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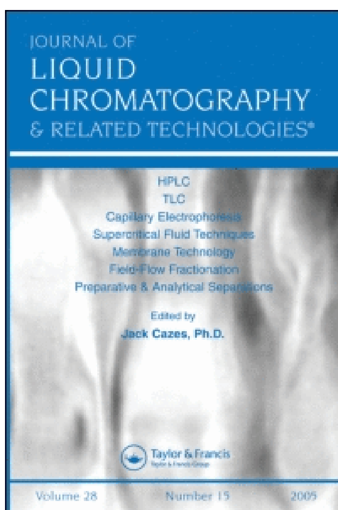

Journal of Liquid Chromatography & Related Technologies
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To cite this Article Inoue, Koichi , Nomura, Chihiro , Mizuno, Yasuomi , Yoshimi, Yukiko , Tsutsumiuchi, Kaname , Hino, Tomoaki and Oka, Hisao(2008) 'Separation of Major Safflowers from *Carthamus* Yellow using High-Speed Countercurrent Chromatography', Journal of Liquid Chromatography & Related Technologies, 31: 7, 1047 — 1059
To link to this Article: DOI: 10.1080/10826070801925011
URL: http://dx.doi.org/10.1080/10826070801925011

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Journal of Liquid Chromatography & Related Technologies®, 31: 1047–1059, 2008
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ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070801925011

Separation of Major Safflowers from *Carthamus* Yellow using High-Speed Countercurrent Chromatography

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Abstract: High-speed countercurrent chromatography (HSCCC) has been successfully applied to the separation of safflower A (SF-A) and safflower B (SF-B) from *Carthamus* yellow, *Carthamus tinctorius* L. A 25 mg quantity of *Carthamus* yellow was separated using a two-phase solvent composed of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.5% aqueous trifluoroacetic acid solution (2/2/1/5, V/V). The total separation time was 6 hr with the total elution volume of 720 mL. HSCCC fractions were analyzed using LC/MS/MS with scan and daughter scan modes, and DAD monitored by wide absorbance from 200 to 500 nm. The separation yielded 1.5 mg of SF-A (95% > purity) and 1.1 mg of SF-B (95% > purity). These results present a successful application of HSCCC to the preparative purification of major safflowers.

Keywords: Carthamus yellow, Safflower, High-speed countercurrent chromatography, Liquid chromatography-tandem mass spectrometry

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INTRODUCTION

Safflower, *Carthamus tinctorius* L., is a well known herb and natural colorant matter. The yellow safflower pigment has been used as a natural food colorant for a long time all over the world. Recently, in an effort to become more health conscious, many food manufacturers have been switching from artificial dye to natural food colorants. Due to this trend toward the use of natural pigments, *Carthamus* yellow has received growing importance as a food colorant.

Carthamus tinctorius L. is a widely used traditional plant medicine having many biological effects. The chemical components from *Carthamus tinctorius* L. have been identified to be flavonoids, polyacetylenes, serotonin derivatives, steroids, flavanone, and acetylenic glucosides.[1–6] In addition, the studies reported that the chemicals from *Carthamus tinctorius* indicated various biological effects.[7,8] Therefore, it is important to establish global standards for the safety assessments and analytical methods of food additives such as *Carthmaus* yellow. It was reported that the yellow pigment has numerous components with safflomin A (SF-A) and safflomin B (SF-B) being the major ones.[9,10] The structures of SF-A and SF-B are shown in Figure 1 from references.[9–11] Reversed-phase thin-layer chromatography (TLC) and liquid chromatography with electrospray-mass spectrometry (MS) and photodiode array detection (DAD) can be used for the integrated analytical methods of these components.[12,13] On the other hand, no fully validated analytical method has been reported using the reference

