin could be prepared by hydrolysis of β,β-dimethylacrylalkannin or β-acetoxyisovalerylalkannin, or a mixture of both (data not shown).

Acids are inevitably applied to prepare the A/S and their derivatives in the published articles (Jain and Mathur, 1965; Papageorgiou, 1978, 1979). In this study the influence of acids such as HCl on the e.e. values of the main shikonin derivatives from the roots of *L.e.* was also studied for the first time in Approaches I and II. As proved (Table 3, Fig. 6b, d), HCl used to displace Cu^{2+} from Cu-complex did not affect the alteration of the the A/S enantiomeric ratio of shikonin derivatives tested, but the very slight alternations were inside the limits of experimental error. As a result, the conclusion was drawn that acids such as HCl made no difference to A/S and A/S derivatives with regard to the absolute configurations and the e.e. values.

As shown in Tables 1 and 3, a good example was shikonin prepared from the roots of *L.e.* according to Approach III, which had e.e. values of 72.8% (Fig. 7), which were between 99.3% e.e. α-methylbutyrylshikonin and 49.6% e.e. acetylshikonin. The tendency was also observed in the roots of *A.e.* and *O.c.*, which had the 94.4% e.e. of alkannin and the 89.5% e.e. of shikonin. Interestingly, the careful analysis and calculation of the results obtained demonstrated that the e.e. value of A or S prepared by

Table 2. CD data of main constituents isolated from the roots of three Chinese Boraginaceae plants and their hydrolysis products

Compound	Determined items					
	C (g/mL) $\times 10^{-4}$	$[\theta]_{365\,nm}$	$[\theta]_{315\,nm}$	$[\theta]_{275\,nm}$	$[\theta]_{215\,nm}$	Absolute configuration
β,β-Dimethylacrylalkannin[a]	1.552	−2064.9	+950.0	−1739.5	−17915.9	*S*
Acetylalkannin[a]	1.657	−935.6	+284.0	−422.1	−3939.9	*S*
β-Acetoxyisovalerylalkannin[a]	1.634	−2255.7	+764.4	−1812.8	−13573.3	*S*
Alkannin[a]	1.411	−3324.9	+1338.9	−2987.8	−12922.4	*S*
α-Methylbutyrylshikonin[b]	1.644	+2926.1	−2069.8	+1816.0	+9682.5	*R*
Acetylshikonin[b]	1.739	+1617.8	−1010.2	+882.1	+4304.5	*R*
β-Hydroxyisovalerylshikonin[b]	1.782	+1060.4	−728.7	+798.0	+9306.2	*R*
β,β-Dimethylacrylshikonin[b]	1.402	+2364.2	−973.2	+1853.9	+18353.3	*R*
Shikonin[b]	1.323	+3086.1	−1914.8	+3263.6	+12929.2	*R*
β,β-Dimethylacrylshikonin[c]	1.452	+2384.2	−983.4	+1863.7	+18373.8	*R*
Acetylshikonin[c]	1.753	+1419.3	−996.8	+796.6	+3983.1	*R*
Shikonin[c]	1.361	+3484.5	−1774.0	+3064.3	+13121.8	*R*

[a] The compounds isolated from the roots of *A.e.* according to Approach I or their hydrolysis products by Approach III. [b] The compounds isolated from the roots of *L.e.* according to Approach I or their hydrolysis products by Approach III. [c] The compounds isolated from the roots of *O.c.* according to Approach I or their hydrolysis products by Approach III. *C*, concentration; [θ], molecular ellipticity.

