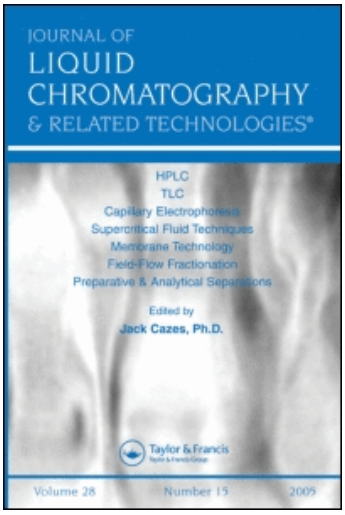

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Y. Ikai[a]; H. Oka[a]; J. Hayakawa[a]; N. Kawamura[a]; K. Harada[b]; M. Suzuki[b]; H. Nakazawa[c]; Y. Ito[d]
[a] Aichi Prefectural Institute of Public Health, Nagoya, Japan [b] Faculty of Pharmacy Meijo University Tempaku, Nagoya, Japan [c] Faculty of Pharmaceutical Science Hoshi Unversity, Tokyo, Japan [d] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, Bethesda, MD, USA

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ISOLATION OF COLISTIN A AND B USING HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

Yoshitomo Ikai,[1] Hisao Oka,[1] Junko Hayakawa,[1] Norihisa Kawamura,[1] Ken-ichi Harada,[2] Makoto Suzuki,[2] Hiroyuki Nakazawa,[3] Yoichiro Ito[4]

[1] Aichi Prefectural Institute of Public Health
Tsuji-machi, Kita-ku
Nagoya 462, Japan

[2] Faculty of Pharmacy
Meijo University
Tempaku, Nagoya 468, Japan

[3] Faculty of Pharmaceutical Science
Hoshi Unversity
Ebara 2-4-41, Shinagawa-ku
Tokyo 142, Japan

[4] Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, MD 20892, USA

ABSTRACT

High-speed countercurrent chromatography was successfully applied to the isolation of colistin-A and colistin-B from a commercial colistin preparation. As the first step, TLC and HPLC analysis conditions for the colistin components were

established. Using these techniques, a two-phase solvent system composed of n-butanol-0.04M aqueous trifluoroacetic acid (TFA) (1:1) was selected for high-speed CCC, where the concentration of TFA was the major factor in controlling the partition coefficients of the colistin components. Yields of colistin-A and colistin-B were 9 mg each from 20 mg of the commercial sample, and the purity of each component was over 90 %. Fast atom bombardment (FAB) mass spectrometry was utilized to confirm the nature of the isolated components.

INTRODUCTION

Colistin (CL) is a peptide antibiotic produced by *Bacillus polimyxa var. colistinus* that exhibits an antibacterial activity against gram-negative organisms.[1] CL is a mixture of many components including colistin A (CL-A) and B (CL-B) as the main components.

As shown in Fig. 1, CL-A and CL-B are both linear-ring peptides and they differ only in their *N*-terminal fatty acids.[2] CL is used for domestic animals such as calf and swine as a feed additive for the purpose of preventing diseases and/or improvement of feed conversion efficiency. However, such usage of antibiotics may involve a risk to human health if they remain in the meat.

Therefore, it is desirable to monitor the residues in livestock products using a simple, rapid, and reliable analytical method. Although chemical methods have been advanced to determine and identify a number of antibiotics, an analytical method for CL has never been developed. Therefore, we wanted to establish an HPLC method for residual CL in food.

For this purpose, we have first undertaken the purification of CL-A and CL-B from a commercial CL preparation to obtain the standards which are not otherwise available. We have chosen high-speed countercurrent chromatography (HSCCC) to separate the CL components, since the method has been successfully used for the separation of bacitracin, a peptide antibiotic with a similar ring structure.[3]

In the present study, our effort has been focused on (1) establishing the optimal TLC and HPLC conditions for CL analysis which are essential for optimization of HSCCC conditions, (2) analysis of the HSCCC fractions, and (3) estimation of the purity of isolated components.

