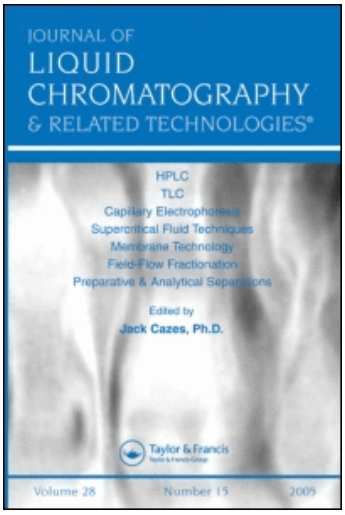


**Journal of Liquid Chromatography & Related Technologies**
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Online publication date: 05 November 1999

**To cite this Article** Rasooly, Avraham and Ito, Yoichiro(1999) 'TOROIDAL COIL COUNTERCURRENT CHROMATOGRAPHY SEPARATION AND ANALYSIS OF STAPHYLOCOCCAL ENTEROTOXIN A (SEA) IN MILK', Journal of Liquid Chromatography & Related Technologies, 22: 9, 1285 — 1293
**To link to this Article: DOI:** 10.1081/JLC-100101732
**URL:** http://dx.doi.org/10.1081/JLC-100101732

J. LIQ. CHROM. & REL. TECHNOL., 22(9), 1285–1293 (1999)

# TOROIDAL COIL COUNTERCURRENT CHROMATOGRAPHY SEPARATION AND ANALYSIS OF STAPHYLOCOCCAL ENTEROTOXIN A (SEA) IN MILK

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## ABSTRACT

Countercurrent Chromatography (CCC) utilizes continuous partitioning of solute between two immiscible solvent phases without a solid support. The absence of solid support makes CCC a suitable method for food analysis because it permits analysis of crude and complex materials that are not amenable to conventional solid-supported chromatography. CCC was evaluated for its ability to separate Staphylococcal enterotoxin A (SEA), a common cause of food poisoning, from milk. Although many foods can be analyzed for SEA directly by Western blot analysis, milk samples generally require some purification because the high concentration of milk proteins distorts SDS-PAGE mobility. Milk samples containing SEA were separated by

toroidal coil CCC and the fractions were analyzed by Western immunoblotting. Fractions containing SEA were pooled, concentrated by ultrafiltration, and rechecked by Western immunoblotting. Concentrating the fractions increased the sensitivity of Western immunoblotting by approximately an order of magnitude.

## INTRODUCTION

Food-borne microbial diseases are very common diseases.[1] One of the most frequent diseases is gastroenteritis resulting from ingestion of food contaminated with Staphylococcal enterotoxin A (SEA) produced by the bacterium *Staphylococcus auras.* SEA is a potent gastrointestinal toxin; amounts as low as 100ng are sufficient to cause symptoms of intoxication.[2] Therefore, it is critical that the food testing methods be able to identify and measure very low levels of SEA.



The most commonly used assay for SEA is an enzyme-linked immunosorbent assay (ELISA), in which monovalent or polyvalent SEA antibodies are reacted with the toxin. Although the ELISA procedure is simple, sensitive, and rapid some problems have been reported. For example, ELISA fails to measure SEA in heat-treated materials[3] and false positive tests may result from cross-reactivity with food antigens.[4,5]

Western immunoblotting is another immunological analysis method, in which antigens are separated by SDS-PAGE, transferred to a membrane, and then reacted with antibodies. Western immunoblotting has several advantages over ELISA, including the ability to detect heat-treated toxins.[6,7] However, only a small test volume (20 μL-100 μL) can be evaluated in a Western blot, limiting the ability to measure small amounts of SEA in food samples without additional, time-consuming purification steps. Moreover, some foods such as milk, contain a high concentration of protein that distorts SDS-PAGE mobility and, therefore, require some purification before Western analysis.

Countercurrent Chromatography (CCC) is a chromatographic method that utilizes the continuous partitioning of solute between two immiscible solvent phases without a solid support.[8-10] High efficiency of such partitioning is achieved by two-phase distribution in a high-speed rotating coil. Use of a long coiled inert Teflon tubing and an aqueous-aqueous polymer phase system makes partitioning of proteins possible without risk of sample loss and denaturation.

CCC, unlike other chromatography methods, is capable of handling crude complex mixtures and relatively large volumes. Since *S. aureus* is a common food contaminant, SEA testing must be able to accommodate food matrices

consisting of very different mixtures of proteins, fats, sugars, nucleic acids, and dyes at various pH values and salt concentrations. Because CCC is relatively insensitive to these variables, it is a good potential tool for food analysis. However, the utility of this method has not yet been recognized.

