

# Counter-Current Chromatographic Separation of Nucleic Acid Constituents with a Polar Volatile Organic-Aqueous Two-Phase Solvent Systems with ELSD Detection

Yoichi Shibusawa<sup>\*1</sup>, Go Morikawa<sup>1</sup>, Akio Yanagida<sup>1</sup> and Yoichiro Ito<sup>2</sup>

<sup>1</sup>Division of Pharmaceutical and Biomedical Analysis, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

<sup>2</sup>Bioseparation Technology, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

**Abstract:** Highly hydrophilic volatile organic/aqueous two-phase solvent systems containing an organic salt such as, acetonitrile/800 mM and 1200 mM ammonium acetate (1 : 1, v/v) were efficiently utilized for high-speed counter-current chromatography (HSCCC) to separate hydrophilic compounds. The retention of the upper and the lower stationary phases in the column of the cross-axis coil planet centrifuge (CCC instrument) was studied by changing the flow-rate of the mobile phase (1.0–3.0 ml/min). Using the acetonitrile/800 mM ammonium acetate two-phase solvent system, the stationary phase was retained at 46.3% relative to the total column capacity of 65 ml by the reversed-phase elution mode at a flow-rate of 1.0 ml/min. The best retention of the stationary upper phase of 51.5% was obtained by the solvent system of the acetonitrile/1200 mM ammonium acetate at the above flow-rate. With the acetonitrile/800 mM ammonium acetate system the base line separation of adenine and adenosine monophosphate (AMP) detected by evaporative light scattering detector (ELSD) and UV was achieved with lower phase mobile at a flow-rate of 1.0 ml/min within 70 min.

**Keywords:** HSCCC, hydrophilic compounds, AMP, ELSD.

## 1. INTRODUCTION

High-speed counter-current chromatography (HSCCC) is a powerful separation technique used in both analytical and preparative separations of a wide variety of compounds using various aqueous/organic two-phase solvent systems [1-4].

HSCCC utilizes a two immiscible solvent phases, one as a stationary phase and the other as a mobile phase and the separation is highly dependent on the partition coefficient values (K) of the solutes, i.e., the ratio of the solute concentration between the mobile and the stationary phases. The K value is therefore the most important parameter in HSCCC. Optimization of the solvent composition to adjust the K value of the target compound is essential for the successful HSCCC separation.

A great number of two-phase solvent systems have been used for the separation of a variety of compounds by HSCCC [5]. However, the most of hydrophilic compounds have a high tendency to partition to the aqueous phase of the organic/aqueous two-phase systems. The polar two-phase solvent systems composed of 1-butanol/water are available for the HSCCC separation of polar compounds such as sugars [6] and water-soluble vitamins [7]. The CCC separation of polar biotic compounds was also performed using more hydrophilic organic/aqueous two-phase solvent

systems composed of ethanol/ammonium sulfate and acetonitrile/sodium chloride [8]. Recently HSCCC separation of polar nucleic acid constituents has been performed using a hydrophilic solvent system composed of 1-propanol/potassium phosphate buffer [9]. However, these hydrophilic solvent two-phase systems, such as 1-butanol/water, ethanol/ammonium sulfate, acetonitrile/sodium chloride and 1-propanol/potassium phosphate are nonvolatile systems. If we use an evaporative light scattering detector (ELSD) for detecting some hydrophilic compounds with no ultraviolet-visible absorption or using a mass spectrometer to detect a small amount of compounds, we have to use a volatile two-phase solvent system in HSCCC.

The two-phase solvent systems containing ammonium acetate used for the counter-current chromatography were reported an enantiomer separation [10], flavonol glycosides separation detected with ESI-MS [11]. Recently, ELSD was applied for detecting ginsenosides for the high-speed counter-current chromatography [12].