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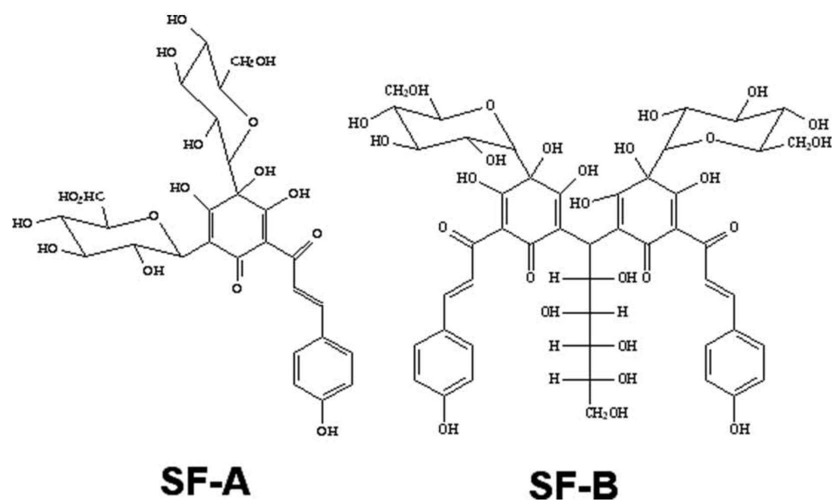

Figure 1. Structural formulae of the main compounds as safflomin A (SF-A: MW. 612) and safflomin B (SF-B: MW. 1062) in *Carthamus* yellow.

standards of SF-A and -B. Therefore, an efficient and effective isolation of major safflowers from *Carthamus* yellow is required.

High-speed countercurrent chromatography (HSCCC) has been used for the separation and isolation of natural components.[14–18] This HSCCC approach of separating ability can prove many reference standards such as antibiotic and food color additives to be on a scale suitable for HPLC analysis.[14,19–24] Therefore, this advanced HSCCC approach is useful for obtaining pure safflowers. In this study, major safflowers such as SF-A and -B were purified using HSCCC and each component was identified using liquid chromatography with tandem mass spectrometry (LC/MS/MS).

EXPERIMENTAL

Reagents

Carthamus yellow was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) and Wako Chemical Co., Inc. (Osaka, Japan). The yellow safflower pigment for food additives (*Carthamus* yellow 70%, ethanol 20%, and dextrin 10%) was obtained from San-Ei Gen F.F.I., Inc. (Osaka, Japan). HPLC grade water, *n*-hexane, ethyl acetate, acetonitrile, *n*-butanol, methanol, formic acid (FA; 99%, LC/MS-grade), and trifluoroacetic acid (TFA; 98%) were obtained from Wako Chemical Co., Inc. HPLC grade *tert*-butyl methyl ether was obtained from Sigma-Aldrich (St. Louis, MO, USA).

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HPLC Analysis of Carthamus Yellow

High performance liquid chromatography (HPLC) was performed using a LC-20AB pump, SPD-20AV detector, CTO-20AC column oven with injector, and C-R8A recorder system (Shimadzu Co., Kyoto, Japan).

The column was a TSK-GEL ODS 100 V (4.6 × 150 mm, 3.0 μm, Tosoh Co., Tokyo, Japan) for the separation of major safflowers from *Carthamus* yellow. The mobile phase for HPLC analysis consisted of 0.1% FA in water (Solvent A), 0.1% FA in acetonitrile (Solvent B), with flow rate of 1.0 mL/min. Column temperature was 40°C. The sample volume of 10 μL was injected. HPLC linear gradient was as follows: 13% Solvent B at 0 min, 60% B at 20 min, 60% B at 25 min, and 13% B at 25.1 min. The elution of major safflowers was monitored by visible absorbance 405 nm.

Concentrated solutions (1.0 mg/mL) of *Carthamus* yellow (Kanto Chemical Co. and Wako Chemical Co.) were prepared in methanol/water (50/50, V/V), and dilutions were made at 50 μg/mL as required by the addition of methanol/water (50/50, V/V).