The purpose of this study is to evaluate the use of toroidal coil CCC in combination with Western immunoblotting to overcome the sample size limitations of Western immunoblotting and the problem of interfering proteins within the sample. We chose milk as a test substance because it has been implicated in SEA food poisoning in the United States[2,11] and cannot be analyzed reliably by direct immunoblotting.[6,7]

Our results demonstrate that CCC effectively separates SEA from other milk protein, yielding a sample that can be readily analyzed by Western blotting.



## EXPERIMENTAL

### Apparatus

The present studies employed a toroidal coil centrifuge to perform CCC extraction of SEA from milk extract. The head of a commercial floor model of the centrifuge (CRU 5000, Damon/IEC Division, Needham, MA, USA) was modified in such a way that the solvent can be continuously eluted through the rotating column without the use of a rotary seal device which may produce complications such as leakage and contamination. The detailed design of the apparatus is reported elsewhere.[12,13] A compact commercial model of the toroidal coil centrifuge is currently available through Pharma-Tech Research Corporation, Baltimore, Maryland.

The separation column was prepared from a single piece of 60 m long and 0.55 mm ID PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a nylon pipe (1.5mm OD and 7m long) which was coiled around the periphery of the centrifuge bowl forming multiple turns. The total column capacity was 13 mL.

### Sample Preparation

Low fat dry milk (Carnation) was diluted 1:10 with distilled water. The sample was spiked with pure SEA (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 100 ng/mL and used for the CCC separation.

**Reagents**

Polyethylene glycol (PEG) 1000 was purchased from Sigma and dibasic potassium phosphate from Mallinckrodt, Paris, KY, USA.

**Preparation of Two-Phase Solvent System and Analyte Solution**

The polymer phase system, composed of PEG1000 and dibasic potassium phosphate, each at 12.5% (w/w) in distilled water, was thoroughly equilibrated in a separatory funnel at room temperature. After the two clear layers were formed, the two phases were separated and each stored in a glass bottle.

An equal weight of distilled water was added to the milk sample. Then, an appropriate amount of PEG1000 and $K_2HPO_4$ was added to the above mixture to adjust the phase composition (in the sample solution) to meet the composition of the polymer phase system used for separation. The mixture was centrifuged at 1000g to separate two clear phases from the bulk of the precipitates. The partition coefficient (K) of the SEA in this solvent system is about 1; i.e., each phase contained SEA at a similar concentration. In each trial 0.5 mL of the lower phase was loaded into the column.

**CCC Procedure**

In each separation the column was first completely filled with the PEG-rich upper stationary phase. This was followed by injection of a test solution through the injection port. The column was rotated at 1200 rpm while the phosphate-rich lower phase was eluted through the column at a flow rate of 0.2mL/min. The effluent from the outlet of the column was continuously monitored with a UV detector (Uvicord S, LKB Instruments, Stockholm, Sweden) and collected into test tubes using a fraction collector (Ultrorac, LKB Instruments). After 2-3 hours of elution, the centrifuge was stopped and the column contents were pushed out by $N_2$ into a graduated cylinder to measure the volume of the stationary phase retained in the column. To reduce the elution volume the eluates were concentrated with a Centricon 10 concentrator (MW 10000) (Amicon, Beverly, MA, USA).

**SDS-PAGE and Immunoblotting**

Eluates were heated at 90°C for 2 min and immediately fractionated by 12.5% SDS-PAGE[14] as described.[6,15] Sample concentration: Lower phase fractions with SEA were concentrated by ultrafiltration using a Centricon 10 concentrator (Amicon, Beverly, MA) at 5000 g.

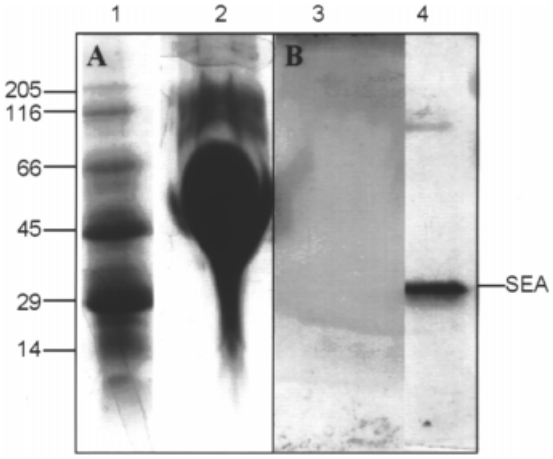


**Figure 1**. SDS PAGE analysis of SEA in milk before CCC separation. A. Coomassie blue stained gel; B. Western immunoblot analysis. Lane 1: molecular weight marker; Lane 2: crude sample of inoculated milk before CCC separation; Lane 3: Western immunoblot of crude sample of inoculated milk before CCC separation; Lane 4: pure SEA.