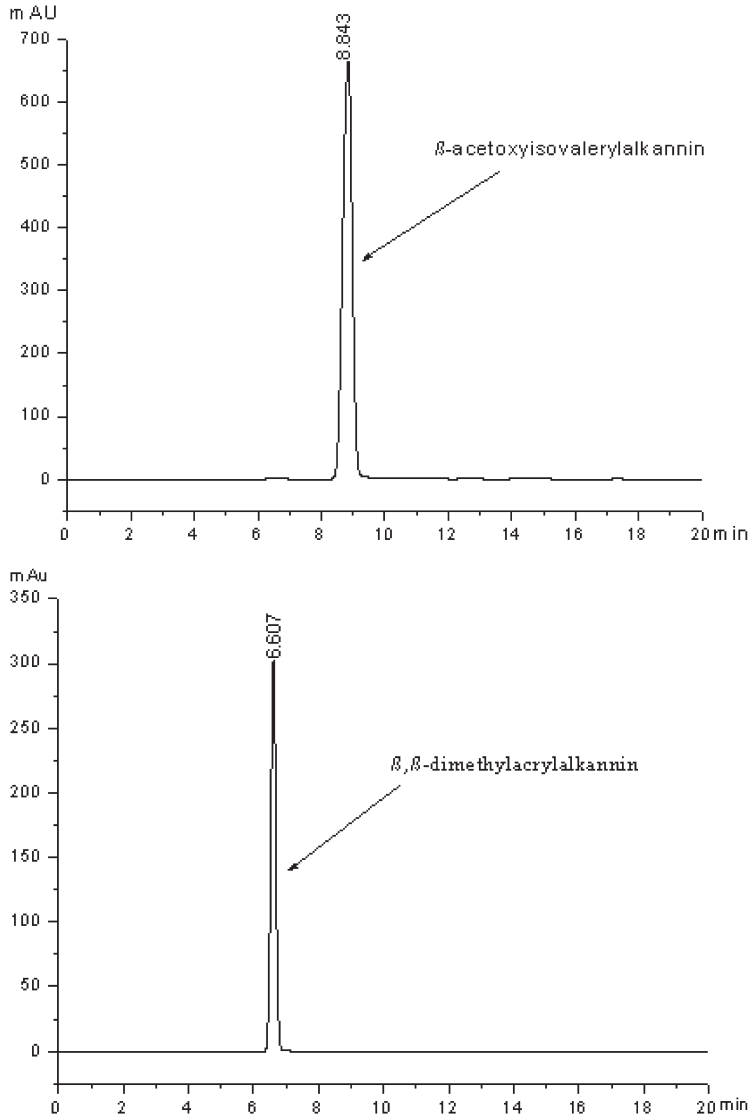

Figure 4. Chromatogram from chiral-HPLC analysis of β-acetoxyisovalerylalkannin and β,β-dimethylacrylalkannin (100%, e.e.) isolated from the roots of *A.e.* according to Approach I.

Table 3. Effect of three approaches on the e.e. values of main constituents of the CO_2 preliminary extract from *Lithospermum erythrorhizon* Sieb. et Zucc

Main constituents	e.e. value		
	Approach I[a]	Approach II[a]	Approach III[a]
α-Methylbutyryl shikonin	98.8%	98.1%	—
Acetylshikonin	49.6%	48.8%	—
β-Hydroxyisovaleryl shikonin	98.5%	97.2%	—
β,β-Dimethylacryl shikonin	99.3%	98.8%	—
Shikonin	—	—	72.8%

[a] Values represent mean values (n = 2); in all cases relative error was lower than 8%.

Approach III positively correlated with the contents of acetyl-A/S. Therefore we came to the conclusion that all the commercial mixtures of alkannin and shikonin came mainly from the Boraginaceae plants themselves; on the other hand, the e.e. values of acetyl- A/S may act as a chemotaxonomic criterion for the determination of the origin of Boraginaceous family distributed extensively in China (Fig. 6a–c), since the commercial root samples were not botanically identified and the samples

