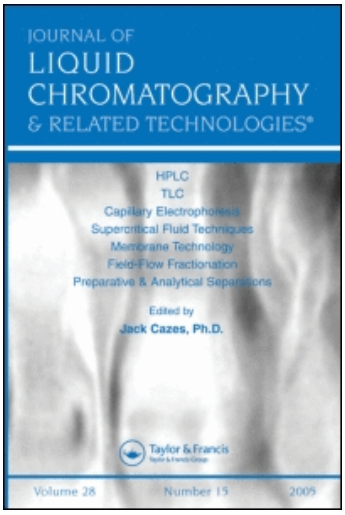


**Journal of Liquid Chromatography & Related Technologies**
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http://www.informaworld.com/smpp/title~content=t713597273

# Application of High-Speed Countercurrent Chromatography/Electrospray Ionization Mass Spectrometry (HSCCC/ESIMS) in Natural Products Chemistry

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**To cite this Article** Kong, Z. , Rinehart, K. L. , Milberg, R. M. and Conway, W. D.(1998) 'Application of High-Speed Countercurrent Chromatography/Electrospray Ionization Mass Spectrometry (HSCCC/ESIMS) in Natural Products Chemistry', Journal of Liquid Chromatography & Related Technologies, 21: 1, 65 — 82
**To link to this Article: DOI:** 10.1080/10826079808001936
**URL:** http://dx.doi.org/10.1080/10826079808001936

J. LIQ. CHROM. & REL. TECHNOL., 21(1&2), 65–82 (1998)

# APPLICATION OF HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY/ ELECTROSPRAY IONIZATION MASS SPECTROMETRY (HSCCC/ESIMS) IN NATURAL PRODUCTS CHEMISTRY

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## ABSTRACT

In this paper, we describe the technique of analytical high-speed countercurrent chromatography coupled with electrospray ionization mass spectrometry (HSCCC/ESIMS) for the first time, and evaluate its performance in terms of chromatography and mass spectrometry. This technique demonstrates the capability and resolving power of HSCCC in the separation of a few natural product mixtures and utilizes ESIMS as a detector with continuous detection and high sensitivity. Application of this HSCCC/ESIMS system is illustrated by the separation and detection of biologically important compounds.

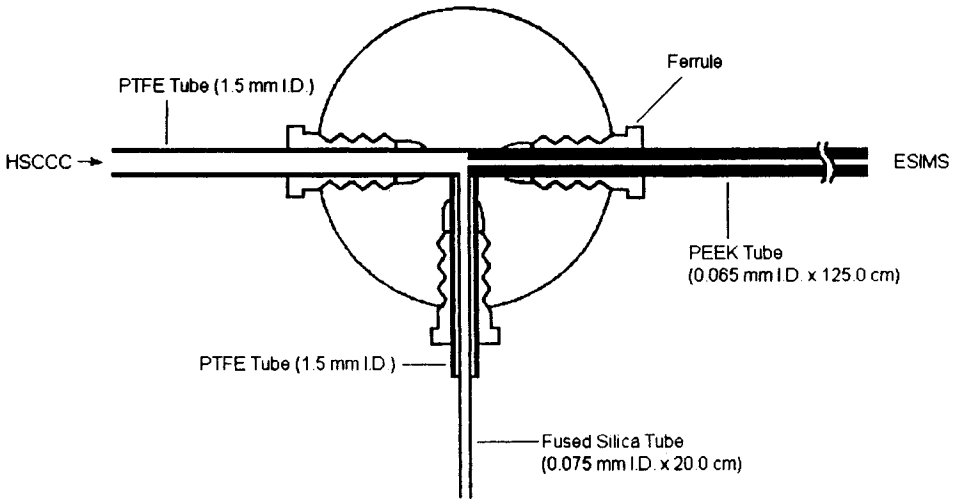



**Figure 1**. Design of T-split.



## INTRODUCTION

High-performance liquid chromatography (HPLC) represents one of the most advanced and powerful separation techniques, which is efficient in separating a variety of compounds and biological mixtures. Many approaches have been attempted toward combining HPLC with various mass spectrometric techniques and thermospray mass spectrometry (TSPMS) has been successfully interfaced with liquid chromatography (LC) at a conventional flow rate.[1,2] Continuous-flow fast atom bombardment mass spectrometry (CF-FABMS) has been coupled with liquid chromatography at both a micro-flow rate and a conventional flow rate.[3-6] Electrospray ionization mass spectrometry (ESIMS) has been successfully interfaced with micro-LC and the flexibility, convenience, sensitivity, cleanliness and ease of maintenance of electrospray mass spectrometry enable its extensive applications to environmental and clinical analyses,[7] but the use of solid supports as stationary phase causes various complications such as adsorptive loss, denaturation of samples and contamination or tailing of solute peaks.

