Separation of Five Isomers of Dihydroxybenzoic Acid by High-Speed Counter-Current Chromatography with Dual-Rotation Elution Method

Dan Liu[1], Zhiguo Su[2], Changhai Wang[1], and **Ming Gu[2,*]**

[1]School of Environmental and Biological Science and Technology, Dalian University of Technology and [2]National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences

Abstract

Five small molecular isomers, 2,3-, 2,4-, 2,6-, 3,4-, and 3,5-dihydroxybenzoic acid, were successfully separated in one step with solvent system *n*-hexane–ethyl acetate–methanol–water (1:5:1.5:5) on high-speed counter-current chromatography (HSCCC). A new method, dual-rotation elution, was successfully used to decrease separation time and increase resolution. Five peak factions were eluted within 850 min. HSCCC became an efficient method to separate small molecular isomers from intermediates and products of organic synthesis, especially with the dual-rotation elution method.

Introduction

The separation of small molecular isomers from intermediates and products of organic synthesis have commonly been performed by high-performance liquid chromatography (HPLC) and capillary gas chromatography (1–3). It is easy to obtain monomeric compounds with high purity by these methods. However, the irreversible adsorptive loss of samples onto the solid support matrix is a problem to be solved.

High-speed counter-current chromatography (HSCCC) is a separation technique based on the partition of solutes between two immiscible liquid phases. One of the liquid phases is retained in the column as a stationary phase and the other liquid phase is applied as a mobile phase, with the aid of gravity and a centrifugal force field. Therefore, it eliminates irreversible adsorptive loss of samples on the solid support matrix (4). Furthermore, the elution methods of HSCCC are flexible, conventional elution, dual-mode elution (5), gradient elution (6), and elution extrusion mode (7), which can be used to separate multiple components with a wide range of polarities and similar structures. HSCCC has been widely used in the separation and purification of nature products (8–10), antibiotics (11), and rare elements (12) with organic–aqueous solvent systems. HSCCC is considered as a suitable alternative for the preparative isolation of two or three isomers from natural products (13–16) and organic synthetic products (17,18).

* Author to whom correspondence should be addressed.

Dihydroxybenzoic acids are small molecular compounds with hydroxyl groups, which make them strongly absorbed onto the solid support matrix during separation on conventional gel chromatography. Dihydroxybenzoic acids have six isomers, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dihydroxybenzoic acid. In this study, five isomers of dihydroxybenzoic acid were selected to study the feasibility of the separation of small molecular isomers on HSCCC. A new dual-rotation elution method on HSCCC was introduced in this experiment.

Experimental

Equipment

The HSCCC (TBE-300) was from Shenzhen Tauto Biotech (Shanghai, China), with three preparative coils connected in series (diameter of tube = 2.6 mm, total volume = 300 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 the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r was the distance from the coil to the holder shaft). The HSCCC systems were equipped with a Model S constant-flow pump, a Model 8823A UV monitor operating at 280 nm, and a Model 3057 recorder.

Reagents and samples

Methanol, ethyl acetate, and *n*-hexane were analytical-grade chemicals from Beijing Chemical Factory (Beijing, China). Reverse osmosis water (18MΩ, Milli-Q, Milipore, Bedford, MA) was used for all solutions and dilutions. Acetonitrile and acetic acid were chromatographic grade chemicals from Promptar Company Ltd. (King of Prussia, PA).

2,3-, 2,5-, and 3,5-Dihydroxybenzoic acid were from Sigma. 2,4-, 2,6-, and 3,4-Dihydroxybenzoic acid were from Fluka.

Measurement of partition coefficient

Partition coefficient (K) of the two-phase system was expressed as the absorbency of solutes in the stationary phase divided by that in the mobile phase. Measurement of the K value was as follows: the two-phase systems were prepared and equilibrated. Four milliliters of upper phase and 4 mL of lower phase

were put in a 10-mL test tube. One milligram of standard sample was weighted accurately and put into the tube. The test tube was shaken vigorously and then thoroughly equilibrated. The upper phase and lower phase were analyzed by HPLC at 280 nm.