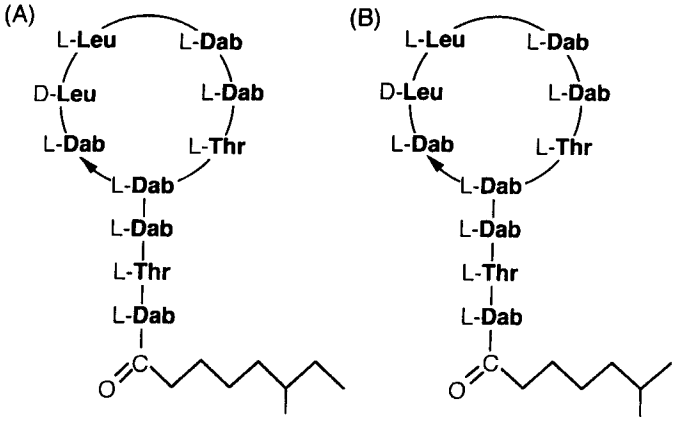

Figure 1. Structures of colistin components. (A) Colistin A, $C_{53}H_{100}N_{16}O_{13}$, MW: 1168. (B) Colistin B, $C_{52}H_{98}N_{16}O_{13}$, MW: 1154. Dab: α, γ-diaminobutyric acid.

EXPERIMENTAL

Materials

Acetone, acetonitrile, ethanol, n-butanol, glycerol, methanol, sodium chloride (NaCl), sodium sulfate (Na_2SO_4), sodium hydroxide, sodium borate, hydrochloric acid (HCl), sulfuric acid (H_2SO_4) and trifluoroacetic acid (TFA) were all analytical grade reagents. Colistin sulfate (19,600 units/mg) was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Fluorescamine and RP-18 TLC plate (Art. 15389) were obtained from E. Merck (Darmstadt, Germany).

The HPLC columns, Kaseisorb ODS-300-5 (5 μm, 250 x 4.6 mm I.D.), Nucleosil 7Ph (7 μm, 250 x 4.6 mmI.D.), Inertsil 5Ph (5 μm, 250 x 4.0 mm I.D.) and Chromatorex 5Ph (5 μm, 250 x 4.6 mm I.D.) were purchased from Tokyo Kasei, Nagel (Duren, Germany), GL-Science (Tokyo, Japan) and Fuji-Silicia (Aichi, Kasugai, Japan), respectively.

TLC Conditions

The samples were separated on a RP-18 TLC plate using an acetone-2 M NaCl aqueous solution (1:1) as a solvent system. The spots on the TLC plate were

detected by the following fluorometric derivatization methods. After spraying a 0.5M borate buffer solution (pH 10.0) followed by a 0.2 % fluorescamine-acetone solution, and the resulting fluorescence was observed under UV light (365 nm).

HPLC Conditions

The separation of the sample was performed on Chromatorex 5Ph HPLC column using acetonitrile-0.01 M TFA aqueous solution (24:76) as a mobile phase. The flow rate of the mobile phase was set at 1.0 mL/min and the effluent from the HPLC column was monitored at the wavelength of 210 nm.

Determination of Partition Coefficient (*K*)

Approximately 1 mg of test sample was weighted in a test tube to which 1 mL of each phase of preequilibrated two-phase solvent system was added, and the test tube was shaken vigorously for 1 min. After settling, 5 μL of the upper and lower layer were injected into the HPLC system to obtain the partition coefficient.

Preparative Separation of CL by HSCCC

The apparatus used was an HSCCC-1A prototype multilayer coil planet centrifuge (Shimadzu, Kyoto, Japan) with a 10 cm orbital radius which produces a synchronous planetary motion at 800 rpm. The multi-layer 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 about 300 mL.

The two-phase solvent system composed of n-butanol and 0.04 M TFA aqueous solution (1:1) was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking and degassing at room temperature. The column was first entirely filled with the upper n-butanol phase, then 20 mg of the sample dissolved in 2 mL of each phase was loaded.

The centrifuge was rotated at 800 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 of different density move toward the head of the coil) at a flow rate of 2 mL/min by HPLC pump. The effluent from the outlet of the column was fractionated into test

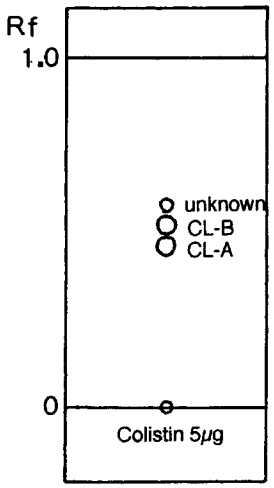

Figure 2. Separation of CL components on TLC. Separation conditions: plate, RP-18 TLC (Merck art 15389); solvent system, acetone-2 M NaCl aq. soln (1:1). Detection procedures: (1) Spray 0.5 M borate buffer (pH 10); (2) Spray 0.2 % fluorescamine-acetone solution; (3) Observe the fluorescence under UV-light (365 nm).