Countercurrent chromatography (CCC) generally designates a family of support-free liquid-liquid chromatography techniques involving two immiscible liquids, prepared by mixing two or more solvents. In this chromatographic

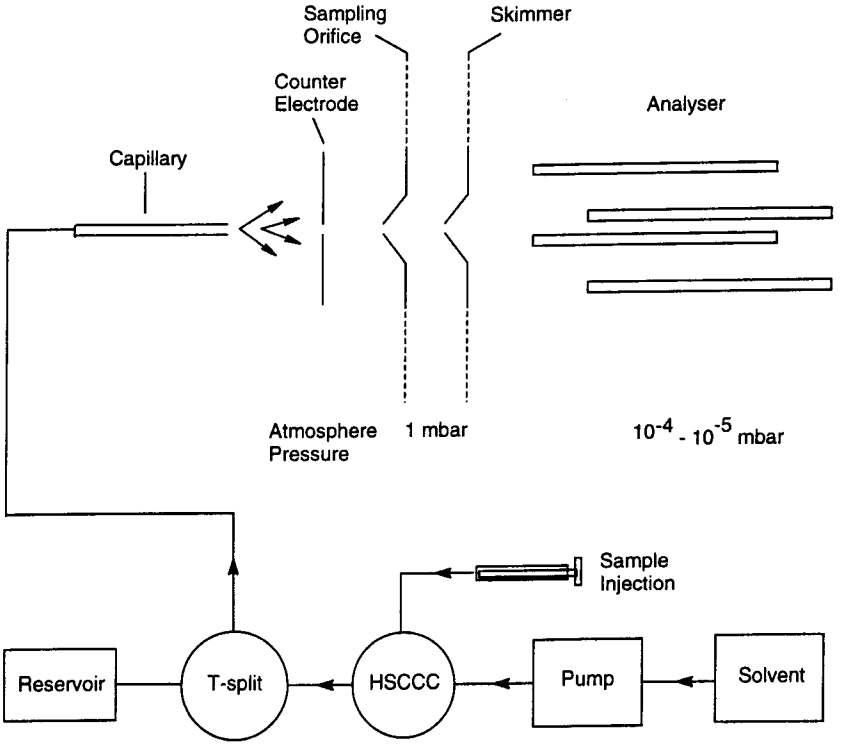
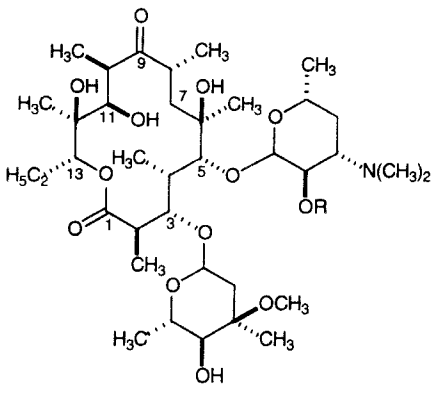



**Figure 2**. Schematic diagram of the HSCCC/ESIMS system.

method, one liquid is employed as the stationary phase while another liquid is pumped through the column as the mobile phase.[8-10] Countercurrent chromatography offers advantages over HPLC in that it is free from all complications arising from the use of solid supports. The performance and efficiency of countercurrent chromatography have been improved recently.[11] Modern high-speed CCC utilizes a multilayer coiled column and a planetary rotation mode. The direction of the development of high-speed CCC ranges from preparative separation to microanalytical separation.

On a preparative scale, HSCCC has been widely used in the separation of natural products. For analytical purposes, high-speed CCC has been developed with a short running time and good resolving power.[12-13] Unfortunately,



Erythromycin A (ErA), R = H

Erythromycin Estolate (ErEst), R = $COCH_2CH_3$

Erythromycin Ethyl Succinate (ErSucc), R = $COCH_2CH_2CO_2C_2H_5$

**Figure 3**. Structures of erythromycins.



HSCCC is still sometimes incompatible with common optical detectors, *i.e.* UV/Vis and refractive index detectors, due to the bleeding of the stationary phase. Stationary phase bleeding produces a chaotic emulsification in the flow stream. Although the emulsification can be minimized by post-column addition of a third solvent to coalesce the two immiscible liquids, this addition of solvent decreases the sensitivity and causes peak broadening.[14]

The coupling of HSCCC with mass spectrometry avoids the solvent problems associated with conventional detectors, and mass spectrometry offers on-line universal detection with high sensitivity. The combination of HSCCC with thermospray MS (HSCCC/TSPMS) has been reported[16-17] and the coupling of HSCCC with fast atom bombardment through a moving belt interface (HSCCC/MBI/FABMS) has been accomplished by our group.[18,19] Other mass spectrometric techniques, including frit/electron ionization (frit/EI), frit/chemical ionization (frit/CI) and frit/continuous flow fast atom bombardment (frit/CF/FAB), also have been coupled with high-speed CCC.[20]

The existing HSCCC/TSPMS interface suffers from high back-pressure, which can rupture the poly(tetrafluoroethylene) (PTFE) tube in the HSCCC column. An HPLC pump has to be inserted between the coil and the TSP