Preparation of two-phase solvent system and sample solutions for HSCCC separation

The two-phase solvent system was prepared by thoroughly mixing organic solvents and water in a separatory funnel at room temperature. The two phases were separated just before use. The optimized solvent system for separation of dihydroxybenzoic acids was *n*-hexane–ethyl acetate–methanol–water (1:5:1.5:5, v/v/v/v). The mixture of 2,3-, 2,4-, 2,6-, 3,4-, and 3,5-dihydroxybenzoic acid was dissolved in the mixture of upper and lower phase in a ratio 1:1 (v/v).

HSCCC separation

The coiled HSCCC column was filled with the stationary phase (upper phase of solvent system). Then the apparatus was forward-rotated at the desired speed, and at the same time, the mobile phase (lower phase of solvent system) was pumped at a selected flow-rate from head-to-tail. If the mobile phase is the denser phase, it must enter through the head of the HSCCC column to move through the lighter stationary phase against the Archimedean forces in a descent, which is called the head-to-tail way. If the mobile phase is the lighter phase, it must enter through the tail of the HSCCC column to move through the denser stationary phase in an ascent, which is tail-to-head. The liquid stationary phase is not retained by the columns if the mobile phase is pumped in the wrong direction. After the mobile phase emerged in the effluent and the hydrodynamic equilibrium was established in the column, 5 mL of the sample solution was injected through the valve. The effluent was monitored with a UV detector at 280 nm, and the peak fractions were collected manually according to the chromatographic profile. The fractions collected were evaporated separately under vacuum and dissolved in 1 mL solvent A for subsequent analysis by HPLC.

HPLC analysis

The reference material and fractions separated by HSCCC were analyzed by an HPLC system (10Avp, Shimadzu, Japan) composed of two pumps, UV detector, oven, system controller, and 20-µL sample loop. The column used was an Ultrasphere C18 column (250 mm × 4.6 mm i.d., 5 µm, Agilent, Palo Alto, CA). The mobile phase was solvent A (acetonitrile–water–acetic acid– trifluoroacetic, 10:89.1:0.8:0.1), and solvent B (acetonitrile–water–acetic acid–trifluoroacetic, 89.1:10:0.8:0.1). The gradient mode was 0–25 min, 0–5% B. The flow-rate was 1.0 mL/min, and the temperature was 40°C. The effluent was monitored at 280 nm.

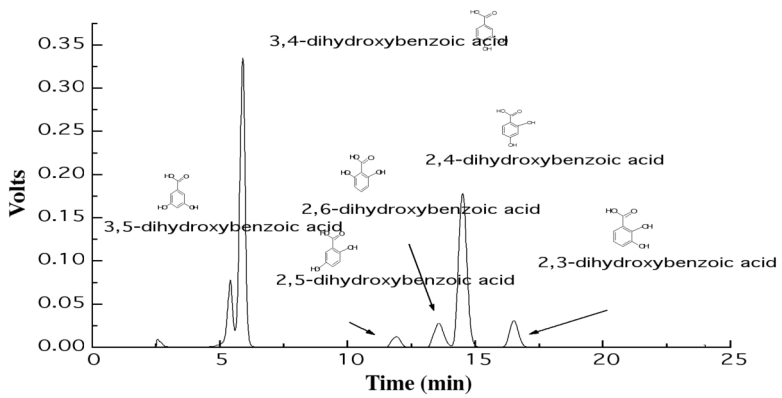

Figure 1. Analysis of six isomers of dihydroxybenzoic acid by HPLC. The column used was Ultrasphere C18 column (250 mm × 4.6 mm i.d., 5 µm, Agilent). The mobile phase was solvent A (acetonitrile–water–acetic acid–trifluoroacetic acid, 10:89.1:0.8:0.1), and solvent B (acetonitrile–water–acetic acid–trifluoroacetic acid, 89.1:10:0.8:0.1) The gradient mode was 0–25 min, 0–5% B. The flow-rate was 1 mL/min, and the temperature was 40°C. The effluent was monitored at 280 nm. Standard samples were dissolved with solvent A and loaded 20 µL.