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HSCCC Isolation of Carthamus Yellow

High-speed countercurrent chromatography (HSCCC) was performed using an HSCCC-1A prototype model (multi-layer coil planet centrifuge, Shimadzu Co., Kyoto, Japan) with a 10 cm orbital radius that produces a synchronous type-J planetary motion at maximum speed of 800 rpm. The multilayer coil was prepared by winding a ca. 160 m length of PTFE tubing onto the column holder with a 10 cm hub diameter and a 15 cm hub length, making six coiled layers with a total capacity of 270 mL.

Four milligrams of the *Carthmaus* yellow pigment was added to the two mutually equilibrated solvent phases (2 mL each; see Table 1) in a test tube, and mixed to equilibrate. After settling, equal volumes of the upper and lower phases were transferred into separate test tubes and diluted, each with an equal volume of water/methanol (5/5, V/V). Each phase was assessed by HPLC and the area of each peak was used to determine the partition coefficient (K) values for each component. The K value was calculated as follows:

$$K = \frac{\text{HPLC peak area of solute in upper phase}}{\text{HPLC peak area of solute in lower phase}}$$

The two phases were mutually saturated by shaking in a separatory funnel and separated immediately before use. Either phase can serve as the mobile or stationary phase depending on the direction of the column rotation.

The two-phase solvent system composed of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.5% aqueous TFA solution (2/2/1/5, V/V, total 2 L) was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking three times each, followed by inverting the vessel and manipulating its stopcock at room temperature. The column was first entirely filled with the upper non-aqueous stationary phase, then a 25 mg of the *Carthamus* yellow from Kanto Chemical Co. dissolved in 1 mL of each phase was loaded. The column was rotated at 780 rpm, while the lower aqueous mobile phase was pumped into the head of the column (the head-tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects with different densities, either lighter or heavier than the surrounding medium, are driven toward the head of the coil) at a flow rate of 2 mL/min by a HPLC pump (LC-6A, Shimadzu Co., Kyoto, Japan). The effluent from the outlet of the column was fractionated into test tubes at 2 min per tube using a fraction collector (Model 2128 Fraction Collector, Bio-Rad Laboratories, Inc., NY, USA). After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing then out of the column with pressurized nitrogen gas under slow coil rotation in the tail-to-head elution mode.

These HSCCC fractions of the *Carthmaus* yellow pigment were analyzed using flow injection analysis (FIA). This system was performed using a LC-

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Table 1. The partition coefficient ratios (K) of major safflowers from *Carthamus* yellow for different two-phase solvent systems

Two-phase solvents	Concentration of acid in water (%)	Ratio	Partition coefficient (K)[a]			
			1	2	3	4
n-Hexane/ethyl acetate/n-butanol/methanol/water	NA[b]	5/5/0/5/5	0	0	0	0
	NA	0/4/1/0/5	0	0	0.03	0.83
	NA	0/0/5/0/5	0.05	0.08	0.37	∞[c]
	0.1% FA[d]	0/0/5/0/5	0.13	0.36	1.81	∞
	0.5% FA	0/0/5/0/5	0.23	0.79	2.61	∞
	0.1% TFA[e]	0/0/5/0/5	0.28	0.92	3.26	∞
tert-butyl methyl ether/n-butanol/acetonirile/water	NA	2/2/1/5	0	0	0.06	2.46
	0.1% FA	2/2/1/5	0.06	0.15	0.59	2.61
	0.5% FA	2/2/1/5	0.10	0.29	0.97	3.14
	1.0% FA	2/2/1/5	0.13	0.39	1.04	3.02
	2.0% FA	2/2/1/5	0.16	0.36	1.17	2.72
	0.1% TFA	2/2/1/5	0.16 ± 0.02[f]	0.34 ± 0.02	1.24 ± 0.11	2.66 ± 0.54
	0.5% TFA	2/2/1/5	0.27 ± 0.01	0.50 ± 0.11	1.54 ± 0.03	3.13 ± 0.12
	1.0% TFA	2/2/1/5	0.26 ± 0.02	0.50 ± 0.06	1.40 ± 0.12	2.82 ± 0.49

[a]Partition coefficient (K) was a value of "area of solute in upper phase/area of solute in lower phase".
[b]Not addition was abbreviated as NA.
[c]Not detection of solute in lower phase means infinitely great.
[d]Formic acid was abbreviated as FA.
[e]Trifluoroacetic acid was abbreviated as TFA.
[f]Values were mean $\pm$ standard deviation (SD, n = 3).