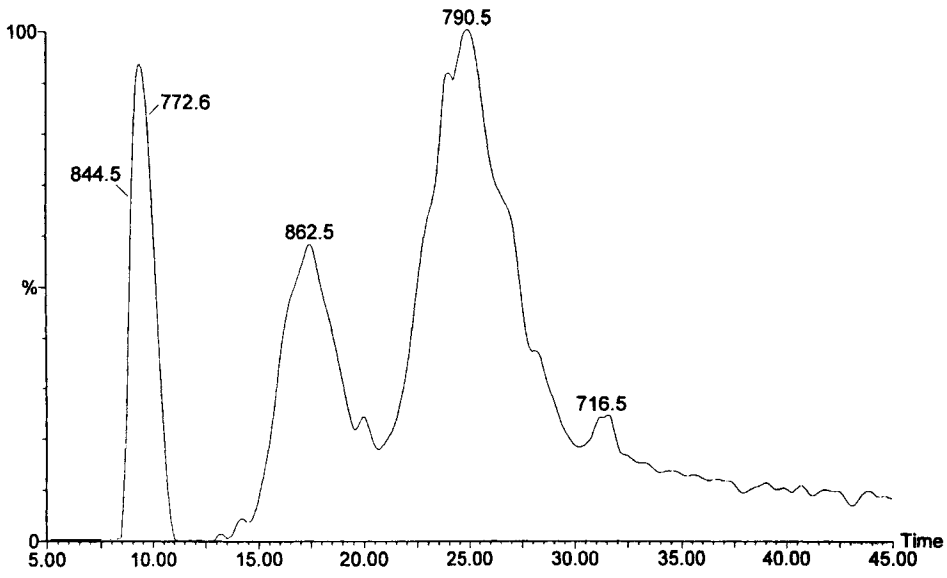



**Figure 4**. Reconstructed ion chromatogram (RIC) of erythromycins.



interface to regulate the back-pressure on the countercurrent instrument. This induces a large dead space in the pump and adversely affects the chromatogram. Although frit-FAB does not generate high back-pressure, it only accepts 1-5 μL/min flow rate. The sample must be split *ca.* 1:200 at 0.8 mL/min, which is the optimal flow rate for our instrument. High-speed CCC/FABMS employing a moving belt interface overcomes these problems, but the sensitivity of existing HSCCC/MS, including TSP, EI, CI and FAB, is relatively low. Also, thermally unstable compounds will decompose during the ionization processes of TSP, EI and CI.

Electrospray ionization mass spectrometry (ESIMS) is known for its low detection limit and is a soft ionization technique suitable for thermally labile compounds. The combination of HSCCC with electrospray MS provides another new analytical method for the first time. Association of high-speed CCC with the collision induced dissociation (CID) technique provides valuable structural information through fragmentation of selected ions. This combination integrates the versatility of high-speed countercurrent chromatography with specific detection and structural characterization of electrospray mass spectrometry and allows the application of high-speed

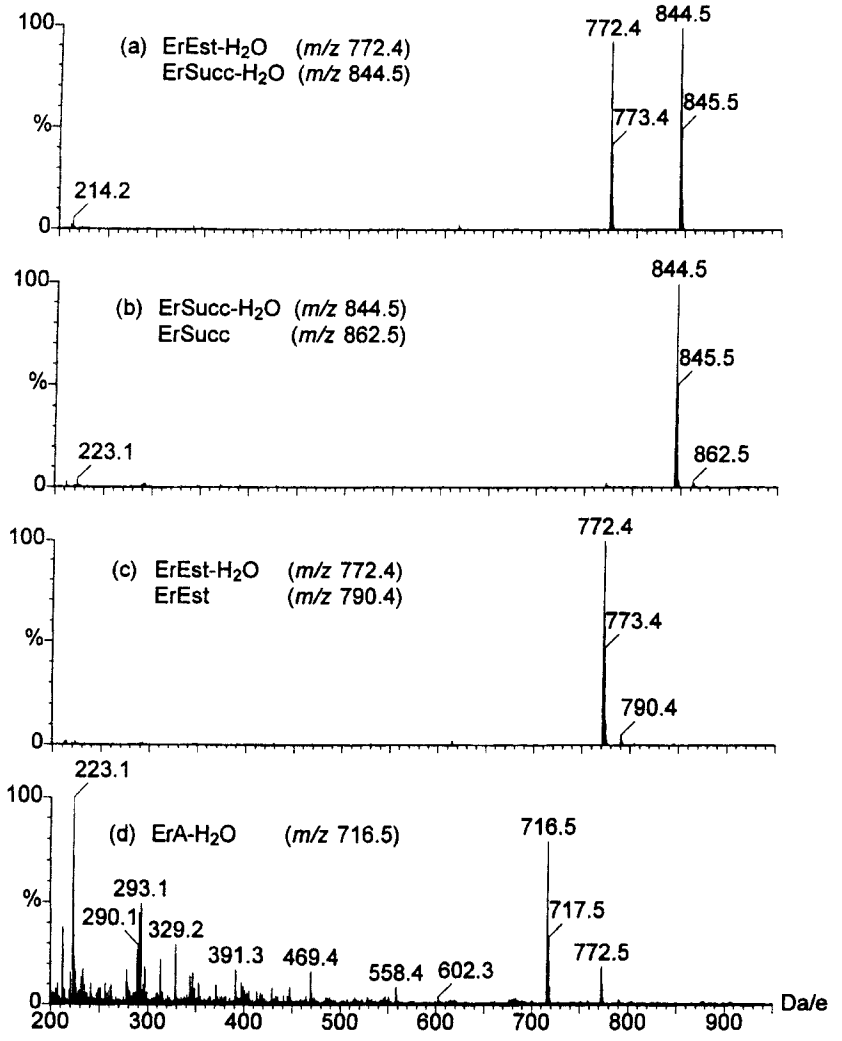



**Figure 5**. Mass spectra of erythromycins, (a) dehydrated erythromycin estolate and dehydrated erythromycin ethyl succinate, (b) erythromycin ethyl succinate, (c) erythromycin estolate, (d) dehydrated erythromycin A.

