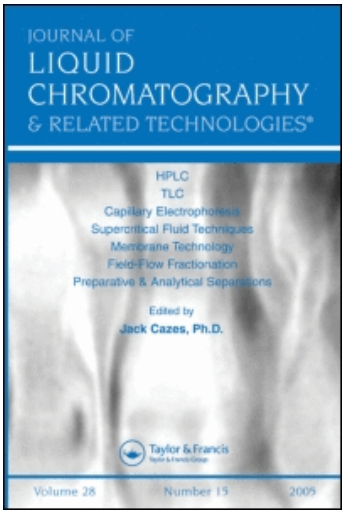

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APPLICATION OF COUNTERCURRENT CHROMATOGRAPHY/THERMOSPRAY MASS SPECTROMETRY FOR THE ANALYSIS OF NATURAL PRODUCTS

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ABSTRACT

The versatility and high resolving power of countercurrent chromatography (CCC) has been demonstrated with a newly developed analytical high speed planet centrifuge system. Interfacing countercurrent chromatography with mass spectrometry (MS) provides a new analytical methodology which integrates the advantages of countercurrent chromatography with the low detection limit and identification capability of mass spectrometry. The

capability of thermospray CCC/MS is evidenced in a preliminary study of plant alkaloids. The technique proved useful in identifying an unknown impurity and in validating the presence of a specific compound in a mixture. The thermospray CCC/MS can become a useful and complementary method to thermospray HPLC/MS for the analysis of nonvolatile or thermally unstable molecules.

INTRODUCTION

The high resolving power of countercurrent distribution has long been recognized (1), but time requirements and inconvenience limit its future applications. Recently, the development of high speed planet centrifuge (2) and analytical high speed countercurrent chromatography have remarkably improved the efficiency of the system, so that separation can be accomplished within a reasonable time period comparable to HPLC (3).

High performance liquid chromatography combined with mass spectrometry (HPLC/MS) represents a powerful analytical methodology. Many different approaches have been attempted in combining HPLC with MS, with their advantages and disadvantages extensively reviewed (4-8). Thermospray is one technique which is well suited

for combined HPLC/MS (9,10). Thermospray can handle high quantities of aqueous solvent at conventional flow rates while providing a soft means of ionization (11,12). Thermospray has been used for numerous environmental and clinical analyses (12-17).

Interfacing countercurrent chromatography with thermospray mass spectrometry provides a new analytical methodology. This combination integrates the versatility of countercurrent chromatography with specific detection of mass spectrometry not available in other detection modes. Also this combination allows for the thermospray interface to be operated using high percentages of water with a volatile buffer, conditions previously shown to result in the best sensitivity (13), without suffering losses in resolution typically observed in HPLC when the mobile phase is switched to higher aqueous percentages. The two phase solvent system commonly employed in CCC offers distinct advantages, allowing the interface to be operated using nearly 100% aqueous solution. This paper describes the preliminary feasibility study of thermospray CCC/MS in the analysis of natural products - plant alkaloids.

MATERIAL AND METHODS

Reagents

Ethanol and n-hexane used for preparation of the two phase solvent systems were glass distilled chromatographic grade purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Experiments were performed with a two phase solvent system composed of n-hexane, ethanol, and water with a volume ratio of 6:5:5. The two phase solvent system was prepared by thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature followed by filtration and degassing with a 5 μm filter.

Analytical High Speed CCC:

A recently developed analytical high speed planet centrifuge equipped with a multi-layer coil column of 0.85 mm i.d PTFE tubing was employed. The system is capable of revolution at 2000 rpm with a 5 cm radius (3). A Waters 6000A HPLC pump (Waters Associates, Milford, MA) was used for the mobile phase. UV detection was achieved with an ISCO Model 1840

(Lincoln, Nebraska) Variable Wavelength UV-Vis absorbance detector. The column was first filled with the stationary phase (upper phase); then the mobile phase (lower phase) was pumped at 0.8 mL/min while the column was spun at 1500 rpm. The sample solution was injected when clean mobile phase was eluted.

