ELSEVIER

Journal of Chromatography A, 791 (1997) 53–63

JOURNAL OF CHROMATOGRAPHY A

Effect of foaming power and foam stability on continuous concentration with foam counter-current chromatography

Hisao Oka[a,*], Masato Iwaya[b], Ken-ichi Harada[b], Hideaki Murata[b], Makoto Suzuki[b], Yoshitomo Ikai[a], Junko Hayakawa[a], Yoichiro Ito[c]

[a]*Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462, Japan*
[b]*Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468, Japan*
[c]*Laboratory of Biophysical Chemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Received 12 May 1997; received in revised form 29 July 1997; accepted 29 July 1997

Abstract

In order to facilitate the application of the foam counter-current chromatography (CCC) technique to various natural products, we have introduced two parameters, i.e., ''foaming power'' and ''foam stability'', which reliably indicate applicability of the sample to foam CCC. In each test, the sample was delivered into a graduated cylinder with a ground stopper followed by vigorous shaking. The foaming power was expressed by the volume ratio of the resulting foam to the remaining solution, and the foam stability by the duration of the foam. A set of data obtained from various samples including bacitracin, gardenia yellow, phloxine B, rose bengale and senega methanol extract revealed a significant correlation between these parameters and the degree of foam enrichment by foam CCC. The overall results indicated that a sample with the following properties may be effectively enriched by foam CCC: the foaming power is greater than 1.0 and the foam stability is over 250 min. We applied these experimental results to the enrichment of microcystins in a cyanobacteria bloom sample extract using foam CCC. © 1997 Elsevier Science B.V.

Keywords: Counter-current chromatography; Foaming power; Foam stability; Gardenia yellow; Bacitracin; Peptides; Dyes; Triterpenoids; Saponins; Xanthenes; Microcystins

1. Introduction

Foam counter-current chromatography (CCC) is based on a unique parameter of foaming capacity or foam affinity of samples in aqueous solution and it has great potential for separations of biological samples [1]. The method utilizes a true counter-current movement between the foam and its mother liquid through a long, narrow coiled tube by the aid of a particular mode of planetary motion generated by a coil planet centrifuge. Samples introduced into the coiled tube are separated according to their foaming capacity or foam affinity. Foam active materials generate foam and quickly move with gas toward one end of the coiled tube while remainders are carried with the liquid stream in opposite direction and eluted through the other end of the coiled tube.

This foam CCC has been successfully applied to continuous enrichment and concentration of hydro-

*Corresponding author.

0021-9673/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0021-9673(97)00825-X

phobic bacitracin components using nitrogen gas and distilled water free of surfactant or other additives. The results indicated that the method may be applied to enrichment of minute amounts of foam active components from a bulk of aqueous solution.

In order to apply this technique to various natural products, it is necessary to develop a set of physico-chemical parameters which reliably indicate its applicability. In this connection we have introduced two parameters, namely, ''foaming power'' and ''foam stability'' which can be obtained by one simple test and using this method we investigated the correlation between these parameters and the degree of enrichment by foam CCC.

2. Experimental

2.1. Reagents

Phloxine B, rose bengale and gardenia yellow were obtained from Wako (Osaka, Japan). Bacitracin and powdered senega were purchased from P-L Biochemicals (Milwaukee, WI, USA) and Nippon Funmatsu Yakuhin (Osaka, Japan), respectively.

2.2. Preparation of crude extract of powdered senega

Powdered senega (30 g) was extracted three times with 150 ml of methanol for 30 min while stirring. The extract was concentrated to 50 ml under reduced pressure. Diethyl ether (30 ml) was added to the extract and the resulting precipitate was collected by filtration. After drying in air the crude extract of powdered senega (5.5 g) was obtained.

2.3. Preparation of extract of microcystins

Water bloom samples were collected in Lake Suwa, Japan, in 1991 and designated as 917S. Lyophilized cells (1.0 g) were extracted three times with 40 ml of distilled water for 30 min while stirring, and extracts combined.

2.4. High-performance liquid chromatography (HPLC) conditions

A chromatograph equipped with constant flow pumps (LC-100P, Yokogawa, Tokyo, Japan) was used with variable-wavelength UV–Vis detection (LC-100U, Yokogawa). The separation was performed under the following conditions:

2.4.1. Bacitracin

HPLC column: Capcell Pak C_{18} (5 μm, 150×4.6 mm I.D., Shiseido, Tokyo, Japan); mobile phase: methanol–0.04 *M* disodium hydrogenphosphate (62:38) solution; flow-rate: 1 ml/min; detection: 234 nm.