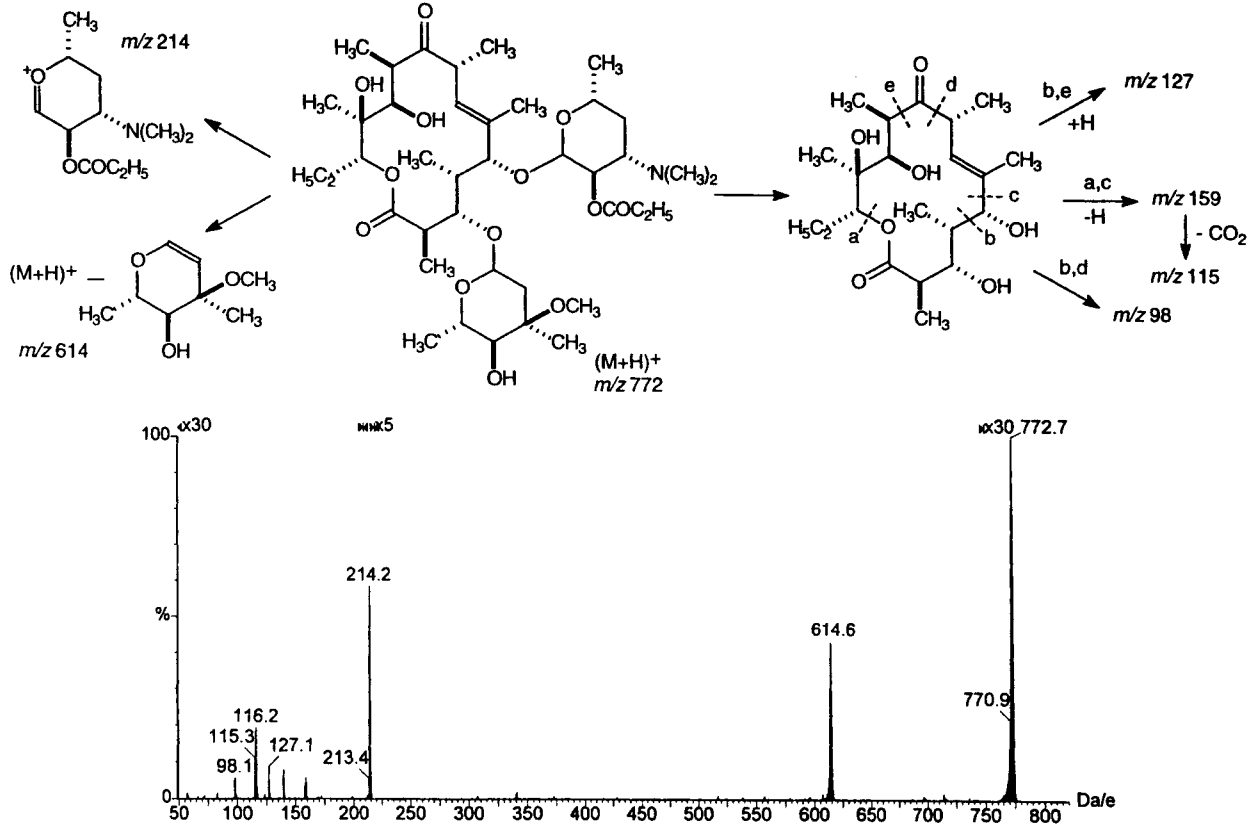



**Figure 6**. ESIMS/CID/MS spectrum of dehydrated erythromycin estolate $(M+H)^+$ ion at *m/z* 772.

CCC/ESIMS to a variety of samples, such as natural products, drugs, peptides, and other biological materials. In the present paper, coupling of high-speed CCC with electrospray MS has been developed and has been employed in the separation, detection and characterization of two sets of natural products.

## MATERIALS AND METHODS

### General

HPLC grade hexane, ethyl acetate and methanol were purchased from Burdick and Jackson (Baxter, McGaw Park, IL) and used without further purification. The water was purified using a Milli-Q water purification system (Millipore Corp., Marlborough, MA). Erythromycins were purchased from Sigma Co. (St. Louis, MO) and used without purification. Didemnins A and B and nordidemnin A were isolated and purified via a known procedure.[21,22]



### High-Speed Countercurrent Chromatography (HSCCC)

High-speed CCC separation was performed on a P.C., Inc. (Potomac, MD) High Speed Countercurrent Chromatograph. A small-volume analytical coil, which was wound with 30.0 m of 0.85 mm i.d. poly(tetrafluoroethylene) (PTFE) tubing, in two layers, on a core of 8.59 cm radius ($\beta$ = 0.85) was installed on the HSCCC. The volume of this coil was 17.0 mL. The system was capable of rotational speeds up to 1,400 rpm. The coil was first filled with stationary phase. The mobile phase was pumped by a Beckman 114M HPLC pump (Beckman, Fullerton, CA) through the coil at 0.8 mL/min while the coil was spun at 1,200 rpm.