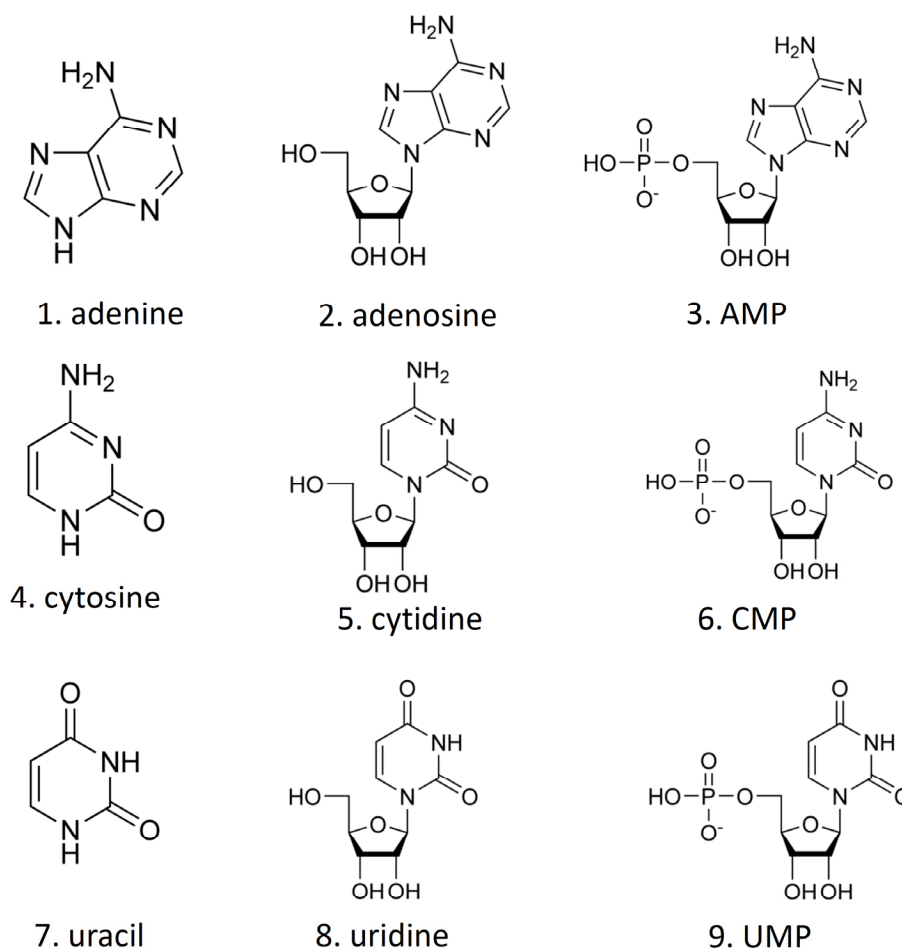
In the present study, new hydrophilic volatile solvent systems composed of acetonitrile/800 mM and 1200 mM ammonium acetate have been utilized for the separation of some polar nucleic acid constituents by HSCCC detected by ELSD using the cross-axis coil planet centrifuge (CCC instrument).

## 2. EXPERIMENTAL

### 2.1. Reagents

Standard compounds of adenine, adenosine, adenosine 5'-monophosphate monohydrate (AMP), cytosine, cytidine,

\*Address correspondence to this author at the Tokyo University of Pharmacy and Life Science, Division of Pharmaceutical and Biomedical Analysis, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan; Tel: +81-426-76-4537; Fax: +81-426-76-4537; E-mail: sibusawa@toyaku.ac.jp



**Fig. (1).** Chemical structures of 9 nucleic acid constituents.

cytidine 5'-monophosphate disodium salt (CMP), uracil, uridine and uridine 5'-monophosphate disodium salt (UMP) were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). The chemical structures of 9 nucleic acid constituents were shown in Fig. (1). Acetonitrile and ammonium acetate used for preparation of the two-phase solvent system were obtained from Kanto Chemical Co. (Tokyo, Japan). All other chemicals were of reagent grade.

## 2.2. Measurement of Partition Coefficient

Ammonium acetate solutions at 650 mM, 700 mM, 800 mM and 1200 mM were prepared by dissolving 50.1g (650 mmol), 54.0 g (700 mmol), 61.7 g (800 mmol) and 92.5 g (1200 mmol) of ammonium acetate ( $\text{CH}_3\text{COONH}_4$ ) in water making the total volume of 1000 ml. The ammonium acetate solution was mixed with the same volume of acetonitrile. The partition coefficients ( $K_{800}$ ,  $K_{1200}$ ) of 9 different nucleic acid constituents such as nucleobases, nucleosides and nucleotides were measured in the solvent systems composed of acetonitrile/800 mM and 1200 mM ammonium acetate solution (1 : 1, v/v) prior to their HSCCC separation. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. The  $K_{800}$  and  $K_{1200}$  values of above standard compounds in this solvent system were determined spectrophotometrically by the following procedure: 1 ml of each phase was delivered into a test tube to which 1 mg of each test compound was added. The