2.4.2. Gardenia yellow

HPLC column: LiChrosorb RP-18 (5 μm, 125×4.0 mm I.D., E. Merck, Darmstadt, Germany): mobile phase: acetonitrile–water; gradient rate: acetonitrile 5–45%, linear 20 min; flow-rate: 1 ml/min; detection: 254 nm for the detection of geniposide and at 435 nm for the detection of crocin.

2.4.3. Senega

HPLC column: Cosmosil $5C_{18}$-AR (5 μm, 150×4.6 mm I.D., Nakarai, Osaka, Japan); mobile phase: acetonitrile–0.01 *M* trifluoroacetic acid (TFA) (65:35) solution; flow-rate: 1 ml/min; detection: 315 nm.

2.4.4. Microcystins

HPLC column: Cosmosil $5C_{18}$-AR (5 μm, 150×4.6 mm I.D., Nacarai); mobile phase: methanol–0.05 *M* phosphate buffer (pH 3.0) (58:42); flow-rate: 1 ml/min; detection: 238 nm.

2.5. Measurement of foaming power and foam stability

The sample solution (20 ml) was delivered into a 100 ml graduated cylinder with a ground stopper and the cylinder vigorously shaken for 10 s. The foaming power was expressed by the volume ratio of the resulted foam to the remaining solution, and the foam stability by the duration of the foam.

2.6. Foam CCC

The apparatus used for the present study is a multilayer coil planet centrifuge that was designed and fabricated at the National Institutes of Health, Bethesda, MD, USA. The design of this prototype was reported in detail elsewhere [1].

The separation column consists of 10 m×2.6 mm I.D. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) coaxially wound around the holder hub making a total capacity of 50 ml. As schematically illustrated in Fig. 1, the coiled column is equipped with four flow tubes, the liquid outlet and the N_2 inlet at the head end and the foam outlet at the tail end while the sample inlet opens at the middle portion of the coil. A needle valve is placed on the liquid outlet line to regulate the liquid flow through the head of the coil.

Each experiment was initiated by introducing N_2 through the head of the coil at 5.76 kg/cm^2. Then, the column was rotated at 500 rpm followed by continuous introduction of the sample solution through the sample inlet tube by connecting it to the reservoir bottle pressured at 2.8 kg/cm^2. Due to the effect of the Archimedean screw force induced by the planetary motion, the sample solution quickly flows toward the head of the coil against the N_2 flow. This gas–liquid counter-current generates foam which is quickly carried with the N_2 stream toward the tail of the coil and collected through the foam outlet. The liquid flow through the coil can be regulated by a needle valve located at the liquid outlet (Fig. 1). Effluent from the foam and liquid outlets was separately collected and subjected to HPLC analysis.

In the present study, both N_2 and sample solution were introduced into the coil through their respective inlets under constant pressure (see Fig. 1) while the effluent flow through the head of the coil is restricted with a needle valve set on the liquid outlet line. If the valve is completely closed, both liquid and foam move toward the tail of the coil and elute together through the foam outlet without enrichment. As the opening of the valve becomes wider, the gas–liquid counter-current through the coiled column is enhanced, and the generated foam is subjected to skimming on the tail side of the coil resulting in enrichment of the foam active material eluting through the foam outlet. On the other hand, increasing valve opening results in a higher flow-rate of the sample solution and this is accompanied by a suppressed N_2 flow, tending to reduce the degree of foam enrichment. Thus, the greatest degree of enrichment of foam active compounds may be achieved by optimizing the valve opening.

Using the present device, five samples have been subjected to foam separation. For each sample, a series of studies was performed to examine the degree of foam enrichment of target components attained at various sample concentrations by manipulating the needle valve opening from 5–100% (14 turns).

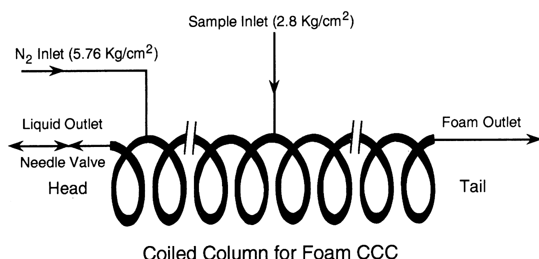

Fig. 1. Design of the coiled column for foam CCC.