Results and Discussion

Analysis of six isomers of dihydroxybenzoic acid by HPLC

The mixture of six isomers of dihydroxybenzoic acid was analyzed by HPLC. The mobile phase was solvent A (acetonitrile–water–acetic acid–trifluoroacetic, 10:89.1:0.8:0.1, v/v/v/v) and solvent B (acetonitrile–water–acetic acid–trifluoroacetic, 89.1:10:0.8:0.1, v/v/v/v). Dihydroxybenzoic acid with highest polarity was eluted first because reversed-phase chromatography is a hydrophobic interaction chromatography. The elution sequence was 3,5-, 3,4-, 2,5-, 2,6-, 2,4-, and 2,3-dihydroxybenzoic acid (Figure 1). Five isomers of dihydroxybenzoic acid were selected to be separated by HSCCC, except 2,5-dihydroxybenzoic acid, which does not have UV adsorption at 280 nm.

Table I. *K* Values of Five Isomers of Dihydroxybenzoic Acid in Different Solvent Systems

Solvent system*	DC† of upper phase	DC† of lower phase	$K_{2,3}$	$K_{2,4}$	$K_{2,6}$	$K_{3,4}$	$K_{3,5}$
n-H–EA–M–W (1:5:1:5)	8.329	69.01	7.99	18.86	0.18	2.72	2.69
n-H–EA–M–W (1:5:1.5:5)	9.095	64.51	4.34	12.61	0.26	2.82	2.35
n-H–EA–M–W (1:5:2:5)	9.759	60.02	5.17	8.47	0.46	2.17	2.13

* *n*-H–EA–M–W = *n*-hexane–ethyl acetate–methanol–water; † DC = dielectric constant.

Selection of two-phase solvent system of HSCCC

Partition coefficient (*K*) is the most important parameter in solvent system selection of HSCCC, whose ideal value is ~ 1 to achieve an efficient separation and a suitable run time. If the *K* value of compounds are very close, it is impossible to get a reasonable resolution. If the *K* value is much more than 1, the component will be eluted as excessively broad peaks and at extended elution time. Dihydroxybenzoic acids are moderate

polar compounds which are dissolved easily in ethyl acetate. Solvent systems composed of *n*-hexane, ethyl acetate, methanol, and water were commonly used in the separation and purification of a wide range of polar solutes in HSCCC (19–22).

In this experiment, a series of solvent systems were tried, and *K* values were calculated (Table I). In the solvent system 1:5:1:5, the *K* value of 2,4-dihydroxybenzoic acid was much higher, which leads to a broad peak. The polarity of the solvent system was supposed to decrease by increasing the proportion of methanol, because methanol is the "best solvent" for modifying the polarity of a solvent system (23). By increasing the proportion of methanol, the polarity of lower phase was decreased and that of upper phase was increased (Table I), which led to the *K* values of the compounds to change. The *K* value of 2,4-dihydroxybenzoic acid decreased. Because 2,4-dihydroxybenzoic acid has the greatest affinity for the upper phase of this solvent system among all isomers studied, the solubility of 2,4-dihydroxybenzoic acid decreased in upper phase and increased in lower phase. On the contrary, 2,6-dihydroxybenzoic acid has the greatest affinity for lower phase, whose *K* value increased with increasing the proportion of methanol in solvent system. Comparing with 2,6- and 2,4-dihydroxybenzoic acid, *K* values of 2,3-, 3,5-, and 3,4-dihydroxybenzoic acid were more complex with the changing of polarity. In the solvent system 1:5:2:5, the *K* values of 2,3-, 2,4-, and 2,6-dihydroxybenzoic acid were suitable for the separation; however, *K* values of 3,4- and 3,5-dihydroxybenzoic acid were very close, and it was difficult to achieve a reasonable resolution. Finally, the solvent system at ratio of 1:5:1.5:5 was used to separate five isomers of dihydroxybenzoic acid; the retention of stationary phase was ~ 55%.