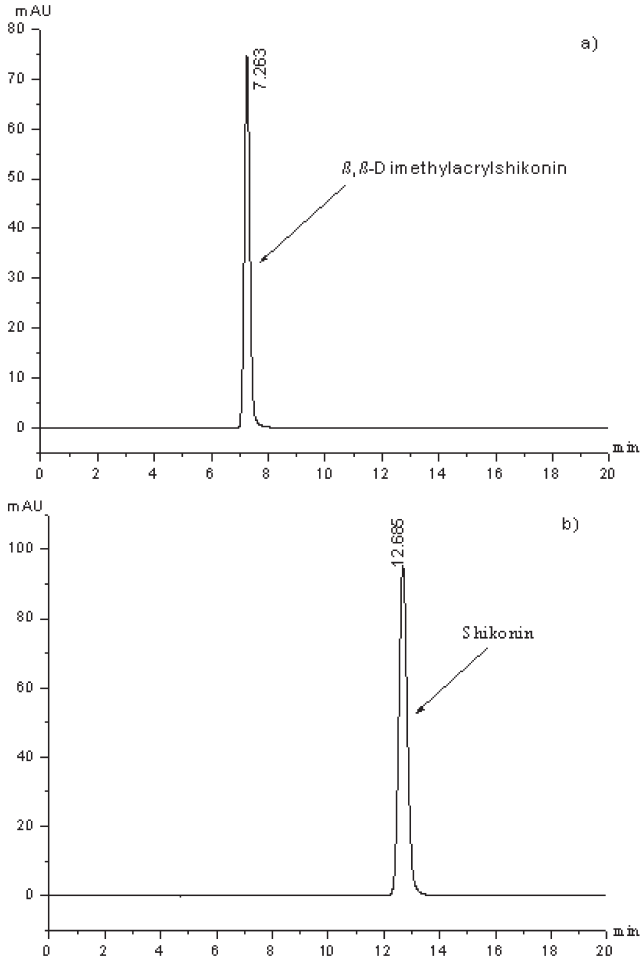

Figure 5. Chromatogram from chiral-HPLC analysis of (a) *β,β*-dimethylacryl- shikonin isolated by silica gel column from the roots of *O.c.* according to Approach I, and (b) shikonin prepared from *β,β*-dimethylacrylshikonin by hydrolysis.

possessing S : A proportions of 75:25 from China must be *L.e.* and not *A.e.* and *O.c.*

Conclusions

In the present research seven main A/S derivatives were isolated from the roots of three kinds of Boraginaceous plants extensively distributed in China, and then analyzed for their e.e. values and absolute configurations using chiral HPLC and CD, respectively. The results showed that *A.e.* roots grown in Xinjiang area mainly consisted of alkannin derivatives, while *L.e.* and *O.c.* roots cultivated in Liaoning and Yunnan regions respectively contained primarily shikonin derivatives. This amended the observations reported by Assimopoulou (Papageorgiou *et al*., 1999) that all Boraginaceous species that are grown in Europe mainly contain alkannin derivatives, whereas those grown in Asia mainly contain shikonin derivatives.

In addition, the influences of hydrolysis conditions and acids on the e.e. values and absolute configurations of the main A/S ester derivatives isolated from the roots of three Boraginaceous plants were also investigated. It was found that, during hydrolytic treatments like Approach I and acid treatment such as Approach