Stationary phase retention varied from 60% to 80%, as measured by the amount of stationary phase eluted in the mobile phase during equilibration. High-speed CCC experiments were performed with a two-phase solvent system of hexane-EtOAc-MeOH-$H_2O$. The ratios were 4:7:4:3 (v/v) for erythromycins and 1:4:1:4 (v/v) for didemnins.

The solvent systems were allowed to equilibrate for 24 hr in a separatory funnel at room temperature, then filtered and degassed. The stationary phase was the heavy phase and the mobile phase was the light phase. When the high-speed CCC/ESIMS system attained equilibrium, the samples were injected and chromatographed.

## T-Split

A tee (1/16 inch, 0.25 mm bore) was purchased from Alltech. This tee was placed between the high-speed countercurrent chromatograph and the electrospray mass spectrometer. A PEEK tube (i.d. 0.065 mm, length 125.0 cm) was used to connect the tee and electrospray mass spectrometer. The high-speed CCC was connected to this tee through a PTFE tube. A fused-silica tube (i.d. 0.075 mm), covered with a short PTFE tube, was hooked onto the tee.

The effluent from the high-speed CCC can be split at a desired ratio by adjusting the length of the fused-silica tube. When the length of the fused-silica tube was 20.0 cm, the effluent from high-speed CCC at 800 μL/min was split at a 1:7 ratio, and the minor portion (100 μL/min) was introduced into the mass spectrometer.



## Electrospray Ionization Mass Spectrometry (ESIMS)

ESIMS spectra were acquired with a Micromass (Manchester, U.K.) Quattro mass spectrometer, with quadrupole-hexapole-quadrupole configuration. Data were acquired in the threshold continuum mode and the mass scale was calibrated with CsI. The resolution was adjusted to resolve isotopic peaks at *ca. m/z* 600.

The mass range was scanned from *m/z* 200 to 1500 for HSCCC/ESIMS and *m/z* 50 to 1500 for high speed HSCCC/ESIMS/CID/MS in 4.9 sec. MS/CID/MS spectra were obtained on the $(M+H)^+$ ions with argon employed as the collision gas.

## Erythromycins and Didemnins

A mixture of erythromycin A (ErA), erythromycin estolate (ErEst) and erythromycin ethyl succinate (ErSucc) (equal amounts, 20 nmol) was dissolved in 20 μL MeOH. Erythromycins were chromatographed using hexane-EtOAc-MeOH-$H_2O$ (4:7:4:3, v/v) solvent system and analyzed by electrospray mass spectrometry. Didemnins (didemnins A and B and nordidemnin A, in equal amounts, 5.0 nmol) were dissolved in 20 μl methanol. This mixture was also separated and analyzed by HSCCC/ESIMS using hexane-EtOAc-MeOH-$H_2O$ (1:4:1:4, v/v) solvent system. The HSCCC /ESIMS system was operated at a flow rate of 800 μL/min.

## RESULTS AND DISCUSSION

### HSCCC/ESIMS System

The high speed countercurrent chromatograph was equipped with a prototype, small-volume analytical coil (17.0 mL). When the coil rotated at *ca.* 1,200 rpm, the stationary phase retention varied from 60% to 80% and was stable for several hours with hexane-EtOAc-MeOH-$H_2O$ solvent systems.

The optimum flow rate with respect to stationary phase retention and peak broadening was 800 μL/min. Although the electrospray mass spectrometry system could operate at a flow rate of up to 1.0 mL/min employing the megaflow ion source and probe, a T-split illustrated in Figure 1 was made and placed between the high-speed countercurrent chromatograph and the electrospray mass spectrometer.



The effluent from the high-speed CCC column at 800 μL/min was divided by the T that split the flow stream at a 1:7 ratio, and a flow of approximately 100 μL/min was introduced into the electrospray MS system through a PEEK tube whose length and I.D. were fixed. To adjust the split ratio, the length of the fused-silica tube was varied.

The HSCCC/ESIMS system, including HPLC pump, injection valve, HSCCC, T-split, and electrospray mass spectrometer, is shown in Figure 2. The HSCCC/ESIMS system was stable for several hours once it was equilibrated. The baseline in the total ion chromatogram (TIC) was smooth, and band broadening of chromatographic peaks was not observed. The mass spectrometer was able to analyze the solutes eluted from the HSCCC and gave valuable structural information on them. The performance of this HSCCC/ESIMS system was demonstrated by the analysis of erythromycins and didemnins.

### Analysis of Erythromycins Using HSCCC/ESIMS

Erythromycins are important macrolide antibiotics whose structures are shown in Figure 3.[23] These compounds are extensively used in the treatment of Gram-positive bacterial infections. Erythromycins have weak chromophores and low extinction coefficients, and can't be detected by conventional optical methods.