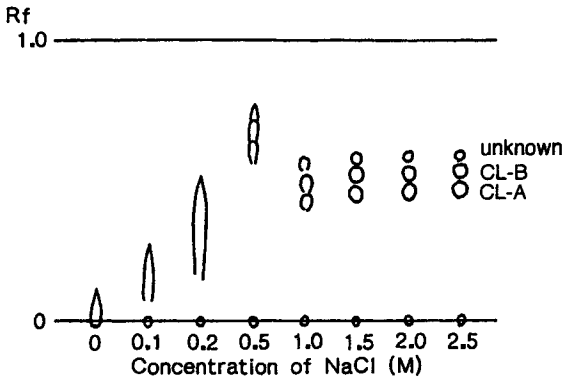

Figure 3. Influence of concentration of NaCl on the separation of CL components. Solvent system, acetone-NaCl aq. soln; other conditions, see Fig. 2.

tubes at 2 mL per tube with a fraction collector. When the separation was complete, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas combined with slow rotation of the coil in the tail-head elution mode.

FABMS Analysis

The FAB mass spectra were obtained in a JMS- AX505W double-focusing mass spectrometer (JEOL, Tokyo, Japan). A xenon ion gun was operated at 5kV. The matrix used was glycerol - 1N HCl (1:1).

RESULTS AND DISCUSSION

Optimization of TLC Conditions

Several TLC conditions for CL have already been reported,[4-8] but most of them are not applicable to this study because of insufficient separation. Only Thomas *et al.*[8] separated CL into 3 spots using a laboratory made silica gel TLC silanized plate and a solvent system consisting of acetone and HCl-NaCl aqueous solution. Similar or more improved results may be achieved using the conventional reversed phase TLC plates.

In order to optimize the separation conditions, a series of preliminary studies was carried out using various organic solvents, aqueous solutions of various salts, and 2 types (C_8 and C_{18}) of TLC plates. The most satisfactory separation was given by a combination of C_{18} TLC plate and acetone-2M NaCl aqueous solution (1:1) as shown in Fig. 2.

In this solvent system, the concentration of NaCl was the most influential factor for the separation. The spots of CL components were not separated without the salt in the solvent system due to extreme tailing, and the shape of the spots was improved by increasing the salt concentration as shown in Fig. 3. The tailing of the spot has been attributed to the influence of silanol groups on the stationary phase, and the salt added to the solvent system can reduce the interaction between the silanols and the analyte.

Optimization of HPLC Conditions

Many HPLC conditions have been reported concerning the separation of CL components.[9-13] Most of them utilize C_{18} HPLC columns and describe satisfactory separations. Their operation conditions require a highly concentrated salt such as NaCl and Na_2SO_4 in the mobile phase to control the peak tailing. We supposed that the tailing is attributed to an interaction between the primary amino groups of CL and residual silanol groups in the stationary phase as described above.

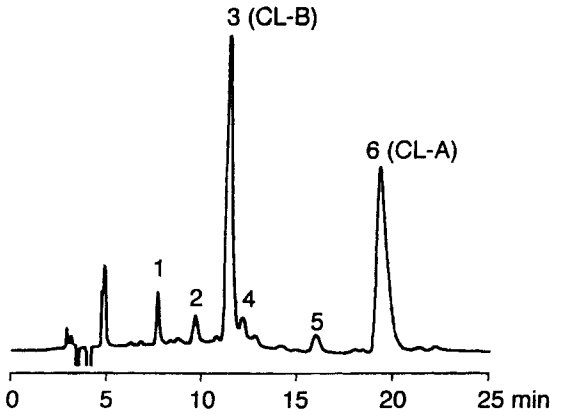

Figure 4. HPLC profile of commercial CL under the optimal conditions. Conditions: Column, Chromatorex Ph (5 μm, 250 x 4.6 mm ID); Mobile phase, acetonitrile-0.01M TFA aqeous solution. (24:76); flow rate, 1.0 mL/min; detection: 210 nm.; sample size, 5 μg.