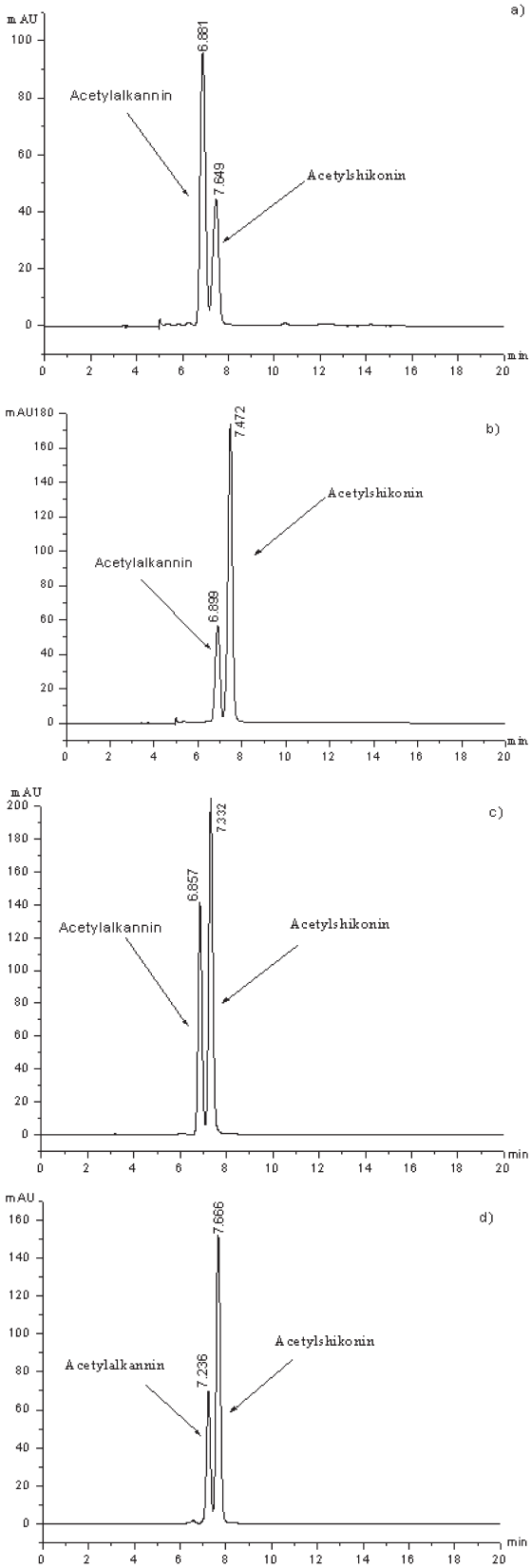

Figure 6. Chromatogram from chiral-HPLC analysis of acetyl-A/S isolated from three kinds of Boraginaceae roots according to Approach IL (a) acetylalkannin from *A.e.*, (b) acetylshikonin from *L.e.*, (c) acetylshikonin from *O.c.* and (d) acetylshikonin from *L.e.* after HCl treatment.

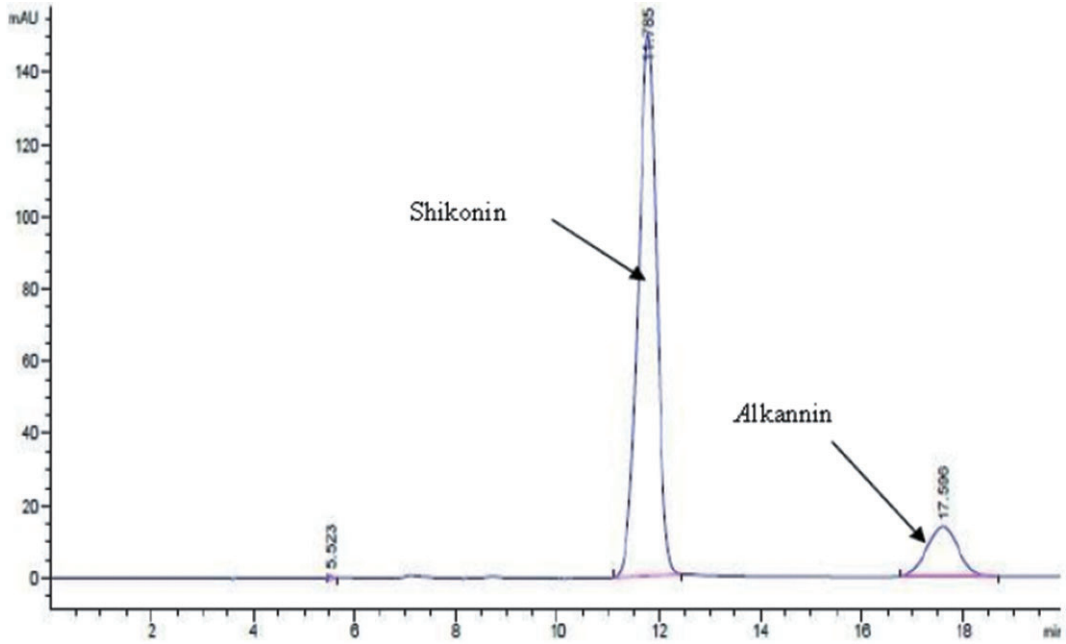

Figure 7. Chromatogram from chiral-HPLC analysis of A/S prepared from the extract of the roots of *L.e.* according to Approach III.

II, the e.e. values and absolute configurations were not affected significantly.

Finally, the results of the comparative study on three treatments of methanolic solution from the roots of Boraginaceous plants demonstrated that the e.e. values and absolute configurations of commercial A/S depended on the specific Boraginaceous family, and were positively correlated to acetyl-A/S, which may act as a chemotaxonomic criterion for determination of the origin of the Boraginaceous family, which is distributed extensively in China. More importantly, a novel approach for preparing the 100% e.e. A or S was found through hydrolysis of a specific acyl derivative from the roots of Boraginaceous species.