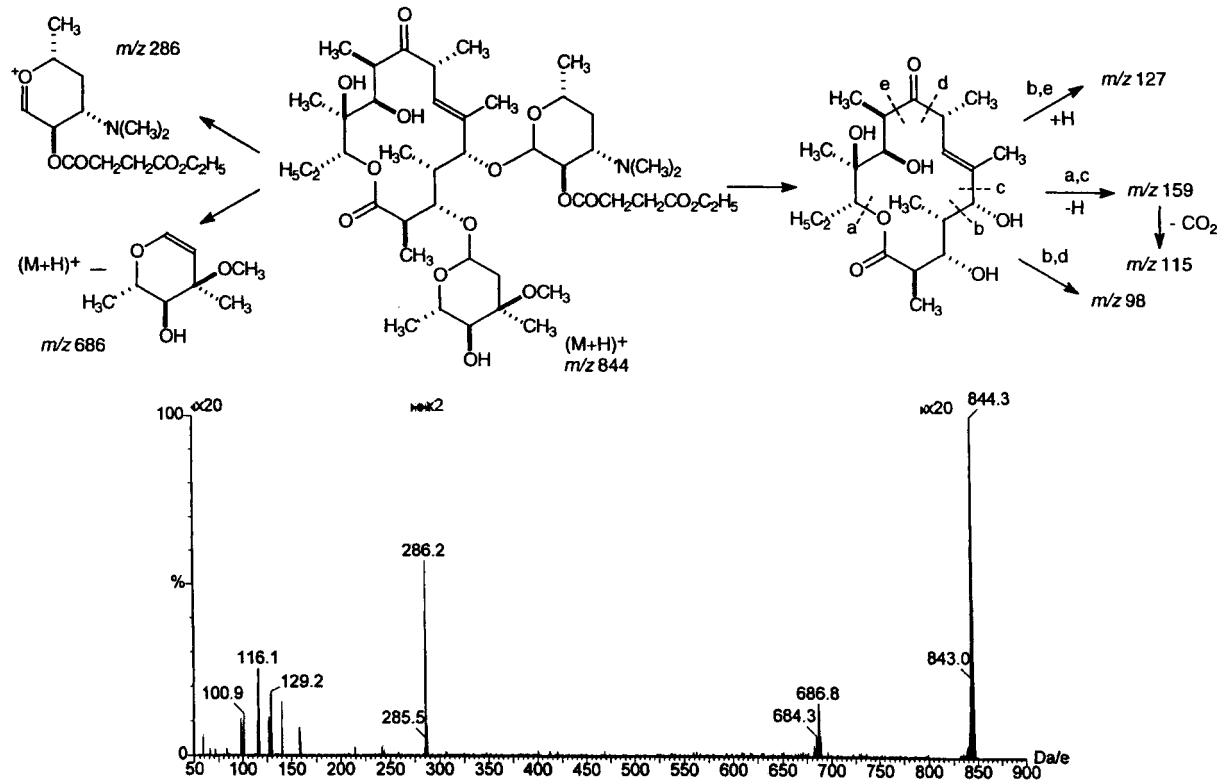



**Figure 7**. ESIMS/CID/MS spectrum of dehydrated erythromycin ethyl succinate $(M+H)^+$ ion at *m/z* 844.

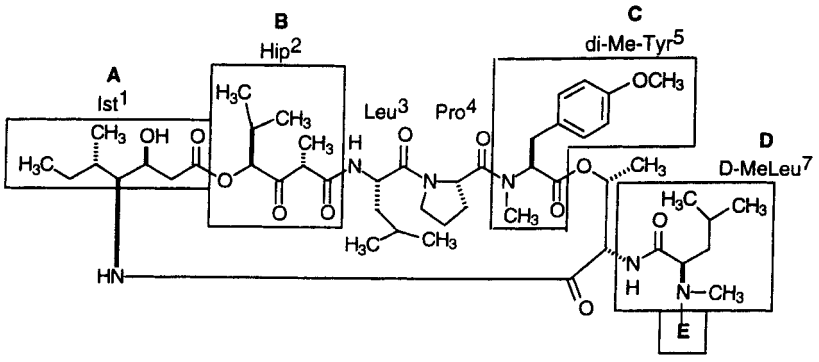
A mixture of erythromycins — erythromycin A (ErA, MW 733), erythromycin estolate (ErEst, MW 789) and erythromycin ethyl succinate (ErSucc, MW 861) — was analyzed using the HSCCC/ESIMS system. The analysis was carried out by using a 4-7-4-3 ratio of hexane-EtOAc-MeOH-$H_2O$. Figure 4 shows the reconstructed ion chromatogram (RIC), with numbers indicating the molecular ions found in the peaks. Each peak in the RIC was identified from the molecular ion and characteristic fragment ions in the corresponding mass spectrum. Peaks at retention time (rt) 17.5 min and 25.5 min were identified as ErSucc and ErEst respectively.

The mass spectra corresponding to these two peaks in the RIC are illustrated in Figure 5 (b and c). ErSucc gave ions at *m/z* 862 $(M+H)^+$ and *m/z* 844 $(M+H-H_2O)^+$ (Figure 5, b) and ErEst gave ions at *m/z* 789 $(M+H)^+$, *m/z* 772 $(M+H-H_2O)^+$ and *m/z* 614 (M-desosaminyl) (Figure 5, c). The mass spectra of ErSucc and ErEst indicate that the $(M+H-H_2O)^+$ ions at *m/z* 844 and *m/z* 772 were more abundant than the molecular $(M+H)^+$ ions at *m/z* 862 and *m/z* 789.