contents were thoroughly mixed and allowed to settle at room temperature. After two clear layers were formed, an aliquot of each phase was diluted with methanol (2 to 100 times) to determine the absorbance at 260 nm using a Jasco V 530 spectrophotometer (Jasco, Tokyo, Japan).  $K_{800}$  and  $K_{1200}$  values were expressed as the solute concentration in the upper phase divided by that in the lower phase.

## 2.3. HSCCC Apparatus

The cross-axis CCC instrument (Pharma-Tech Research Corporation, Baltimore, MD, USA) holds a pair of horizontal rotary shafts, symmetrically mounted one on each side of the rotary frame at a distance of 10 cm from the centrifuge axis. A spool-shaped column holder is mounted on each rotary shaft at an off-center position of 5 cm from its mid-point. The multilayer coil separation column was prepared from a 2.0 mm I. D. polytetrafluoroethylene (PTFE) tube (Tokyo Rikakiki, Tokyo, Japan) by winding it onto a 10 cm diameter holder hub forming two layers of left-handed coils between a pair of flanges spaced 5 cm apart. A pair of columns mounted on the rotary frame was connected in series with a flow tube (PTFE, 0.85 mm I. D.) to give a total capacity of 65 ml. The revolution speed of the apparatus is regulated at 500 rpm with a speed control unit. The coiled column rotates about its own axis as it synchronously revolves around the central axis of the centrifuge, producing an efficient mixing of the two phases while retaining a sufficient amount of the stationary phase in

the coiled column. The system permit elution of the solvent through the rotating column without the conventional rotary seal which would cause leakage of the solvent.

This cross-axis CCC instrument was connected to the Hitachi HPLC instrument (Hitachi, Tokyo, Japan) consisting of a model L-7100 pump, a Rheodyne 7166 sample injector (Rheodyne, CA, USA) and a model L-7455 diode-array detector (Hitachi) and type 2000ES evaporated light scattering detector (Alltech Associates Inc., Deerfield, IL, USA) as shown in Fig. (2).

#### 2.4. Measurement of Stationary Phase Retention

In each measurement, the coil was first filled with the upper or lower stationary phase of acetonitrile/800 mM or 1200 mM ammonium acetate (1 : 1, v/v) and the apparatus was rotated at the desired revolution speed (500 rpm) while the other mobile phase was pumped into the column at a flow-rate of 1.0-3.0 ml/min. The elution was continued until the total elution volume exceeded the column capacity (65 ml). Then the centrifuge was stopped and the column contents were emptied into a 100-ml capacity glass graduated cylinder by connecting the inlet of the column to a pressured nitrogen line. The percentage retention of the stationary phase relative to the total column capacity was calculated from the volume of the stationary phase recovered from the column.

#### 2.5. High-Speed Counter-Current Chromatography

The separation of nucleic acid constituents was performed with the two-phase solvent systems composed of acetonitrile/800 mM and 1200 mM ammonium acetate (1:1, v/v) on a reversed-phase partition mode (mobile phase: aqueous lower phase; stationary phase: organic upper phase). For the separation of a synthetic mixture of standard compounds, the coiled PTFE column (capacity: 65 ml) of the cross-axis CCC instrument was first entirely filled with an organic upper phase as a stationary phase at a flow-rate of 0.5 ml/min. After the upper stationary phase was eluted from the column, the lower phase was set the bottle of the mobile

phase. Then, the column was rotated at 500 rpm while an aqueous lower mobile phase was pumped into the column at a flow-rate of 1.0 ml/min. After the hydrodynamic mixing between the upper stationary phase and lower mobile phase reached a state of equilibrium in the column, a sample solution containing a set of the standard compounds (each about 0.5-1.0 mg) in a 0.5 ml (0.25 ml each phase) was injected and eluted with the lower mobile phase. Effluent from the outlet of the column was monitored by absorbance at 260 nm and was monitored by evaporative light scattering detector (ELSD).