20AD pump, SPD-20AV detector, SIL-20AC autosampler, and C-R8A recorder system (Shimadzu Co., Kyoto, Japan). The solvent for FIA was 0.1% FA in water/0.1% FA in acetonitrile (50/50, V/V) with flow rate of 1.0 mL/min. These HSCCC fractions were monitored at 405 nm.

LC/MS/MS Analysis of CARTHAMUS Yellow Components

LC analyses were performed with a Waters Ailiance 2695 system (Waters, Milford, MA, USA). LC separation was achieved on a TSK-GEL ODS 100 V (2.0 × 150 mm, 3 μm: Tosoh Co., Tokyo, Japan) maintained at 40°C, and the mobile phase consisted of 0.1% FA in water (Solvent A) and 0.1% FA in acetonitrile (Solvent B). LC linear gradient was as follows: 13% Solvent B at 0 min, 60% B at 15 min, 90% B at 15.1 min, 90% B at 20 min, and 13% B at 20.1 min, with flow rate of 0.2 mL/min. The sample was injected with an injection volume of 10 μL. The separated compounds were detected with a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The mass spectrometer was operated with an electrospray source in positive ionization mode. The ionization source conditions were: capillary voltage of 3.0 kV, extractor of 4 V, RF lens of 0 V, source temperature of 110°C and desolvation temperature of 400°C. The cone and desolvation gas flows were 50 L/hr and 850 L/hr, respectively, and were obtained from the nitrogen source (N_2 Supplier Model 24S, Anest Iwata Co., Yokohama, Japan). Argon was used as collision gas and was regulated at 0.35 mL/hr and the multipliers were set to 650 V.

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RESULTS AND DISCUSSION

Partition Coefficients of Carthamus Yellow Components

The *Carthamus* yellow usually contains several dye components, which have different structures and are mixed together proportionally. These compounds could be separated on C_{18} based column due to their polarities, hence, reversed-phase high performance chromatography (HPLC) was used for the analysis of major components.[13] In the HPLC analysis of *Carthamus* yellows (50 μg/mL for Kanto and Wako Co., and 100 times dilution for San-Ei Gen F.F.I.), the chromatogram showed the four main peaks at absorbance of 405 nm (Figure 2). UV-vis absorption spectra of the four peaks were shown in Figure 3, in which maximum absorption wavelengths were observed at 405 nm. Therefore, we decided to monitor the components of *Carthamus* yellows at 405 nm in HPLC and to measure the partition coefficient (K) of the four peaks using HPLC chromatograms to select a two-phase solvent system of HSCCC.

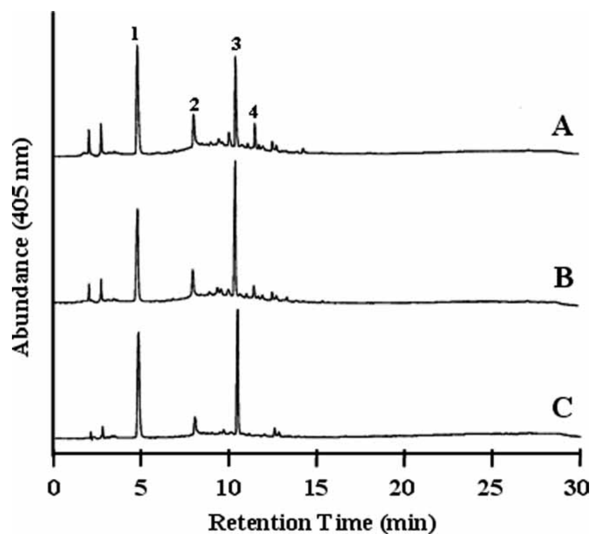

Figure 2. HPLC chromatograms of *Carthamus* yellow standards. A: *Carthamus* yellow standard for food analysis (Kanto), B: *Carthamus* yellow standard for food analysis (Wako), C: Yellow safflower pigment for food additives (*Carthamus* yellow 70%, ethanol 20%, and dextrin 10%, San-Ei Gen F.F.I.).