Separation of five isomers of dihydroxybenzoic acid by HSCCC

Five isomers of dihydroxybenzoic acid were separated on HSCCC with solvent system *n*-hexane–ethyl acetate–methanol–water (1:5:1.5:5) in conventional elution method, at a flow-rate of 1.0 mL/min and at a revolution of 700 rpm (Figure 2). Each fraction was collected and analyzed by HPLC; the elution sequence of the dihydroxybenzoic acids was 2,6-, 3,5-, 3,4-, 2,3-, and 2,4-dihydroxybenzoic acid. 2,3- and 2,4-dihydroxybenzoic acid were eluted as excessively broad peaks and at extended elution times. Dual-mode elution composed of two elution directions of mobile phase, a method suitable for the separation of multiple components at a wide range of polarity, was applied to separate these five isomers of dihydroxybenzoic acid. The chromatogram of HSCCC separation is shown in Figure 3A. At 0–600 min, the lower phase of the solvent system was used as mobile phase from head to tail at a flow-rate of 1.0 mL/min; after 600 min, the lower phase of the solvent system was replaced by the upper phase as mobile phase that was pumped from tail to head. In the whole run, the column was forward-rotating at 700 rpm. The sequence of five peaks was 2,6-, 3,5-, 3,4-, 2,4-, and 2,3-dihydroxybenzoic acid, which was a different elution sequence from conventional elution. Dual-mode elution separated five isomers of dihydroxybenzoic acid with spiculate peaks and half of the elution time of conventional elution.

A new dual-rotation elution method composed of forward-rotation and counter-rotation was tried consequently to separate five isomers of dihydroxybenzoic acid as follows. Mobile phase was pumped from head to tail at a flow-rate of 1.0 mL/min.

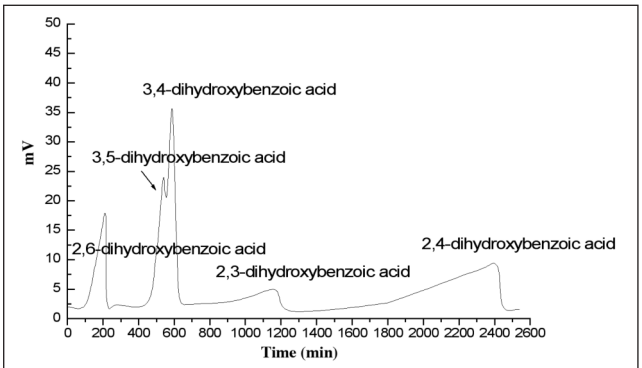

Figure 2. Separation of five isomers of dihydroxybenzoic acid by HSCCC. Experimental conditions for HSCCC were as follows: apparatus, TBE-300 with 300 mL column; retention of stationary phase, 55%; solvent system for HSCCC, solvent (*n*-hexane–ethyl acetate–menthanol–water, 1:5:1.5:5); mobile phase, lower phase; elution mode, head to tail; flow rate, 1.0 mL/min; revolution, 700 rpm; temperature, 28°C; detection wavelength, 280 nm; sample loading, 1 mg of each other in 5 mL lower phase.