3. Results and discussion

3.1. Introduction of foaming parameters

In foam CCC the sample solution is introduced from the sample inlet at the middle portion of the coiled column where it is immediately mixed with the N_2 stream and the foam generated moves toward the foam outlet at the tail. Since the coiled column consists of a 10 m long tube, the foam must travel through a 5 m long narrow coiled path before it reaches the foam outlet. As the foam travels through the decreasing pressure gradient along the coil, every bubble is expanded while the excess fluid is removed by the centrifugal force. Therefore, we assume that the foam must be subjected to a repetitive process of coalescence, eruption and regeneration before reaching the foam outlet at the tail. Consequently, successful foam CCC using nitrogen gas and distilled water free of surfactant or other additives requires strong foam-producing capability and foam stability of

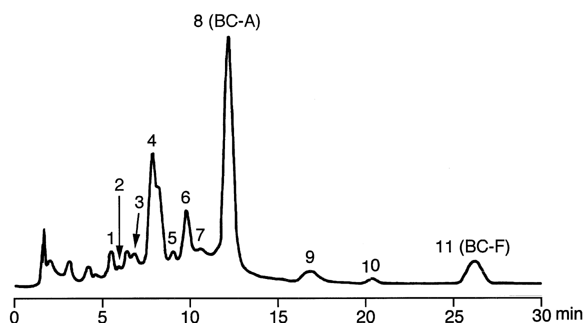

Fig. 2. HPLC separation of commercial bacitracin complex. HPLC conditions: see Section 2.4.

analytes. A lack of either capacity would result in its failure.

3.2. Foam CCC vs. foaming parameters

In order to correlate the foaming parameters measured by our simple test method to the foam productivity in foam CCC, we selected the following five samples because of their strong foaming capacity: bacitracin, gardenia yellow, rose bengal, phloxine B and senega methanol extract.

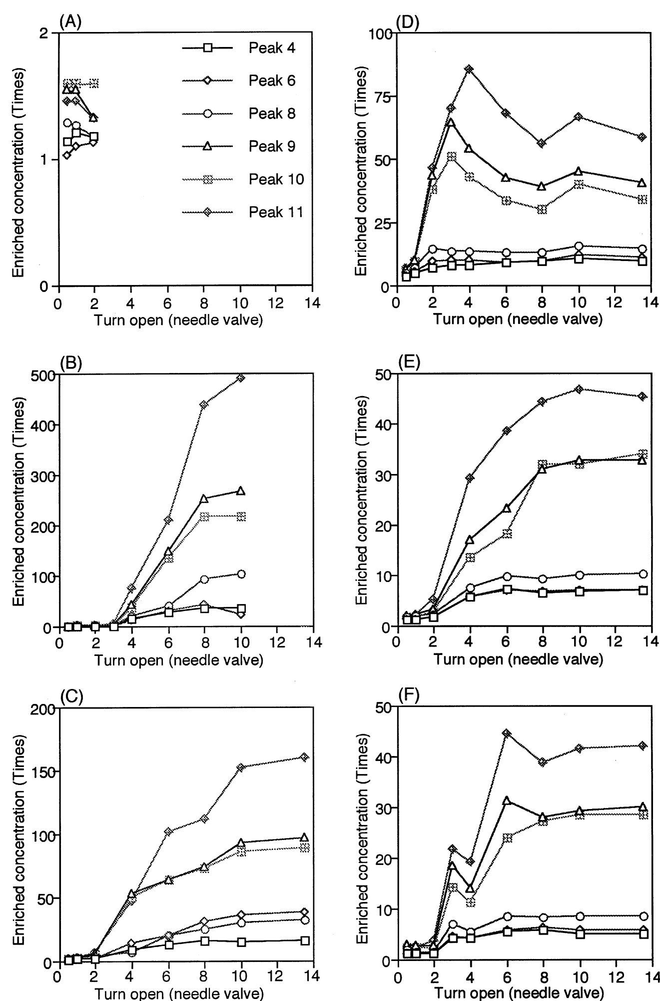

Fig. 3. Enrichment of bacitracin components in foam fractions using foam CCC with continuous sample feeding. (A) Bacitracin 50 ppm solution, (B) bacitracin 100 ppm solution, (C) bacitracin 120 ppm solution, (D) bacitracin 150 ppm solution, (E) bacitracin 200 ppm solution, (F) bacitracin 300 ppm solution.

3.2.1. Bacitracin

Bacitracin (BC) is a basic cyclic peptide antibiotic consisting of more than fifteen components including two major components, BC-A and BC-F, while the structures of other components are unknown [1]. Fig. 2 shows an HPLC chromatogram of bacitracin complex. In the present study, we paid the special attention to six peaks, i.e., peaks 4, 6, 8, 9, 10 and 11 to evaluate foam enrichment in foam CCC.

According to the procedure described in Section 2.6, foam CCC of bacitracin was performed using 100 ml sample solution at various concentrations ranging from 50 ppm to 300 ppm. For each sample concentration, the experiment was repeated by changing the needle valve opening from 3% (0.5 turn) to 100% (14 turns or full opening). These experimental results are summarized in Fig. 3.