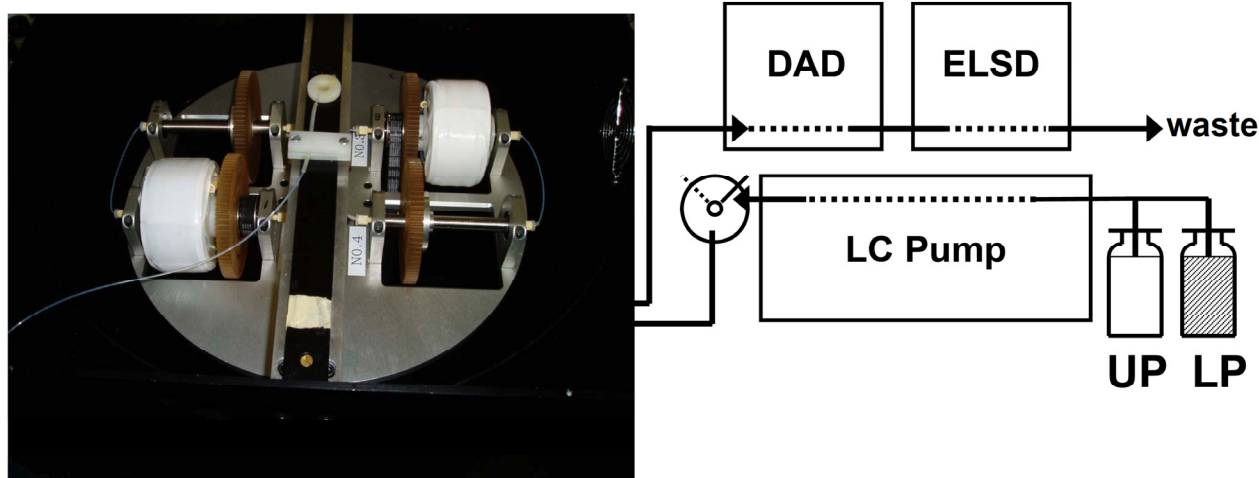
### 3. RESULTS AND DISCUSSION

#### 3.1. Solvent Systems and Partition Coefficients of Nucleic Acid Constituents

We have tested the solvent system composed of acetonitrile/650 mM, 700 mM, 800 mM and 1200 mM ammonium acetate two-phase solvent systems. Acetonitrile forms two-phase system against 650 mM or higher concentration of the ammonium acetate. However, retention of the upper organic phase of the acetonitrile/700 mM ammonium acetate was only about 20% of the total column capacity (65 ml) at flow-rate of 1.0 ml/min. As the concentration of the ammonium acetate is increased to 800 mM, however, the retention of the upper phase was improved to about 46%. Over 50% of retention of upper phase was obtained by the solvent systems composed of acetonitrile/1200 mM ammonium acetate. Commonly, the retention of the stationary phase is very important in the HSCCC experiment to separate substances. Then, the solvent systems containing 800 mM and 1200 mM ammonium acetate were selected for the subsequent experiment.

In the HSCCC technique, successful separation requires a proper choice of two-phase solvent systems. In order to achieve efficient separation of nucleic acid constituents, the partition coefficient ( $K_{800}$ ,  $K_{1200}$ ) values of these compounds were determined in two solvent systems, acetonitril/800 mM ammonium acetate (1 : 1, v/v) and acetonitrile/1200 mM ammonium acetate (1 : 1, v/v). The  $K_{800}$  and  $K_{1200}$  values of

## Cross axis CCC instrument



**Fig. (2).** HSCCC system connected to cross-axis CCC instrument. Cross axis CCC instrument: cross-axis coil planet centrifuge; DAD: diode-array detector; UP: upper phase; LP: lower phase.

the nucleic acid constituents were determined spectrophotometrically according to a standard test tube procedure as described in the Experimental section.