Thermospray CCC/MS

The effluent from the CCC (0.8 mL/min) was introduced into a Waters 6000A pump through a zero dead volume tee fitted with a reservoir. The Waters pump was necessary to achieve the solvent pressure required for thermospray. Also, since the CCC pump would show flow rate variations, the thermospray Waters pump was operated at 0.7 mL/min with the resevoir providing extra solvent or venting excess solvent from the CCC system. The effluent from the Waters pump was mixed coaxially (14) with 0.3 M ammonium acetate added at 0.3 mL/min to provide the volatile buffer for ion evaporation ionization. This solvent system (total of 1 mL/min) passed through a UV detector (280 nm) and into the thermospray interface. At lower CCC flow

rates (0.3 mL/min) the pressure drop across the thermospray vaporizor was sufficiently low to permit direct coupling of the CCC effluent to the thermospray interface without the use of the Waters HPLC pump. Post column addition of buffer and UV detection of the CCC effluent was maintained as described above.

The thermospray interface (Vestec, Houston, TX) was installed on a Finnigan 4500 quadrupole mass spectrometer. The interface included a temperature controller and readout. The temperature zones monitored were the vaporizer, source and aerosol (just past the ion exit cone). Electrical cartridge heaters were used in the source and the vaporizer was directly heated. The thermospray interface was operated at a source temperature of 250°C and a vaporizer temperature to maximize the HPLC solvent clusters (about 240°C). The solvent cluster has been shown to co-maximize with the analyte being analyzed (13). This interface did not require any splitting of the LC effluent. The large volume of solvent was pumped out of the source with a liquid nitrogen cold trap prior to a mechanical

rough pump. Both negative and positive ion detection using ion evaporation ionization or filament on chemical ionization (CI) were employed for the analysis of the alkaloids. The filament was operated at 1000 V with a 0.15 mA emission current. The mass spectrometer was scanned from m/z 180 to m/z 600 in 2 seconds. The mass calibration of the quadrupole was verified daily with polyproplene glycol (AMW 1000).

RESULTS AND DISCUSSION

A thermospray CCC/mass spectrometric system that integrates the versatility of countercurrent chromatography with the low detection limit of mass spectrometry has been developed in this laboratory. Our preliminary study with a mixture of plant alkaloids demonstrates that thermospray CCC/MS offers certain advantage which would be complementary to HPLC/MS as a new analytical methodology.

As shown in Figure 1, (+)-vincamine is the major alkaloid of Vinca minor (18). Clinical studies have demonstrated that i.v. administration of vincamine reduces the arterial blood pressure and increases

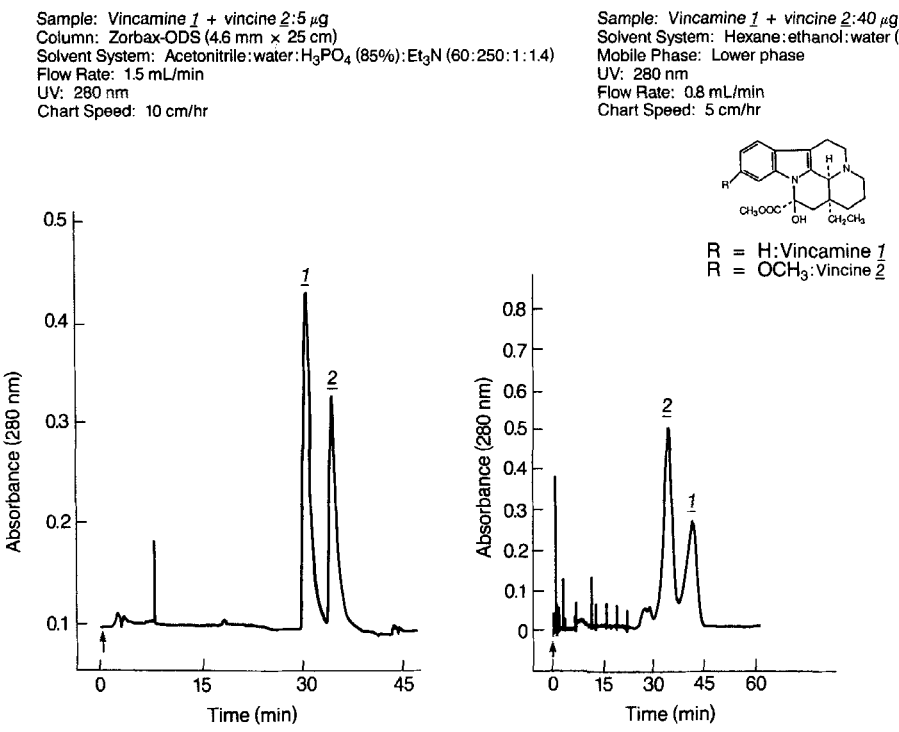

Figure 1. HPLC/UV chromatogram (left) and CCC/UV chromatogram (right) showing the major components present in the alkaloid. Note that in the CCC/UV chromatogram a small peak eluting before vincine was identified as an isomer of vincine not separated by HPLC.