At a concentration of 50 ppm (Fig. 3A), no significant foam enrichment was observed, and the valve opening more than 14% (2 turns) yielded no foam fraction. At 100 ppm (Fig. 3B), we observed excellent foam enrichment (nearly 500 times) (peak 11) at 71% valve opening (10 turns) although the valve opening of over 86% (12 turns) failed to yield a foam fraction. As the sample concentration was increased the foam enrichment was gradually decreased, nevertheless a significant enrichment of over 40 times (peak 11) was attained in a wide range of valve openings above 43% (6 turns) even at the maximum sample concentrations of 200–300 ppm (Fig. 3E and Fig. 3F). Fig. 4 shows the relationship between the degree of enrichment and the sample

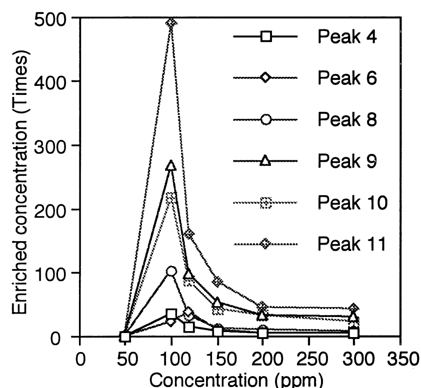

Fig. 4. Influence of the concentration of bacitracin on the enriched concentration.

concentration in foam CCC depicted at the optimum point. For example at 50 ppm we selected the results at 17% valve opening (2 turns), at 100 ppm at 71% (10 turns) and so forth. The diagram clearly indicates that the significant enrichment of bacitracin components is attained with the sample concentrations at 100 ppm or greater. From the above results, we may conclude that 100 ppm is near the lower limit of bacitracin concentration which provides efficient foam enrichment by the present method. This lower limit is used to correlate the results obtained by our foaming test as described below.

Fig. 5 shows the foaming power (left) and the foam stability (right) of the bacitracin complex measured by the simple test (see Section 2.5). Between 50 and 100 ppm the foaming power in-

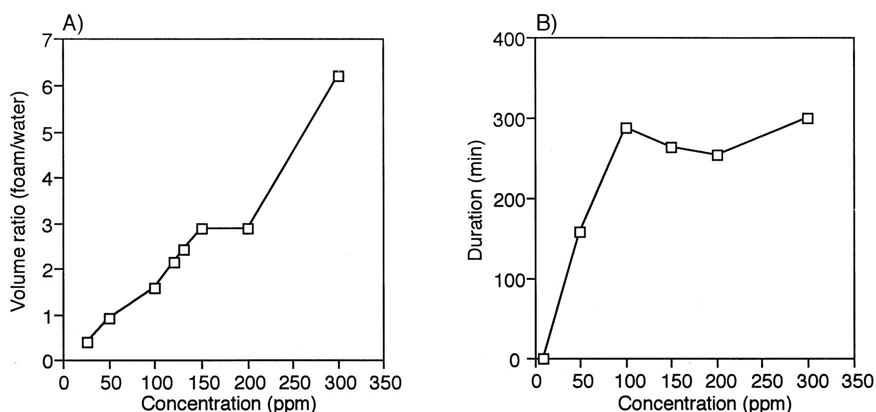

Fig. 5. Foaming power and foam stability of bacitracin complex. (A) Foaming power, (B) foam stability.

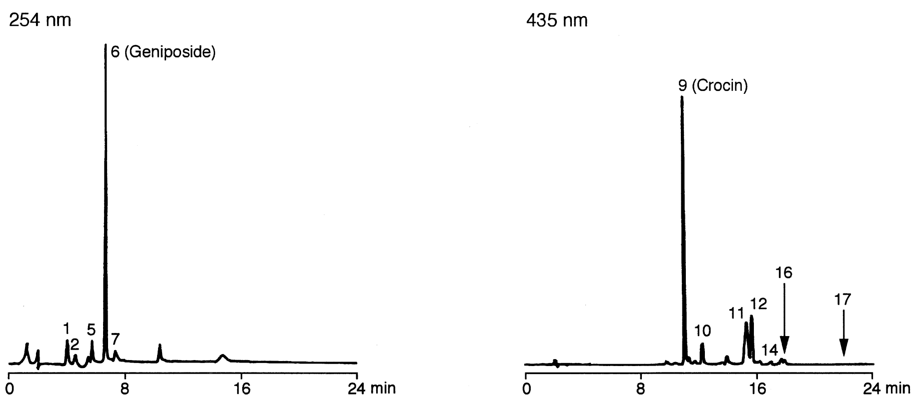

Fig. 6. HPLC separation of gardenia yellow components. HPLC conditions: see Section 2.4.