### Quantitation and Densitometry of the Western Blot

The filter was scanned by a Hewlett Packard 4C scanner and the bands were quantitated by using an NIH Image software. To determine the amount of SEA recovered, the SEA bands were compared to standard SEA bands of known concentrations.

## RESULTS

### Western Blot Analysis of SEA in Milk

To evaluate toroidal coil CCC as a tool for measuring SEA in food we attempted to separate SEA from milk spiked with SEA (100 ng/1mL). Samples were separated on 12.5% SDS-PAGE as described in Experimental. SDS-PAGE analysis of the starting material is shown in Figure 1. The Coomassie blue-stained gel (Figure 1 A lane 2) showed a large amount of proteins in the MW 14-80 kD range. The high concentration of these proteins overloaded the gel, resulting in distorted mobilities. As shown in Figure 1B, Western analysis of this gel (lane 3) fails to detect SEA. One way to overcome this problem is to dilute the sample.[6,7] However this decreases the sensitivity of the detection.

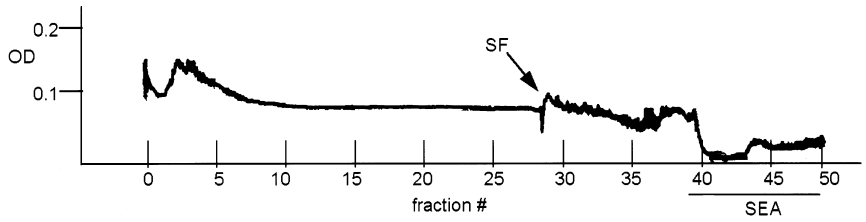


**Figure 2**. Chromatogram of CCC separation of native SEA added to milk. SF: solvent front.

As an alternative, we sought to separate and partially purify the toxin from the food matrix. Unlike chromatography on solid supports, CCC separation is relatively insensitive to crude material and can accommodate large volumes. Therefore, this method seemed suitable for separating the crude material analyzed in Figure 1.



## CCC Separation of SEA in Milk

The samples were applied to a CCC system and SEA was separated as described in Experimental. The solvent system was 12.5%(w/w) PEG 1000 and 12.5%(w/w) $K_2HPO_4$ in water because the partition coefficient of the target toxin was near 1. The lower phase was pumped at a flow rate of 0.2mL/min and the run was monitored at 280 nm (Figure 2). No major peaks were detected in the chromatogram. Given that the low concentration of SEA is well below the level of spectrophotometric detection we did not expect to see a SEA peak.

To determine if SEA was present in any of the resulting fractions we used Western immunoblots. A portion of each fraction was separated by SDS-PAGE (Figure 1). As seen in Figure 3, SEA is found predominantly in fractions 40-48. Although the SEA signal was strong, SEA was spread over 8 fractions (a volume of about 2 mL). There was no distortion of gel mobility as was seen in the original material (Figure 1). These results indicate that this CCC protocol is suitable for SEA separation from milk.

## Concentration of SEA

To increase the sensitivity of the assay, fractions with high amounts of SEA (fractions 40-48) were pooled and concentrated by ultrafiltration as described in Experimental. The final volume was approximately 100 μL. This concentrated sample was then separated by SDS-PAGE.