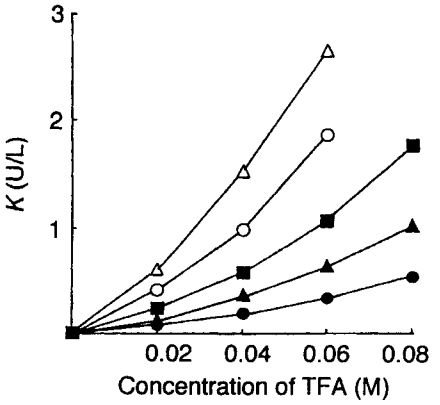

Figure 5. Influence of concentration of TFA on the partition coefficient of CL components. Keys: ●, Peak No. 1 of Fig. 5; ▲, peak No. 2; ■, peak No. 3 (CL-B); ○, peak No. 5; Δ, peak No. 6 (CL-A).

However, too high a concentration of salt in the mobile phase shortens the column life and causes other complications in the HPLC system such as clogging of the transfer tubing. Therefore, we intended to establish new HPLC conditions for CL analysis without salt in the mobile phase.

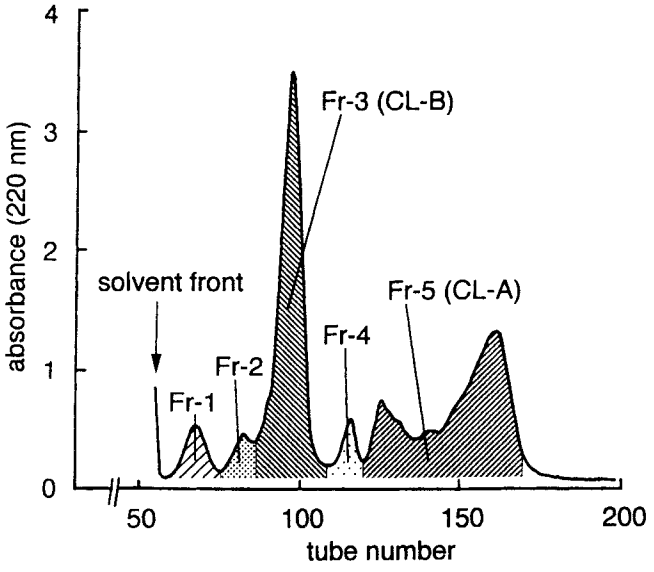

Figure 6. HSCCC elution curve of commercial CL. Conditions: apparatus, HSCCC-1A; revolution, 800 rpm ; solvent system, *n*-butanol-0.04M TFA aq. soln (1:1); mobile phase, lower phase; Flow rate of mobile phase, 2.0 mL/min; detection, 220 nm; sample size, 20 mg.

A combination of acetonitrile and aqueous TFA is frequently used as a mobile phase for the HPLC separation of peptides and proteins. We attempted to separate CL components using various ratio of acetonitrile and 0.01 M TFA aqueous solution as mobile phases, but satisfactory results could not be obtained on conventional C_{18} HPLC columns because of peak tailing. Therefore, we tried other types and excellent separations were obtained by using wide-pore ODS column (Kaseisorb ODS-300-5) and 3 kinds of phenyl type columns (Nucleosil 7Ph, Inertsil 5Ph and Chromatorex 5Ph). Among them, we chose a Chromatorex 5Ph, because a maximum number of peaks was observed. Fig. 4 shows the HPLC profile of commercial CL under the optimal conditions.

Selection of Two-Phase Solvent System for HSCCC

HSCCC requires a mutually equilibrated immiscible solvents (two-phase solvent system), one as the stationary phase and the other as the mobile phase, where the analytes are partitioned between these two phases. Generally, the two-phase solvent system for HSCCC consists of an organic solvent, water and some modifiers.