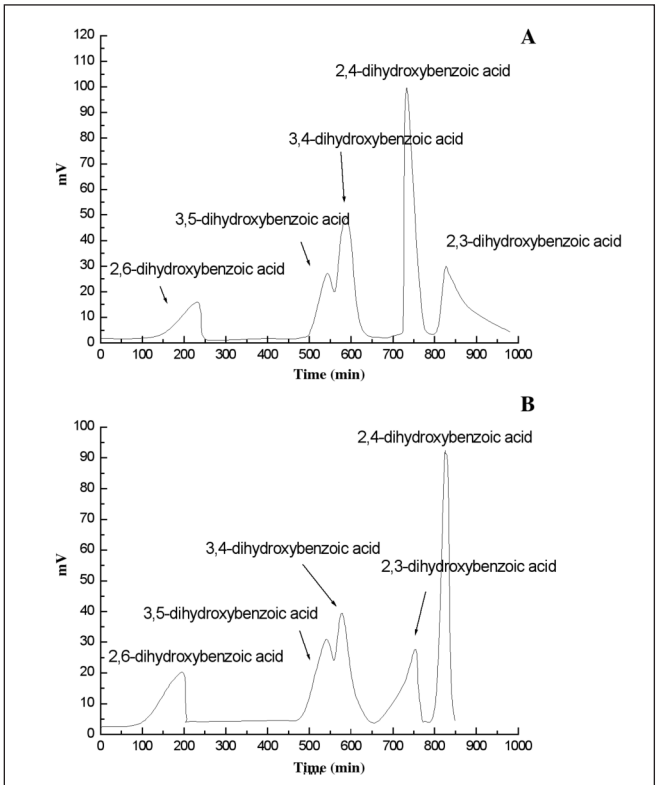

Figure 3. Separation of five isomers of dihydroxybenzoic acid on HSCCC with dual-mode method (A) and dual-rotation elution method (B). Experimental conditions for HSCCC were as follows: apparatus, TBE-300 with 300 mL column; retention of stationary phase, 55%; solvent system for HSCCC, solvent (*n*-hexane–ethyl acetate–menthanol–water: 1:5:1.5:5); flow rate, 1.0 mL/min; revolution, 700 rpm; temperature, 28°C; detection wavelength, 280 nm; sample loading, 2 mg of each other in 5 mL lower phase. Dual-mode method (A): 0–600 min, lower phase as mobile phase pumped from head to tail, forward-rotate; after 600 min, upper phase as mobile phase pumped from tail to head, foward-rotate. Dual-rotation elution method (B): 0–600 min, lower phase as mobile phase, forward-rotate; after 600 min, upper phase as mobile phase, counter-rotate.

HSCCC was forward-rotated at 700 rpm (in the period of 0–600 min, the lower phase was used as mobile phase); HSCCC was counter-rotated at 700 rpm after 600 min, and the upper phase was used as mobile phase. Five peak factions were eluted within 850 min (Figure 3B) and analyzed by HPLC. The sequence of peaks by this elution method was 2,6-, 3,5-, 3,4-, 2,3-, and 2,4-dihydroxybenzoic acid. The elution sequence between 2,3- and 2,4-dihydroxybenzoic acid was, interestingly, reversed once again (same as that of conventional elution mode) after changing both the rotation mode and mobile phase at 600 min.

As a result, K' values (K value of component in counter-rotation mode) of 2,4- and 2,3-dihydroxybenzoic acid were the reciprocal of their K values. $K'$of 2,4-dihydroxybenzoic acid was lower than that of 2,3-dihydroxybenzoic acid. It was supposed that with the changing of the rotation mode and mobile phase, resolution of these two compounds was decreased. If the compounds have been separated inside the column, and the resolution factor was far more than 1.5 between adjacent peaks, dual-rotation elution can replay dual-mode elution to shorten of elution time with an acceptable resolution factor. In this experiment, it is quite clear from Figures 2 and 3A that during conventional elution mode at 0–600 min, the 2,3- and 2,4-dihydroxybenzoic acid were separated inside the column and the residue column volume was not big enough; 2,4-dihydroxybenzoic acid was eluted later than 2,3-dihydroxybenzoic acid.

By this new dual-rotation elution method, five isomers of dihydroxybenzoic acid were separated at a reasonable resolution.

Acknowledgment

The authors are grateful for the support given by the National Natural Science Foundation of China (20606032, 20636010, 50773083).