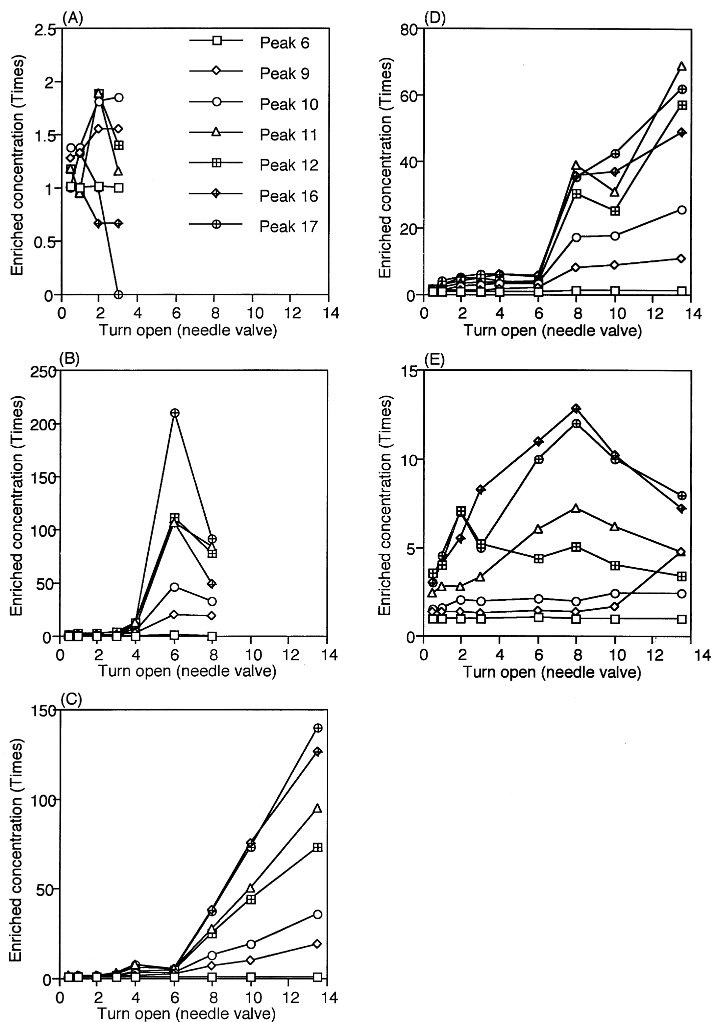

Fig. 7. Enrichment of gardenia yellow components in foam fractions using foam CCC with continuous sample feeding. (A) Gardenia yellow 100 ppm solution, (B) gardenia yellow 250 ppm solution, (C) gardenia yellow 300 ppm solution, (D) gardenia yellow 500 ppm solution, (E) gardenia yellow 2500 ppm solution.

creases from 0.9 to 1.6 and the foam stability from 150 to 300 min. If these parameters directly reflect the results of foam CCC, we can assume that a sample having the foaming power greater than 1.0 and a foam stability over 250 min could be effectively enriched by foam CCC. These minimum requirements of foaming parameters tentatively determined by the bacitracin experiment were tested for their applicability to several other samples.

3.2.2. Gardenia yellow

Gardenia yellow is a natural coloring substance and consists of more than ten components including geniposide and crocin and other minor components of unknown nature [2]. Fig. 6 shows the HPLC analysis of gardenia yellow components monitored at 254 nm for geniposide (left) and at 435 nm for crocin (right). In the present study, we selected seven peaks including 6, 9, 10, 11, 12, 16 and 17 to evaluate foam CCC.

Fig. 7 shows the results of foam CCC of gardenia yellow. At 100 ppm (Fig. 7A), there is no significant foam enrichment and a valve opening of over 25% (3.5 turns) produced no foam fraction. At 250 ppm (Fig. 7B), however, significant foam enrichment of over 200 times is attained with a valve opening of 43% (10 turns). The foam enrichment steadily declines as the sample concentration is further increased from 300 ppm (Fig. 7C) to 2500 ppm (Fig. 7E).

Fig. 8 shows the foaming power of gardenia yellow. At 100 ppm, the parameter failed to meet the minimum requirement previously set at 1.0 for bacitracin complex. However, at a concentration of 250 ppm which gave efficient enrichment, the foaming power became greater than 1.0 and the foam stability was over 480 min, exceeding the minimum requirement values for both parameters, supporting the applicability of the method.

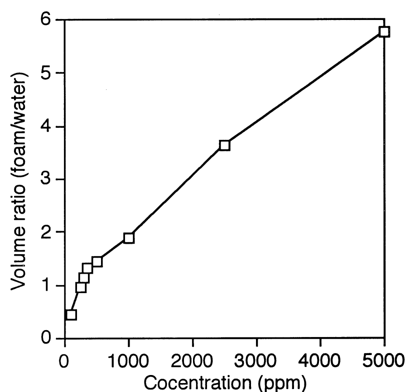

Fig. 8. Foaming power of gardenia yellow.