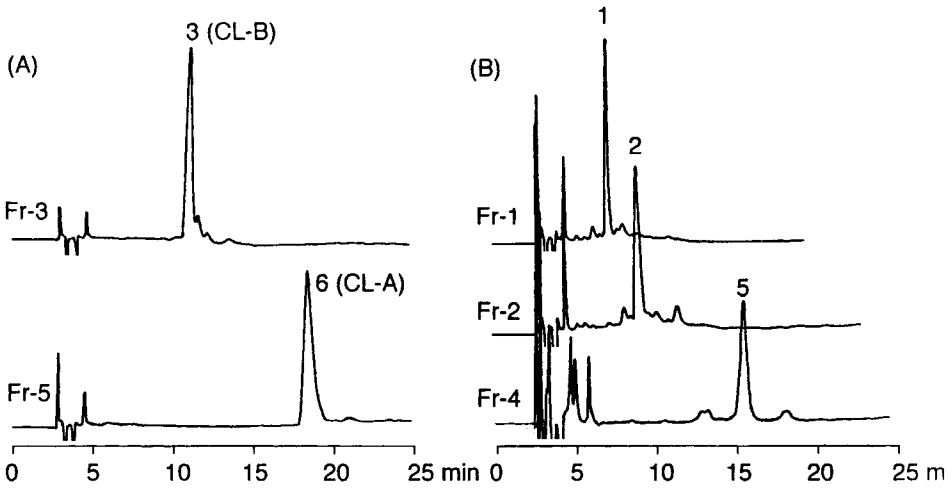

Figure 7. HPLC profiles of isolated components of CL. (A) CLs-A (Fr-5) and -B (Fr-3), (B) Frs-1, -2 and -4. Conditions: see Fig. 4.

The performance of the solvent system can be estimated by measuring the partition coefficient (K) of the analytes, separation factor between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) and settling time which governs the degree of stationary phase retention. It is known that the best separation can be achieved when the K value is close to 1.0, α value is greater than 1.5, and the settling time is shorter than 30 sec.[4]

In consideration of these three parameters, the two-phase solvent system was selected as described below: CL is soluble in water, slightly soluble in alcohols but almost insoluble in nonpolar solvents such as hexane and chloroform. From this property, we selected *n*-butanol and water as a basic solvent composition. However, this binary solvent system *per se* was not suitable for use, because the CL components were partitioned mostly into the aqueous phase. In order to partition the CL components into the butanol phase, various modifiers such as salts (NaCl and Na_2SO_4) and acids (HCl, H_2SO_4 and TFA) were added and, finally, a desirable K value was obtained by using TFA. It was found that the partition of CL components into the butanol phase was increased with the concentration of TFA in the solvent system. We considered the reason as follows: As shown in Fig. 1, both CL-A and CL-B have five free amino groups originated from L-α,γ-diaminobutyric acid (L-Dab) in their structures, and these protonated amino groups in the aqueous solution to form neutral lipophilic ion pairs with TFA anions. As a result, the hydrophobicity of CL components increases with the concentration of TFA. In order to determine the optimal concentration of TFA, K values of 5 components were measured under various TFA concentrations. As shown in Fig. 5, the K value of each CL component

increased with an increased concentration of TFA. When the TFA concentration was 0.04 M, the K values of CL-A and CL-B were 1.5 and 0.6, respectively, where all α values were greater than 1.5, and settling time was 28 sec. From these results, a good separation of LC components is expected by using TFA at 0.04M. Therefore, we selected n-butanol-0.04 M TFA aqueous solution (1:1) as a two-phase solvent system for the HSCCC separation of CL components.

Preparative Separation of CL Components

Using the two-phase solvent system described above, 20 mg of commercial CL were separated by HSCCC. Retention of the stationary phase was 45.0 %. The HSCCC fractions were subjected to TLC analysis, and the UV absorbance was measured in every tube at the wavelength of 220 nm to draw the elution curve. From the TLC analysis, it was found that CL-A and CL-B were eluted at fractions 120 - 170 and 80 -110, respectively. According to the results of the TLC analysis and the elution curve shown in Fig. 6, the collected samples were combined to make 5 major fractions (Fr-1,tube No. 59-74; Fr-2, 75-85; Fr-3, 86-107, Fr-4, 108-120; Fr-5, 121-170) as shown in Fig. 6.

Each fraction was evaporated to 1-2 mL under reduced pressure at 40°C and lyophilized. Yields of CL-B in Frs-3 and CL-A in Fr-5 were 9 mg each and the other minor components were 0.5 - 1.0 mg. HPLC analysis was performed for each fraction. As shown in Fig. 7 (A), the fractions of CL-A and CL-B were almost single components, and the purity of both components was estimated to be over 90 % from the chromatograms. Although the chromatograms of Frs-1, -2 and -4 show mixtures of the minor components, the major peaks 1, 2 and 5 in these chromatograms represent minor impurities of the original sample as seen in the chromatogram of commercial CL in Fig. 4. The structures of these minor components, however, have not been elucidated so far. In order to confirm the structures of isolated CL-A and CL-B and to characterize the structures of the above minor components, FAB mass analysis was carried out for each fraction. The results show that intensive protonated molecules and some fragment ions were observed in the FAB mass spectra of CL-A and CL-B, supporting their structures as shown in Fig. 8. The minor components showed similar mass spectra to CL-A and CL-B, indicating that they are closely related to CL-A and CL-B in their structures. From the protonated molecules in each mass spectrum, the molecular weights of the components from peaks 1, 2 and 5 were estimated as 1140, 1154 and 1168, respectively. However, additional information concerning the structures of those minor components could not be obtained, because of noisy background caused by the impurities in the sample. We are planning to further purify those fractions of minor components by HSCCC for structural characterization.