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To achieve successful HSCCC separation of the components, the two-phase solvent system should satisfy the following requirements: (1) the settling time of the two-phase solvent system with samples should be shorter than 30 seconds; (2) the partition coefficient (K_n) value of target compounds should be close to about 1.0; (3) the separation factor ($\alpha = K_m/K_n$, $K_m > K_n$) should be greater than 1.5; (4) the two-phase solvents are nearly equal volumes of each phase; (5) the two-phase solvents are a volatile solvent system. In this study, K values of the four main compounds were determined in the following two solvent systems: *n*-hexane/ethyl acetate/*n*-butanol/methanol/water (added FA or TFA) and *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (added FA or TFA) at various volume ratios. Table 1 shows that *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.5 or 1.0% aqueous TFA solution (2/2/1/5, V/V) could be used to separate the four main components by evaluation of K values. This solvent composition proves the satisfactory reproducible K values. In addition, Table 2 shows α values of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0, 0.1, 0.5, and 1.0% aqueous TFA solution (2/2/1/5, V/V), indicating that α values of aqueous TFA solutions were greater than 1.5. The settling time is less than 30 sec using *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.5% aqueous TFA solution (2/2/1/5, V/V) system, which ensures a satisfactory retention level of the stationary phase in HSCCC.[25] Based on these results, we

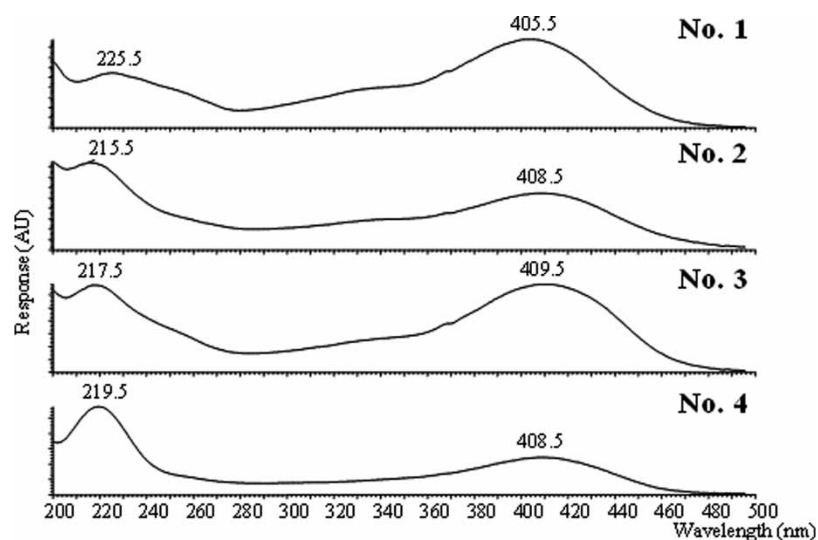

Figure 3. UV-vis spectra of the main components (No. 1–4, see Figure 2) of *Carthamus* yellow standard using HPLC separation.

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decided to use *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.5% aqueous TFA solution (2/2/1/5, V/V) for separation of *Carthamus* yellow.

Isolation of Carthamus Yellow Components by HSCCC

A 25 mg quantity of *Carthamus* yellow was separated using the above solvent system. The retention of the stationary phase was 75.7%. The total separation time was 6 hr with the total elution volume of 720 mL.