3.2.3. Senega methanol extract

Senega methanol extract contains triterpenoid saponins which are typical foaming compounds. Main components are senegin II, II′, III, and IV [3]. Fig. 9 shows a typical HPLC analysis of senega methanol extract. In the present study we selected peaks 2 (senegin IV), 5 (senegin III), 6 (senegin II′), 8 and 10 (senegin II) to measure the enrichment. Fig. 10 shows the results. At a concentration of 50 ppm (Fig. 10A), no foam fraction was collected. Increasing the concentration to 100 ppm (Fig. 10B) yielded a foam fraction enriched by 2–10 times while a valve opening of over 29% (4 turns) failed to elute foam. Further increasing the concentration to 250 ppm (Fig. 10C) produced significant foam enrichment of over 10 times at 57% valve opening (8 turns). At concentrations of 500 to 1000 ppm (Fig. 10C and Fig. 10D), foam enrichment continued but at lower levels (2–5 times). The effects of the valve opening and sample concentration on foam enrich-

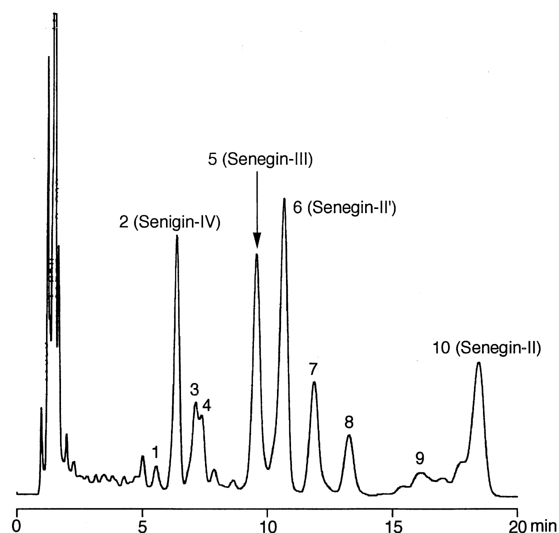

Fig. 9. HPLC separation of senega methanol extract. HPLC conditions: see Section 2.4.

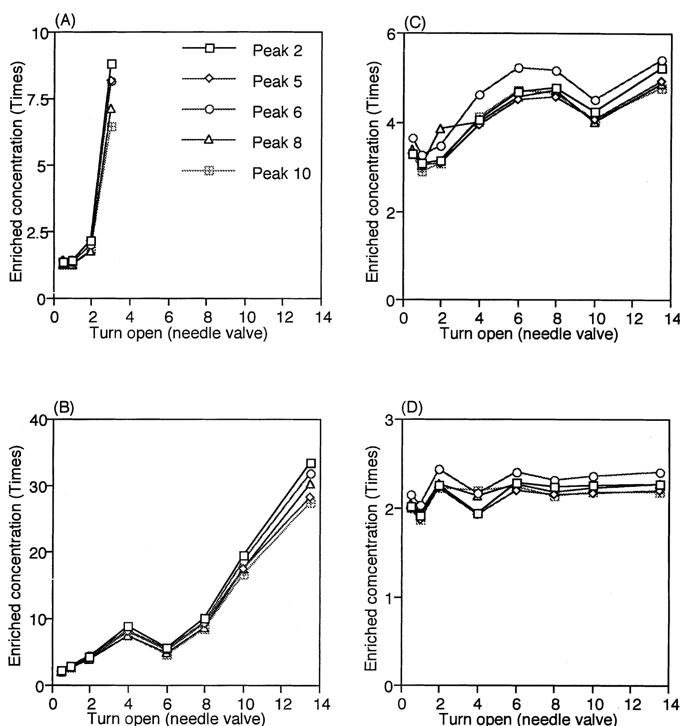

Fig. 10. Enrichment of the components of senega methanol extract in foam fractions using foam CCC with continuous sample feeding. (A) Senega methanol extract 100 ppm solution, (B) senega methanol extract 250 ppm solution, (C) senega methanol extract 500 ppm solution, (D) senega methanol extract 1000 ppm solution.

ment were similar to those observed with the bacitracin complex and gardenia yellow.

The foaming power of senega methanol extract is shown in Fig. 11. The concentration range between 25 to 1000 ppm showed over 480 min of foam stability. Between 25–50 ppm, although the foam stability is over the minimum requirement of 250 min, the foaming power is below 1.0. When the concentration was further increased to 100 ppm, both foaming power and foam stability values exceeded the minimum requirement values, and showed a good correlation with the results obtained by foam CCC.