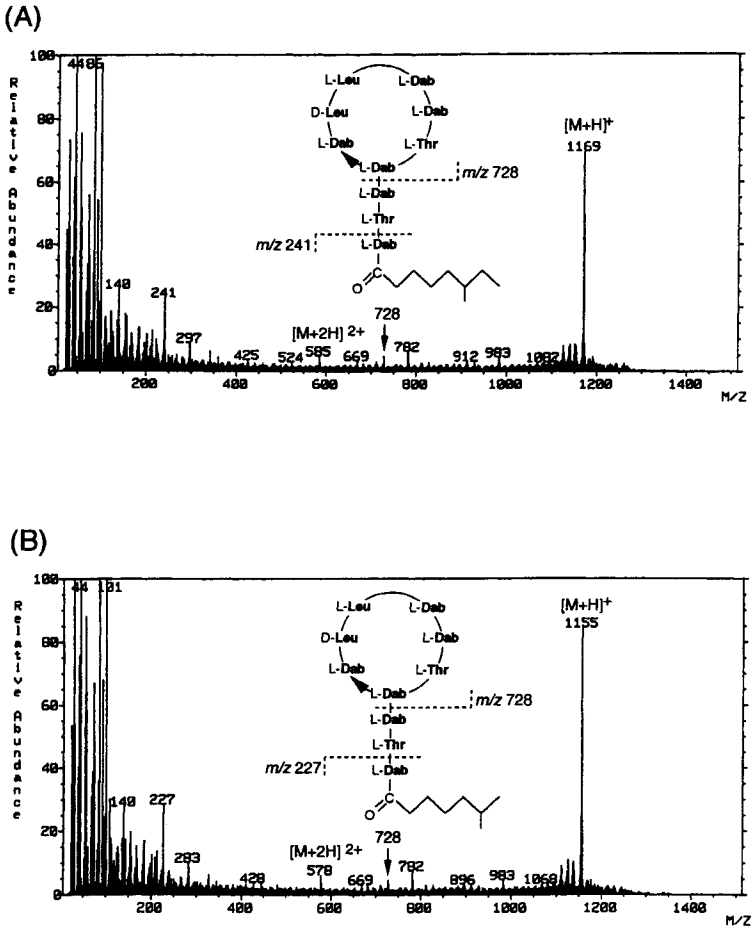

Figure 8. FAB mass spectra and fragmentation patterns of CL-A and CL-B. (A) CL-A, (B) CL-B. Conditions; primary beam, Xe^0; Accel. volt, 5kV (primary), 3kV (secondary); matrix, glycerol-1N HCl (1:1).

CONCLUSION

In order to isolate CL-A and CL-B from a commercial CL preparation, separation conditions of CL components were examined using TLC, HPLC and HSCCC. CL was separable into three spots using a conventional C_{18} TLC plate and acetone-2M NaCl aqueous solution (1:1) as a solvent system. In this solvent system, NaCl was effectively used to control the tailing of the spot. With respect to the

HPLC, CL components were separated into 6 peaks and a phenyl type HPLC column was successfully used to separate CL components, while the conventional ODS column was not satisfactory because of tailing. As a two-phase solvent system for the HSCCC separation of CL components, n-butanol-0.04 M TFA aqueous solution (1:1) was selected. In this solvent system, the concentration of TFA was the most important factor in controlling the separation. Using this solvent system, CL-A and CL-B were efficiently isolated from the commercial CL by HSCCC. Because the purity of both components were over 90 %, they were considered to be useful as working standards for the chemical analysis of residual CL in the livestock products. In this HSCCC preparative separation, some minor components of CL were also isolated in low purity. By FAB mass analysis molecular weights of these minor components were obtained. These results suggest that the HSCCC is useful not only for the isolation of main components but also for efficient concentration of some minor components in CL preparation.

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