Table 2. The separation factor (α) of major safflowers from *Carthamus* yellow using *tert*-butyl methyl ether/*n*-butanol/asetonitorile/water (2/2/1/5, V/V) added TFA

Concentration of TFA in water (%)	Separation factor (α)[a]		
	K_2/K_1	K_2/K_3	K_3/K_4
NA	NC[b]	NC	41
0.1	2.1	3.6	2.1
0.5	1.8	3.1	2.0
1.0	1.9	2.8	2.0

[a]Separation factor (α) was a value of "ratio of partition coefficient (K_m/K_n, $K_m > K_n$)". The K of compound No.1 was abbreviated as K_1.

[b]The NC shows that a value was not calculated using these K.

The HSCCC elution curve of the *Carthamus* yellow components monitored at 405 nm using flow injection analysis (FIA) is shown in Figure 4. Based on this elution curve, the collected fractions were combined into nine pooled fractions (No. A-I, see Figure 4). The amounts of fractions were 13.1 mg (Fraction A), 1.0 mg (Fraction B), 1.5 mg (Fraction C), 0.7 mg (Fraction C), 0.7 mg (Fraction D), 1.5 mg (Fraction E), 0.6 mg (Fraction F), 1.1 mg (Fraction G), 2.8 mg (Fraction H), and 1.1 mg (Fraction I), respectively.

LC/MS/MS Analysis of HSCCC Fractions from Carthamus Yellow

The HSCCC fractions of *Carthamus* yellow were analyzed using LC/MS/MS with scan and daughter scan modes, and DAD monitored by wide absorbance from 200 to 500 nm. The respective LC/DAD (405 nm) chromatograms of the isolated fraction from A to I are shown in Figure 5. Based on these LC/DAD chromatograms of the fractions, isolated components from *Carthamus* yellow were expected to be compound No. 1 from fraction C, No. 3 from fraction G, and No. 4 from fraction I, respectively. The purities were estimated to be 95% (100% by abundance of 405 nm). The UV-vis absorption spectra of the pure

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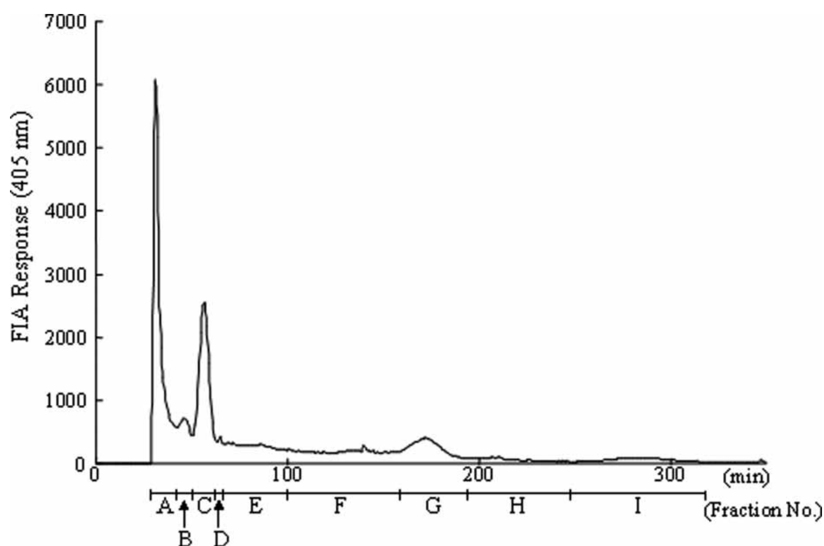

Figure 4. Separation, elution curve, and fraction ranges (A-I) of *Carthamus* yellow by HSCCC. the optimal HSCCC solvent of *tert*-butyl methyl ether/n-butanol/acetonitrile/0.5% TFA (2/2/1/5, V/V). Fraction A from 30 to 41 min, Fraction B from 42 to 50 min, Fraction C from 51 to 61 min, Fraction D from 62 to 72 min, Fraction E from 73 to 100 min, Fraction F from 101 to 156 min, Fraction G from 157 to 191 min, Fraction H from 192 to 259 min, and Fraction I from 260 to 310 min.