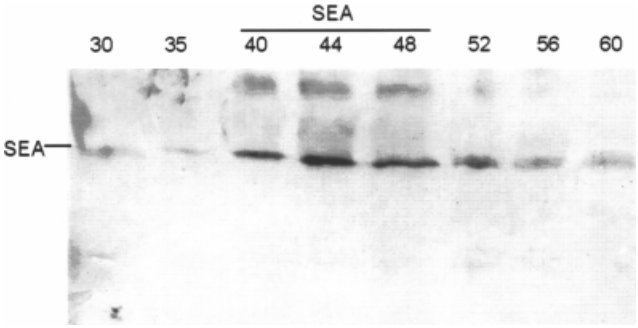


**Figure 3**. Western immunoblot analysis of CCC lower phase fractions after separation of SEA in milk fractions 30-60.

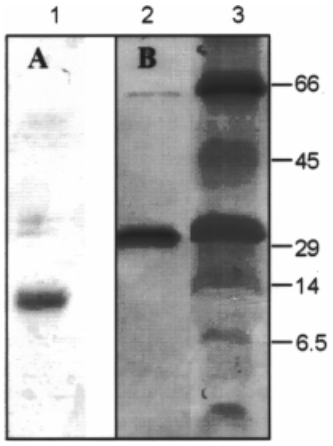


**Figure 4**. SDS PAGE analysis of concentrated lower phase fractions of CCC separation SEA in milk. A. Coomassie blue stained gel; B. Western immunoblot analysis. Lane 1: concentrated fractions of inoculated milk after CCC separation, lane 2: Western immunoblot of concentrated fractions of the original sample, lane 3: molecular weight marker.

Coomassie blue staining of all proteins (Figure 4, lane 1) demonstrated that most proteins were removed from these fractions. An immunoblot of a similar gel had a very intense signal in the lane with the concentrated fractions (Fig. 4, lane 2), while no signal was seen in the crude, unseparated sample (Fig. 1, lane 2).

The blot was analyzed by densitometry (see Experimental), and the signal in the sample separated by CCC separation and concentrated is ~11 times stronger than that of the starting material (not shown).

## DISCUSSION

One of the main advantages of CCC is that it can separate substances from relatively large volumes of crude and complex samples which is important for food analysis. This potential has not yet been recognized in the area of food testing. Recently, we have demonstrated that CCC is effective in separating toxin from mushrooms.[15] We have now continued this study by examining a food that cannot be examined by direct Western immunoblotting.

In this study, we used Western immunoblotting to demonstrate that toroidal coil CCC can separate SEA from milk.

CCC separation increases the reliability of SEA analysis by reducing the measurement background. The currently used methods (ELISA and Western blotting) are both immunological methods, which are prone to false positive results because the antibodies can cross-react with food. Since these ingredients may be removed by CCC, the combination of CCC separation with Western blotting has the potential of being a powerful and reliable assay system.

Eventually, combining CCC separation with other analytical identification methods, such as mass spectrometry, capillary electrophoresis or HPLC, may lead to non-immunological assays for SEA in food.



## ACKNOWLEDGMENTS

We are grateful for the advice and support of Dr. Adrian Weisz who initiated this project. This work was funded in part by the FDA food safety initiative funds.

## REFERENCES

1. D. L. Archer, F. E. Young, "Contemporary Issues: Disease with a Food Vector," Clin. Microbiol. Rev., **1**, 377-398 (1988).

2. M. I. Evenson, M. W. Hinds, R. S. Bernstein, M. S. Bergdoll, Int. J. Food Microbiol., **7**, 311-316 (1988).

3. R. W. Bennett, J. Assos. Off. Agric. Chem., **75**, 6-12 (1992).

4. C. E. Park, M. Akhtar, M. K. Rayman, Appl. Environ. Microbiol., **59**, 2210-2213 (1993).

5. C. E. Park, M. Akhtar, M. K. Rayman, Appl. Environ. Microbiol., **60**, 677-681 (1994).

6. A. Rasooly, **Bacteriological Analytical Manual**, 8th edition, revion A, AOAC International, 1998, pp. 13.33-13.44.

7. A. Rasooly, R. S. Rasooly, Int. J. Food Microbiol., in press (1998).

8. Y. Ito in, **Countercurrent Chromatography: Theory and Practice**, N. B. Mandava, Y. Ito, eds., Marcel Dekker, New York, 1988, chapter 3, pp. 79-442.

9. W. D. Conway, **Countercurrent Chromatography: Apparatus, Theory and Applications**, VCH, New York, 1990.

10. Y. Ito, **Countercurrent Chromatography in Chromatography, V**, J. Chromatogr. Library Part A., E. Heftmann, ed., chapter 2, Elsevier Scientific Publishing Co., Amsterdam, 1992, pp A69-A107.

11. M. I. Halpin-Dohnalek, E. H. Marth, J. Dairy Sci., **72**, 2266-75 (1989).

12. Y. Ito, R. L. Bowman, Anal. Biochem., **85**, 614-617 (1978).

13. Y. Ito, J. Suaudeau, R. L. Bowman, Science, **189**, 999- 1000 (1975).

14. U. K. Laemmli, Nature, **227**, 680-685 (1970).

15. A. Rasooly, Y. Ito, J. Liq. Chromatogr., **21 (1 & 2)**, 93-102 (1998).

Received September 27, 1998
Accepted October 17, 1998
Manuscript 4916

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