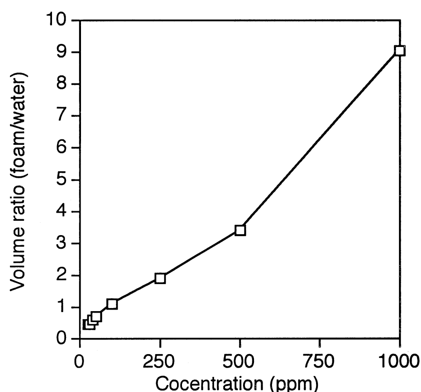

Fig. 11. Foaming power of senega methanol extract.

3.2.4. *Phloxine B and rose bengale*

Many xanthene dyes exhibit strong foaming capacity. Among them we chose rose bengale and phloxine B for the present study. Fig. 12 shows the foaming power (left) and foam stability (right) of rose bengale. At concentrations between 5000 and 50 000 ppm, the foaming power exceeds the minimum requirement but the foam stability is below the critical value for all concentrations. Because the foam stability is greater at lower concentrations, we applied to foam CCC at the concentrations of 10 000 ppm and 25 000 ppm, but we found that no foam was eluted in either case.

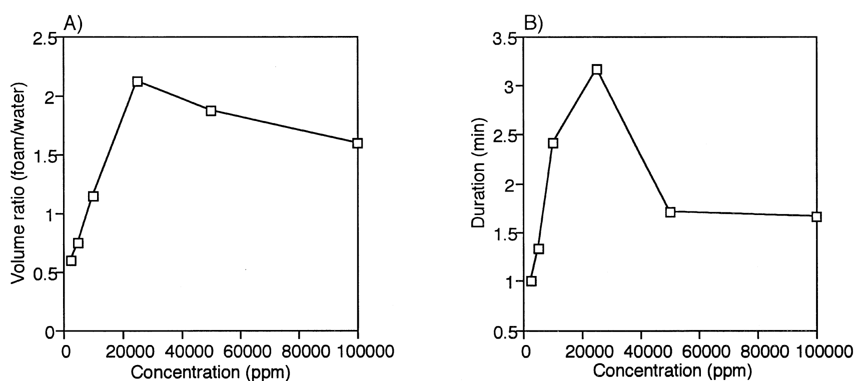

Fig. 12. Foaming power and foam stability of rose bengale. (A) Foaming power, (B) foam stability.

Fig. 13 shows the foaming power and foam stability of phloxine B. At concentrations between 10 000 and 100 000 ppm, the foaming power exceeded the minimum requirement of 1.0, but the foam stability was below the critical value. We tested foam CCC at 25 000 ppm but no foam was eluted.

3.3. Application of foam CCC to enrichment of microcystins

The above method has been tested for enrichment of microcystins. These are hepato-toxic cyclic peptides produced by cyanobacteria with foaming properties [4]. We extracted microcystins from the 917S with distilled water and obtained two extracts with different foaming capacities. The first extract had foaming power of 1.88 and foam stability of 93 min which satisfied only the former requirement. The second extract had a foaming power of 1.32 and the foam stability of 720 min which satisfied both requirements for foam CCC. We applied both samples to foam CCC.

Fig. 14A shows a typical HPLC chromatogram of an extract from cyanobacteria bloom sample 917S containing microcystins. We chose peaks 1 (microcystin RR), 2 (microcystin YR), 3 (microcystin LR), and 4 (microcystin LR-s) to evaluate their foam enrichment. The first extract yielded foam fraction at 14% valve opening (2.0 turns). The HPLC analysis of the original sample, foam fraction and liquid fraction are shown in Fig. 14A–C, respectively. The enriched concentrations of the components are only 3–4 times and polar components with retention times shorter than that of microcystin RR were not eliminated. The second extract produced foam at 29% valve opening (4.0 turns). The HPLC analysis of original sample, foam fraction and liquid fraction are similarly shown in Fig. 15. The enrichment reached

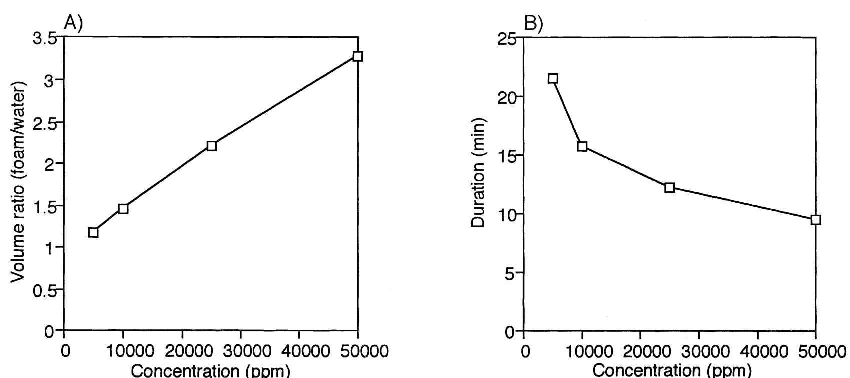

Fig. 13. Foaming power and foam stability of phloxine B. (A) Foaming power, (B) foam stability.