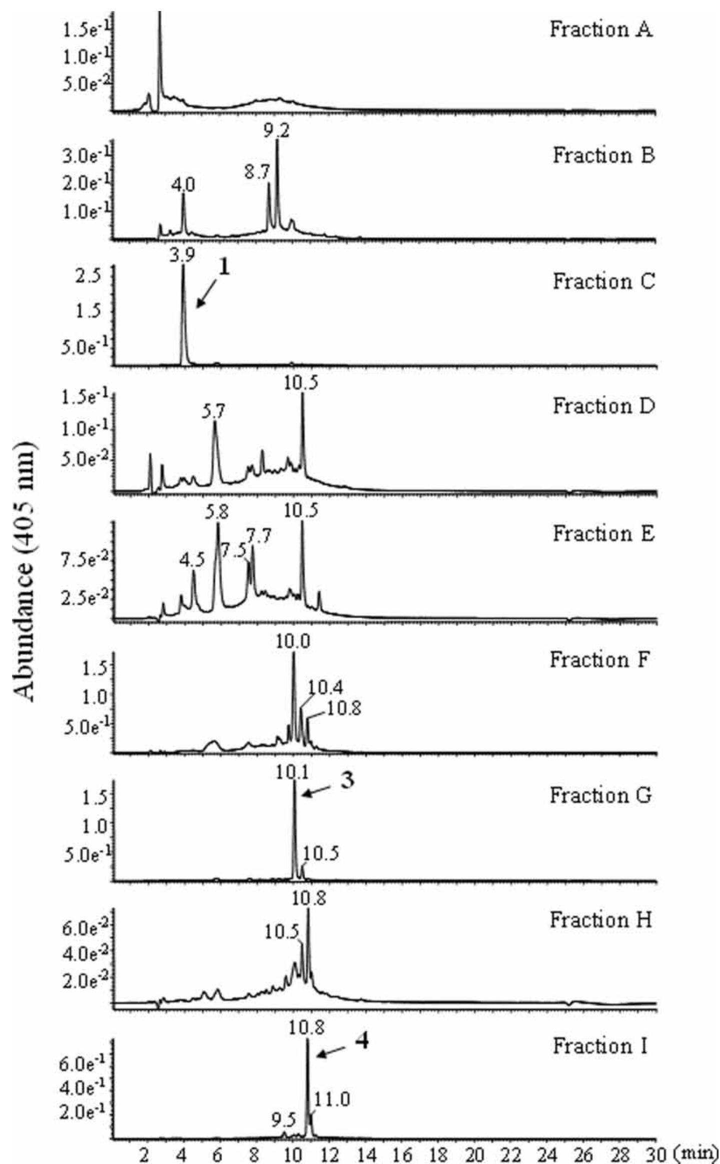

Figure 5. LC/DAD chromatograms of HSCCC fractions (A-I, see Figure 4) from *Carthamus* yellow. These HSCCC fractions were monitored by visible absorbance 405 nm.

components gave the same spectra as shown in Figure 3. Then, the LC/MS/MS spectra of isolated compounds (No. 1, 3, and 4) were measured using MS scan and MS/MS daughter scan modes (Figure 6). Based on UV-vis, MS, and MS/MS spectra of No. 1 (Figure 6, A and B), this compound of No. 1 was