References

1. H. L. Jin, Y. L. Zhang, and Y. Guo. HPLC separation of isomers of tetrahydro-1,5-benzothiazepines and tetrahydro-1,5-benzodiazepines. *Chromatographia* **22:** 1–6 (1986).
2. L. Deng, H. Nakano, and Y. Iwasak. Direct separation of monoacylglycerol isomers by enantioselective high-performance liquid chromatography. *J. Chromatogr. A* **1198–99:** 67–72 (2008).
3. J. Xing, C.Y. Wu, T. Li, Z.L. Zhong, and Y.Y. Chen. Separation of aromatic isomers by capillary gas chromatography with two calixarene polysiloxane stationary phases. *Anal. Sci.* **15:** 785–92 (1995).
4. Y. Ito and R.L. Bowman. Countercurrent chromatography: liquid-liquid partition chromatography without solid support. *Science* **167:** 281–83 (1970).
5. M. Agnely and D. Thiébaut. Dual-mode high-speed counter-current chromatography: retention, resolution and examples. *J. Chromatogr. A* **790(1–2):** 17–30 (1997).
6. G.L. Tian, Y.B. Zhang, T.Y. Zhang, F.Q. Yang, and Y. Ito. Separation of tanshinones from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography using stepwise elution. *J. Chromatogr. A* **904(1–2):** 107–11 (2000).
7. M.J. Berthod, S. Ruiz-Angel, and Carda-Broch. Elution-extrusion countercurrent chromatography. Use of the liquid nature of the stationary phase to extend the hydrophobicity window. *Anal. Chem.* **75(21):** 5886–94 (2003).
8. X. Han, W. Pathmasiri, L. Bohlin, and J.C. Janson. Isolation of high purity 1-[2',4'-dihydroxy-3',5'-di-(3"-methylbut-2"-enyl)-6'-methoxy] phenylethanone from *Acronychia pedunculata* (L.) Miq. by high-speed counter-current chromatography. *J. Chromatogr. A* **1022:** 213–16 (2004).
9. J.Y. Peng, Y.Y. Jiang, G.R. Fan, B. Chen, Q.Y. Zhang, Y.F. Chai, and Y.T. Wu. Optimization suitable conditions for preparative isolation and separation of curculigoside and curculigoside B from Curculigo orchioides by high-speed counter-current chromatography. *Sep. Purif. Technol.* **52:** 22–28 (2006).
10. L. Chen, Y.S Han, F.Q. Yang, and T.Y. Zhang. High-speed counter-current chromatography separation and purification of resveratrol and piceid from *Polygonum cuspidatum*. *J. Chromatogr. A* **907:** 343–346 (2001).
11. K. Harada, M. Suzuki, A. Kato, K. Fujii, H. Oka, and Y. Ito. Separation of WAP-8294A components, a novel anti-methicillin-resistant *Staphylococcus aureus* antibiotic, using high-speed counter-current chromatography. *J. Chromatogr. A* **932(1-2):** 75–81(2001).
12. E. Kitazume, M. Bhatnagar, and Y. Ito. Separation of rare earth elements by high-speed counter-current chromatography. *J. Chromatogr. A* **538(1):** 133–40 (1991).
13. S.Y. Shi, K.L. Huang, Y.P. Zhang, Y. Zhao, and Q.Z. Du. Purification and identification of antiviral components from *Laggera pterodonta* by high-speed counter-current chromatography. *J. Chromatogr. B* **859:** 119–24 (2007).
14. J.J. Lu, Y. Wei, and Q.P. Yuan. Preparative separation of punicalagin from pomegranate husk by high-speed countercurrent chromatography. *J. Chromatogr. B* **857:** 175–79 (2007).
15. L. Li, R. Tsao, and Z.Q. Liu. Isolation and purification of acteoside and isoacteoside from *Plantago psyllium* L. by high-speed counter-current chromatography. *J. Chromatogr. A* **1063:** 161–69 (2005).
16. Q.Z. Du, L. Li, and G. Jerz. Purification of astilbin and isoastilbin in the extract of *smilax glabra rhizome* by high-speed counter-current chromatography. *J. Chromatogr. A* **1077:** 98–101 (2005).
17. R.S.F. Sliva, G.G. leitão, and T.B. Brum. Applications of counter-current chromatography in organic synthesis purification of heterocyclic derivatives of lapachol. *J. Chromatogr. A* **1151:** 197–1202 (2007).
18. Y.F. Luo, Y.B. Xu, and L.J. Chen. Preparative purification of anti-tumor derivatives of honokiol by high-speed counter-current chromatography. *J. Chromatogr. A* **1178:** 160–65 (2008).
19. M. Gao, M. Gu, and C.Z. Liu. Two-step purification of scutellarin from *Erigeron breviscapus* (vant.) Hand. Mazz. by high-speed counter-current chromatography. *J. Chromatogr. B* **838:** 139–43 (2007).
20. M. Gu, X.L. Wang, Z.G. Su, and F. Ouyang. One-step separation and purification of 3,4-phenyllactic acid, salvianolic acid B and protocatechualdehyde from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography. *J. Chromatogr. A* **1140:** 107–11 (2007).
21. W.H. Zhao, C.C. Gao, X.F. Ma, and Y. X. Zhang. The isolation of 1, 2, 3, 4, 6-penta-O-galloyl-beta-d-glucose from *Acer truncatum* Bunge by high-speed counter-current chromatography. *J. Chromatogr. B* **850:** 523–27 (2007).
22. X. Han, X.F. Ma, T.Y. Zhang, Y. B. Zhang, Q.H. Liu, and Ito Y. Isolation of high-purity casticin from *Artemisia annua* L. by high-speed counter-current chromatography. *J. Chromatogr. A* **1151:** 180–82 (2007).
23. A.P. Foucault. "Solvent system in centrifugal partition chromatography". In *Centrifuga Partition Chromatography*. A.P. Foucault ed. Marcel Dekker Inc. New York, NY, 1995, pp. 363.

Manuscript received September 11, 2008.