Interestingly, the first peak (rt 9.5 min) in the RIC spectrum did not belong to any compounds in the mixture. The mass spectrum indicated that this peak contained two compounds with molecular ions at *m/z* 844 and *m/z* 772 (Figure 5, a). These two ions corresponded to the $(M+H)^+$ ions of dehydrated erythromycin ethyl succinate (ErSucc-$H_2O$) and dehydrated erythromycin estolate (ErEst-$H_2O$). These ions, ErSucc-$H_2O$ and ErEst-$H_2O$, were produced from ErSucc and ErEst, by losing a molecule of $H_2O$ during the solvolysis in methanol, rather than from ErSucc and ErEst in the ionization process. Figure 6 shows the collision-induced dissociation (CID) spectrum of the ion *m/z* 772. The fragment ions at low mass helped us to assign the structure of this ion, *m/z* 772 (ErEst-$H_2O$). The CID spectrum of ion *m/z* 844 is illustrated in Figure 7. The structure of ErSucc-$H_2O$ (*m/z* 844) was deduced from the CID spectrum. For both ErSucc and ErEst, the hydroxyl group (-OH) at C-6 has been lost and a carbon-carbon double bond formed.

The peak at rt 31.5 min in the RIC was identified as dehydrated erythromycin A (ErA-$H_2O$, *m/z* 716). The molecular ion of ErA (*m/z* 734) was not detected in this peak. Like ErSucc-$H_2O$ and ErEst-$H_2O$, ErA-$H_2O$ can be produced from ErA by losing a molecule of $H_2O$ during the solvolysis. An attempt to assign the structure of ErA-$H_2O$ failed due to the relatively small amount of sample eluted from the HSCCC. Figure 5, d shows the corresponding mass spectrum. A small amount of ErEst (*m/z* 790, 772) is also shown in the mass spectrum. The RIC indicated that the peak for ErA-$H_2O$




| | A | B | C | D | E |
|---|---|---|---|---|---|
| Didemnin A | Ist | Hip | di-Me-Tyr | D-MeLeu | -H |
| B | Ist | Hip | di-Me-Tyr | D-MeLeu | -Pro-Lac |
| Nordidemnin A | Norsta | Hip | di-Me-Tyr | D-MeLeu | -H- |
| Methylenedidemnin A | Ist | Hip | di-Me-Tyr | X | -- |

**Figure 8**. Structures of the didemnins.



tailed. This may be caused by the strong hydrophilicity of the molecule, and a small amount of ErA-$H_2O$ may be retained in the stationary phase. ErA was not detected during the analysis. After the analysis, the stationary phase was pumped out and continuously analyzed by ESIMS, but ErA was still not detected. This could be attributed to (1) transformation of all erythromycin A into ErA-$H_2O$, or (2) partial transformation of erythromycin into ErA-$H_2O$. In the later case, the unreacted ErA was adsorbed by the stationary phase and could not be detected by electrospray MS.

## Analysis of Didemnins Using HSCCC/ESIMS

Didemnins are cyclic depsipeptides isolated from the marine tunicate *Trididemnum solidum*.[21,22] Their structures are shown in Figure 8. Didemnin A (Did A) is a precursor for the synthesis of other didemnins showing antiviral, antitumor and immunosuppressive activities. However, large-scale purification

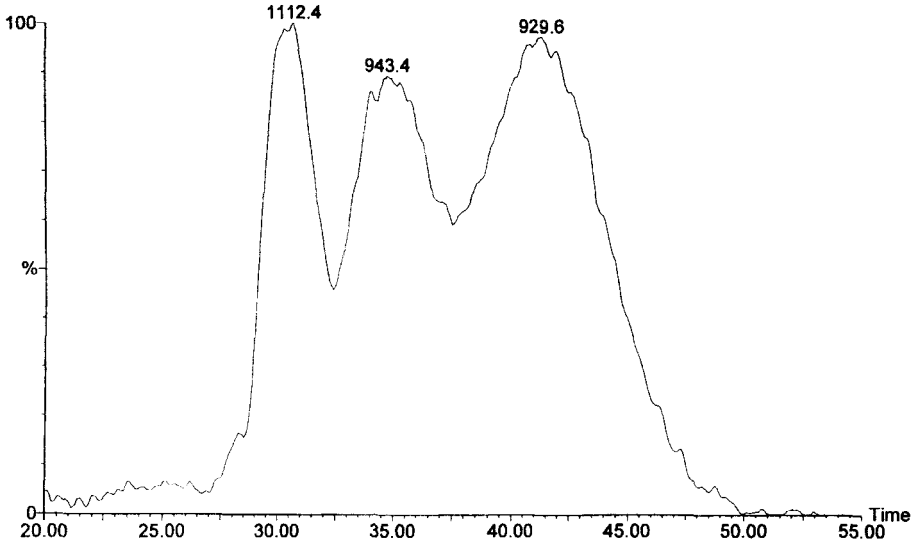



**Figure 9**. Reconstructed ion chromatogram (RIC) of didemnins.



of Did A is difficult due to the presence of nordidemnin A (Nordid A). HSCCC/ESIMS has been successfully applied to the separation and detection of didemnins. The RIC of didemnins is shown in Figure 9, with the number indicating the molecular ions found in the peaks. The identity of each peak in the RIC was deduced from the molecular ion in the corresponding mass spectrum. Peaks at retention time 30.5 min, 35.5 min and 42.0 min corresponded to didemnins B and A and nordidemnin A, respectively.