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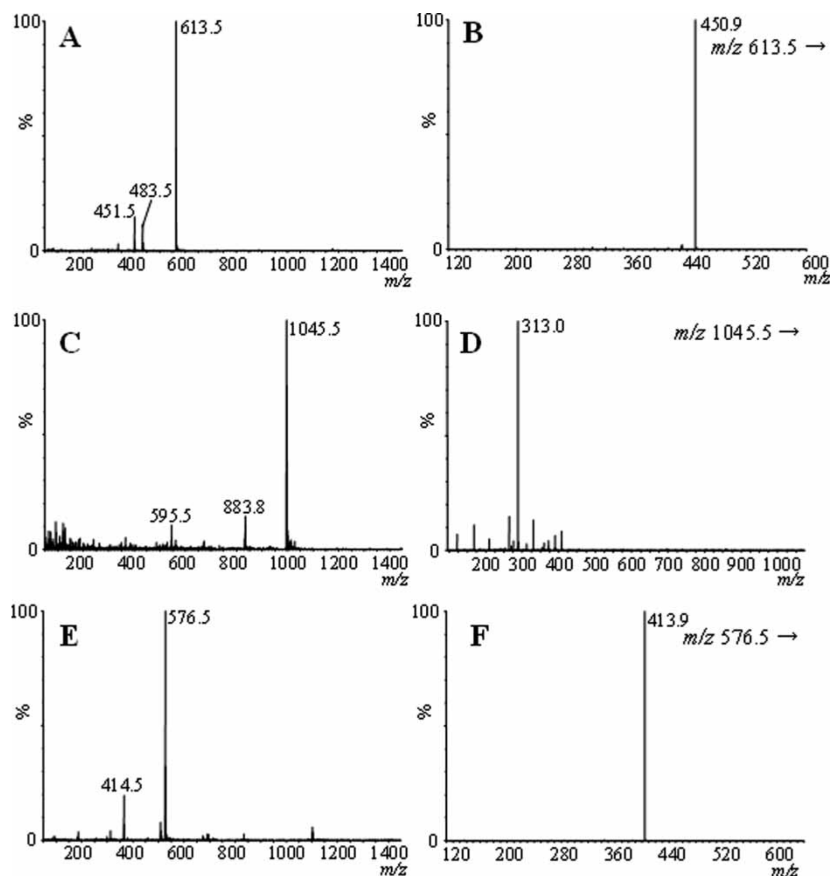

Figure 6. LC/MS/MS spectra of purity components of No. 1 (fraction C), 3 (fraction G), and 4 (fraction I) from Carthamus yellow. A; MS scan mode ranged from m/z 100 to 1500 of fraction C (compound of No. 1), B; MS/MS daughter scan mode from m/z 613.5 of fraction C (compound of No. 1), C; MS scan mode ranged from m/z 100 to 1500 of fraction G (compound of No. 3), D; MS/MS daughter scan mode from m/z 1045.5 of fraction G (compound of No. 3), E; MS scan mode ranged from m/z 100 to 1500 of fraction I (compound of No. 4), B; MS/MS daughter scan mode from m/z 576.5 of fraction I (compound of No. 4), MS cone voltage of 20 V and MS/MS collision energy of 30 eV.

identified to be SF-A. The MS spectrum of SF-A on positive mode shows a main ion at m/z 613.5 $[M+H]^+$ (Figure 6, A). MS/MS spectrum from m/z 613.5 shows a product ion at m/z 450.9 $[M+H\text{-glycerol}]^+$ (Figure 6, B).[11] The spectrum of compound No. 3 shows a main ion at m/z 1045.5 $[M\text{-}H_2O+H]^+$ (Figure 6, C), and MS/MS spectrum from m/z 1045.5 shows a product ion at m/z 313.0 indicating that the compound of No. 3 is SF-B. Therefore, reference standards of SF-A (No. 1) and B (No. 3) was

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able to be obtained for HPLC analysis. In addition, MS spectrum of No. 4 shows a main ion at m/z 576.5 (Figure 6, E). MS/MS spectrum of No. 4 from m/z 576.5 shows a product ion at m/z 413.9 (Figure 6, F). We expect that the compound of No. 4 could be di-dehydration of SF-A.

CONCLUSION

In conclusion, we were able to purify SF-A and SF-B using HSCCC with the two-phase solvent system composed of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.5% aqueous TFA solution (2/2/1/5, V/V). The overall results indicate that this approach of HSCCC separation and LC/MS/MS identification is a powerful technique for the development of analytical methods for the regulation of global standards.

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Received October 10, 2007
Accepted December 15, 2007
Manuscript 6217

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