Table 1 lists the  $K_{800}$  and  $K_{1200}$  values of 9 nucleic acid constituents, such as nucleobases, nucleosides and nucleotides in the two-phase solvent system composed of acetonitrile/800 mM and 1200 mM ammonium acetate (1:1, v/v). If the lower phase is used as a mobile phase, the reciprocal numbers of the partition coefficient values ( $1/K_{800}$ ,  $1/K_{1200}$ ) are useful information for HSCCC separation which are also inserted in the table. The  $K_{800}$  and  $K_{1200}$  values of nucleobase, nucleoside and nucleotide are smaller than 1.0 indicating that all these nucleic acid constituents are more partitioned in the lower aqueous phase in both acetonitrile/800 mM and 1200 mM ammonium acetate solvent systems. The  $K_{800}$  values of nucleosides are smaller than those of nucleobases suggesting that nucleosides bonded to sugar are more hydrophilic than nucleobases. The  $K_{800}$  and  $K_{1200}$  values of nucleotide are smaller than those of nucleosides because nucleotides bonded phosphorus group are more hydrophilic than nucleosides.

### 3.2. Measurement of Stationary Phase Retention

Table 2 shows the percentage retention of the stationary phase for the acetonitrile/800 mM and 1200 mM ammonium acetate two-phase solvent systems using both upper and lower phases as the mobile phase. Each measurement was performed at 1.0, 2.0 and 3.0 ml/min flow-rates at 500 rpm in normal-phase (mobile phase: organic upper phase; stationary phase: aqueous lower phase) and reversed-phase (mobile phase: aqueous lower phase; stationary phase: organic upper phase) partition modes. In both normal and reversed-phase partition modes, the retention of stationary phase was increased as the flow-rate of the mobile phase was decreased from 3.0 to 1.0 ml/min. In case of modern high speed and high performance conventional CCC instruments, the stationary phase retention in normal-phase mode is

always higher than that for reverse-phase mode. However, in the case of the cross-axis CCC instrument, the retention of the stationary phase obtained reversed-phase partition modes are always greater than those obtained in the normal-phase partition mode at flow-rates of 1.0, 2.0 and 3.0 ml/min. The maximum retention of the stationary phase of upper phase of acetonitrile/800 mM ammonium acetate (1 : 1, v/v) was 46.3%. The best retention of 51.5% was obtained using the solvent system composed of acetonitrile/1200 mM ammonium acetate (1 : 1, v/v).

**Table 1. Partition Coefficient Values of Several Nucleic Acid Constituents**

Nucleic Acid Constituents	Compounds	$K_{800}$	$1/K_{800}$	$K_{1200}$	$1/K_{1200}$
nucleobase	adenine	0.53	1.90	0.30	3.32
	cytosine	0.41	2.44	0.15	6.58
	uracil	0.43	2.34	0.38	2.60
nucleoside	adenosine	0.41	2.43	0.38	2.67
	cytidine	0.23	4.29	0.10	9.71
	uridine	0.33	2.99	0.21	4.83
nucleotide	AMP	0.07	14.29	0.02	50.00
	CMP	0.02	45.45	0.01	33.33
	UMP	0.06	16.13	0.02	50.00

$K_{800}$ : partition coefficient in the acetonitrile/800 mM ammonium acetate (1:1).  
 $K_{1200}$ : partition coefficient in the acetonitrile/1200 mM ammonium acetate (1:1)  
 AMP: adenosine 5'-monophosphate.  
 CMP: cytidine 5'-monophosphate.  
 UMP: uridine 5'-monophosphate.

### 3.3. Separation of Nucleic Acid Constituents by HSCCC

The separation of uracil, uridine and UMP was performed by the cross-axis CCC instrument using the

**Table 2. Retention of Stationary Phase on Cross-Axis Coil Planet Centrifuge**

Solvent System	Mode	Stationary Phase	Mobile Phase	Flow-Rate (ml/min)	Retention (%)
CH <sub>3</sub> CN/800 mM CH <sub>3</sub> COONH <sub>4</sub> (1:1)	normal	LP	UP	1.0	39.4
		LP	UP	2.0	33.8
		LP	UP	3.0	31.3
	reversed	UP	LP	1.0	46.3
		UP	LP	2.0	40.9
		UP	LP	3.0	33.8
CH <sub>3</sub> CN/1200 mM CH <sub>3</sub> COONH <sub>4</sub> (1:1)	normal	LP	UP	1.0	47.0
		LP	UP	2.0	40.3
		LP	UP	3.0	35.8
	reversed	UP	LP	1.0	51.5
		UP	LP	2.0	44.1
		UP	LP	3.0	42.9

Cross-axis coil planet centrifuge (column : 2.0 mm x 20 m, 65 mL capacity).