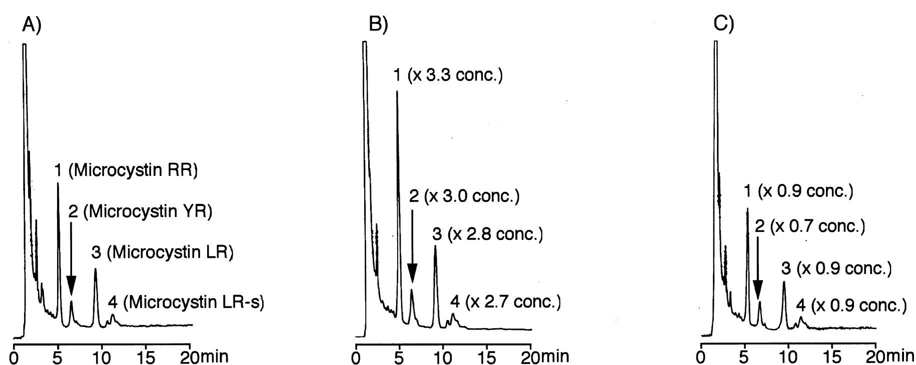

Fig. 14. HPLC analyses of bloom sample 917S extract that does not satisfy the foaming parameters. (A) Original sample, (B) foam fraction, (C) liquid fraction.

10–30 times and polar components are eliminated from the foam fraction indicating that the target compounds are selectively enriched. The HPLC analysis of liquid fraction of both extracts show similar profiles. The above results indicate that the foaming parameters can be effectively applied to a crude mixture containing a large amount of impurities.

4. Conclusions

In order to test whether foam CCC can be applied to various samples, two physical parameters, i.e., "foaming power" and "foam stability" are measured by means of a simple experiment with a graduated cylinder. The correlation between these parameters and foam enrichment in foam CCC has been examined for five samples including bacitracin complex, gardenia yellow, senega extract, phloxine dyes and microcystins. If the foaming power and foam stability exceed 1.0 and 250 min, foam-producing components in the sample will be effectively enriched using foam CCC.

Acknowledgements

This study was partially supported by a grant of the Ministry of Education, Japan. We are grateful to Dr. Naohisa Ishikawa, the Director of Aichi Prefectural Institute of Public Health for his encouragement. We also thank Dr. Henry M. Fales for editing the manuscript.

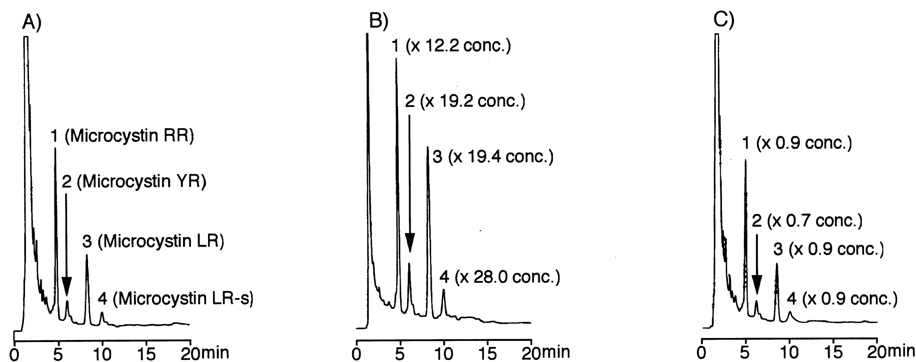

Fig. 15. HPLC analyses of bloom sample 917S extract that satisfies the foaming parameters. (A) Original sample, (B) foam fraction, (C) liquid fraction.

References

[1] H. Oka, in Y. Ito and W.D. Conway (Editors), High-Speed Countercurrent Chromatography, Wiley, New York, 1996, Ch. 5, pp. 107–120.

[2] H. Oka, Y. Ikai, S. Yamada, J. Hayakawa, K.-I. Harada, Y. Yamazaki, M. Suzuki, H. Nakazawa and Y. Ito, in W.D. Conway and R.J. Petroski (Editors), Modern Countercurrent Chromatography (ACS Symposium Series), American Chemical Society, Washington, DC, 1995, Ch. 8, pp. 92–106.

[3] J. Shoji, S. Kawanishi, Y. Tsukitani, Yakugaku Zasshi 91 (1971) 198–202.

[4] K.-I. Harada, in M.F. Watanabe, K.-I. Harada, W.W. Cramichael and H. Fujiki (Editors), Toxic Microcystis, CRC Press, New York, 1995, Ch. 6, pp. 103–148.