Acknowledgements

We gratefully acknowledge National Natural Science Foundation of China (nos 30973604 and 91013012), the Shanghai Program on Key Basic Research (no. 08JC1410800) and Comprehensive Technology Platforms for Innovative Drug R&D (2009ZX09301-007) for financial support.

References

Ai KH, Li FY, Li Y, Wang WB and Wu YY. Studies on the naphthoquinone constituents of *Onosma Confertum W. W. Smithe* and quantitative determination of shikonin. *Acta Botanica Sinica* 1989; **31**: 549–553.

Ai KH, Jin J and Liu H. Studies on CD spectra derivatives of shikonin and alkannin. *Acta Pharmaceutica Sinica* 1993; **28**: 282–285.

Assimopoulou AN and Papageorgiou VP. Study on the enantiomeric ratio of the pharmaceutical substance alkannin and shikonin. *Biomedical Chromatography* 2004a; **18**: 794–799.

Assimopoulou AN and Papageorgiou VP. Study on isohexenylnaphthazarins polymerization in alkaline media. *Biomedical Chromatography* 2004b; **18**: 508–522.

Brockmann H. Die Konstitution des Alkannins, Shikonins und Alkannans. *Annalen der Chemie, Justus Liebigs* 1936; **521**: 1–47.

Fukui H, Tsukada M, Mizukami H and Tabata M. Formation of stereoisomeric mixtures of naphthoquinone derivatives in *Echium lycopsis* callus cultures. *Phytochemistry* 1983; **22**: 453–456.

Jain AC and Mathur SK. Pigment of *Arnebia hispidissima*. *Bulletin of the National Institute of Sciences of India* 1965; **28**: 52–56.

Liang RH, Xie MY and Shi YF. Making a comparison between supercritical CO_2 extracted *Amebia Euchroma (Royle) John* pigment and organic solvents. *Food Science* 2004; **25**: 130–132.

Liu J, Zhou W, Li SS, Sun Z, Lin BZ, Lang YY, He JY, Cao XH, Yan TD, Wang L, Lu JM, Han YH, Cao Y, Zhang XK and Zeng JZ. Modulation of orphan nuclear receptor Nur77-mediated apoptotic pathway by acetylshikonin and analogues. *Cancer Research* 2008; **68**: 8871–8880.

Manabe A, Tokumori T, Sumida Y, Yoshida T, Hatano T, Yazaki K and Okuda T. Application of supercritical fluid extraction to components of crude drugs and plants. III. Extraction of pigments from *Lithospermum* root and *licorice* root. *Yakugaku Zasshi* 1987; **107**: 506–510.

Mellidis AS and Papageorgiou VP. Naphthazarins from *Onosma heterophylla*. *Journal of Natural Products* 1987; **50**: 618–619.

Papageorgiou VP. Study on the structure of the components of *Alkanna Tinctoria Tausch*. *Chimika Chronika* 1978; **7**: 45–54.

Papageorgiou VP. Naphthoquinones from roots of *Macrotomia cephalotes* DC. *Planta Medica* 1979; **37**: 259–263.

Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D and Nicolaou KC. The chemistry and biology of alkannin, shikonin and related naphthazarin natural products. *Angewandte Chemie, International Edition English* 1999; **38**: 270–300.

Papageorgiou VP, Assimopoulou AN, Samanidou VF and Papadoyannis IN. Analytical methods for the determination of alkannins and shikonins. *Current Organic Chemistry* 2006; **10**: 583–622.

Wang WJ, Dai M, Zhu CH, Zhang JG, Lin LP, Ding J and Duan WH. Synthesis and biological activity of novel shikonin analogues. *Bioorganic and Medicinal Chemistry Letters* 2009; **19**: 735–737.

Xuan YY and Hu X. Naturally-occurring shikonin analogues—a class of necroptotic inducers that circumvent cancer drug resistance. *Cancer Letters* 2009; **274**: 233–242.