The mass spectra of these compounds are illustrated in Figure 10. Due to the soft ionization of ESIMS, the mass spectra mainly provided molecular ions, and fragmentation of didemnins was not observed. The first eluted didemnin was Did B, which afforded ions at *m/z* 1112 $(M+H)^+$ and *m/z* 1134 $(M+Na)^+$ (Figure 10, a). The small peak at *m/z* 941 may be attributed to the $(M+H)^+$ ion of methylenenordidemnin A, which was produced through the reaction of Nordid A with formaldehyde.

The second peak in the RIC was Did A. The characteristic signals for Did A were ions at *m/z* 943 $(M+H)^+$ and *m/z* 965 $(M+Na)^+$ (Figure 10, b). Nordid A was the most polar and was retained by the stationary phase longer. It gave an

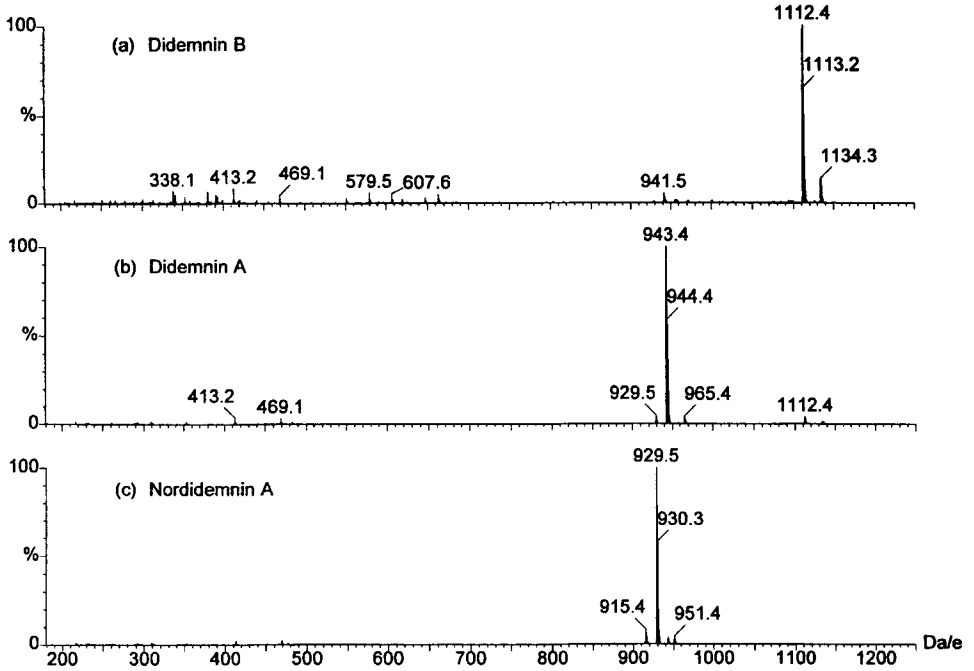



**Figure 10**. Mass spectra of didemnins, (a) didemnin B, (b) didemnin A, (c) nordidemnin A.



$(M+H)^+$ ion at *m/z* 929, an $(M+Na)^+$ ion at *m/z* 951 and a bisnordidemnin A ion at *m/z* 915 (Figure 10, c). The didemnin mixture was resolved well enough that each peak in the RIC was only slightly overlapped with the other peaks. Thus, Did A can be separated from Nordid A by high-speed CCC.

The HSCCC/ESIMS system has been successfully applied to the separation and detection of erythromycins and didemnins. Scale-up purification of Did A from Nordid A is possible by high-speed CCC. High-speed CCC can be directly compatible with electrospray MS without any modification of the mass spectrometer.

This technique is easy to use and maintain. The results presented in this paper indicate that this technique has broad applications in dealing with natural products.

## CONCLUSION

Our study demonstrates that high-speed CCC/ESIMS can be used for natural products. This technique should effectively enable the direct analysis of relative large, polar, thermally labile compounds in water by utilizing electrospray MS as detector through the production of mass spectra. Detection of UV/Vis-inactive compounds can be achieved by this technique. In addition, the direct analysis of aqueous solutions eliminates tedious sample preparations. High-speed CCC/ESIMS offers a number of advantages over TSP, CI, EI and FAB CCC/MS with respect to the low detection limit, soft ionization process, and access to high molecular weights through the production of molecular ions bearing multiple charges. It also provides a model for CCC separation by preparative work. When high-speed CCC is associated with MS/CID/MS, structural information on selected ions can be obtained. This feature will enable its application to the structure elucidation of natural products.



## ACKNOWLEDGMENTS

The authors thank Dr. J. N. McGuire for technical assistance. This work was supported by a grant from the National Institute of General Medical Sciences. The Quattro mass spectrometer was purchased, in part, with a grant from the Division of Research Resources, National Institutes of Health (RR07141).

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Received March 20, 1997
Accepted May 20, 1997
Manuscript 4418