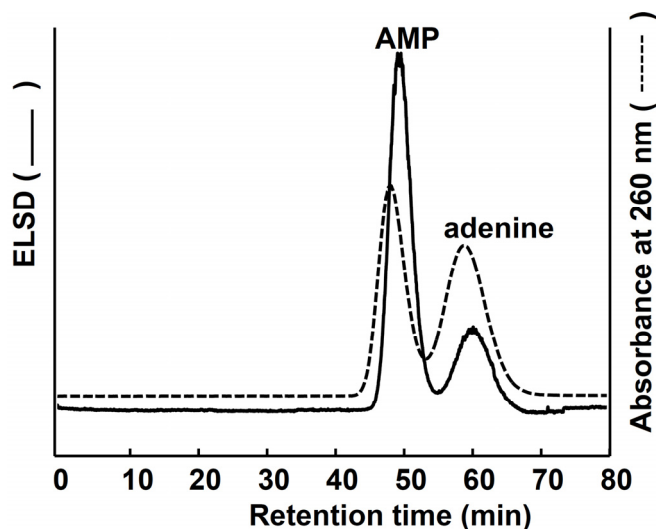
LP: lower phase.

UP: upper phase.

acetonitrile/1200 mM ammonium acetate two-phase solvent system under the best retention of the stationary phase determined by the retention experiment. The HSCCC was performed by the reversed-phase partition mode using the cross-axis CCC instrument. The  $1/K_{1200}$  values of uracil, uridine and UMP are 2.60, 4.83 and 50.0, respectively (Table 1). Then, the elution order of these compounds is expected to be UMP, uridine and uracil. However, the  $1/K_{1200}$  values are not suitable for complete separation of these nucleic acid constituents since the peaks of uridine and uracil are overlapped (data not shown). If it is possible to find a two-phase solvent system in which the large difference between  $1/K$  value of uridine and that of uracil, the resolution of these nucleic acid constituents are improved.

Capability of the cross-axis CCC instrument was demonstrated on the separation of adenine and AMP using an extremely hydrophilic solvent system composed of acetonitrile/800 mM ammonium acetate (1:1, v/v). The separation was performed in the reversed-phase partition mode using the lower aqueous phase as the mobile phase. As described earlier, the separation column was first entirely filled with the stationary upper organic phase and the column was rotated at 500 rpm while the aqueous lower mobile phase was pumped into the column at a flow-rate of 1.0 ml/min. After the hydrodynamic equilibrium was established in the column, 0.5 ml sample solution containing adenine (0.10 mg) and AMP (0.08 mg) was injected through the sample port.

Fig. (3) shows a base line separation of AMP and adenine detected by ELSD and UV at 260 nm where AMP ( $1/K_{800}=14.29$ ) and adenine ( $1/K_{800}=1.90$ ) were eluted from the column. It was found that the solvent system composed of acetonitrile/800 mM ammonium acetate was volatile and it was possible to use the ELSD for the detection of nucleic acid constituents. The upper stationary phase retained in the column was estimated as 40.2% of the total column capacity (65 ml). The separation was completed within 70 min.



**Fig. (3).** Separation of AMP and adenine by HSCCC using hydrophilic solvent system with ELSD and UV detections. Dimension of PTFE multilayer coiled column: 2.0 mm I.D. x 20 m (65 ml capacity); solvent system: acetonitrile/800 mM ammonium acetate (1:1, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.0 ml/min; revolution: 500 rpm.

## CONCLUSION

The overall results of our studies indicate that the present method is capable of separating highly polar compounds such as nucleic acid constituents using the new hydrophilic solvent system composed of acetonitrile/800 mM ammonium acetate (1:1, v/v) by HSCCC. The solvent system is volatile and it was used for the detection of the compounds by evaporative light scattering detector (ELSD). This solvent system could be very useful for the separation of other hydrophilic compounds which have no chromophore by cross-axis CCC instrument coupled with ELSD.

## ACKNOWLEDGMENTS

Declared none.

## CONFLICT OF INTEREST

Declared none.

## ABBREVIATIONS

HSCCC	= High-speed counter-current chromatography
CCC instrument	= Cross-axis coil planet centrifuge
ELSD	= Evaporative light scattering detector
AMP	= Adenosine 5'-monophosphate
CMP	= Cytidine 5'-monophosphate
UMP	= Uridine 5'-monophosphate

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