cerebral blood flow and oxygen consumption (19). The isolation of vincamine has been complicated by the presence of other minor alkaloids. Because of structural similarities, these minor alkaloids always

cochromatographed with vincamine under a variety of chromatographic conditions. Extensive purification provided a few mg of the minor component which was identified as vincine, 11-methoxyvincamine (20). Vincamine and vincine can be efficiently separated with a high speed CCC system employing a two phase system composed of hexane-ethanol-water (6:5:5) (Figure 1).

The thermospray CCC/MS total ion current chromatogram for the analysis of vincamine (1) and vincine (2) using hexane:ethanol:H_2O (6:5:5) as the two phase solvent system is shown in Figure 2. The thermospray spectra acquired for vincamine (mol. wt. 354) displayed a protonated molecular ion at m/z 355 $[M+H]^+$ (Figure 3). Fragment ions were also observed at m/z 337 corresponding to $[M+H-H_2O]^+$. The thermospray spectrum of vincine (mol. wt. 384) showed a protonated molecular ion at m/z 385 $[M+H]^+$ together with a fragment ion at m/z 367 $[M+H-H_2O]^+$ (Figure 4). Neither compound showed sufficient ion current in the negative ion detection mode to record a mass spectrum.

The analytical CCC results showed a small peak just preceding the vincine peak which was not resolved

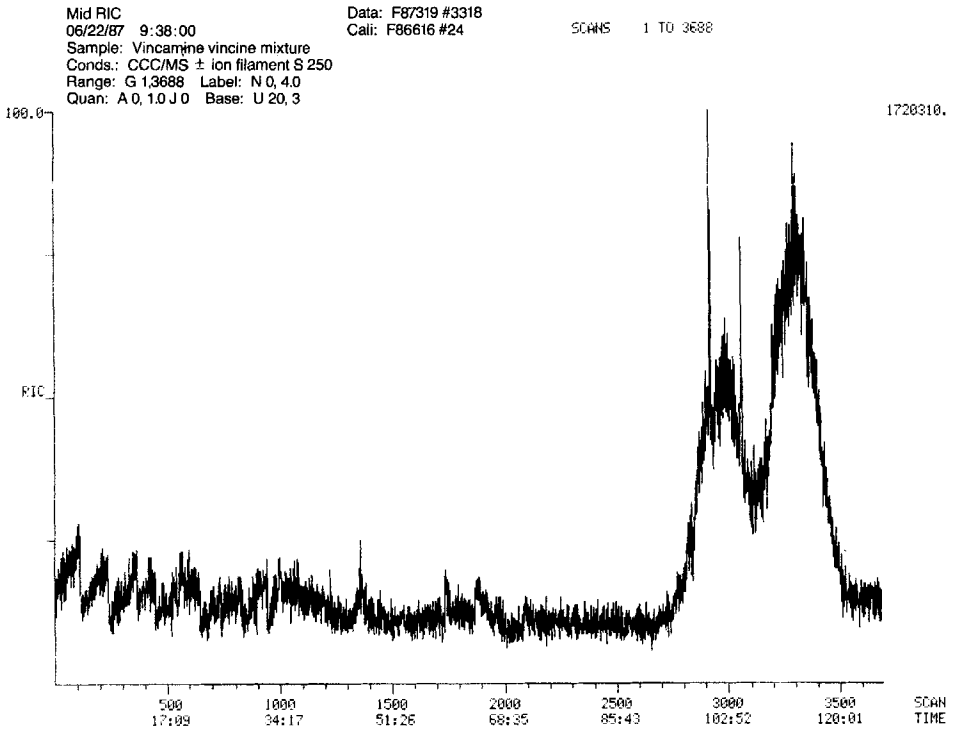

Figure 2. Thermospray CCC/MS total ion current chromatogram for the separation of the alkaloid mixture. The peak at scan number 2850 was an isomer of vincine, the peak at scan number 2950 was vincine, and the peak at scan number 3300 was vincamine.

as well under the CCC/MS conditions. Therefore the mass spectrum of the front shoulder of the vincine of Figure 2 was examined (Figure 5). This showed the presence of a protonated molecular ion at m/z 385

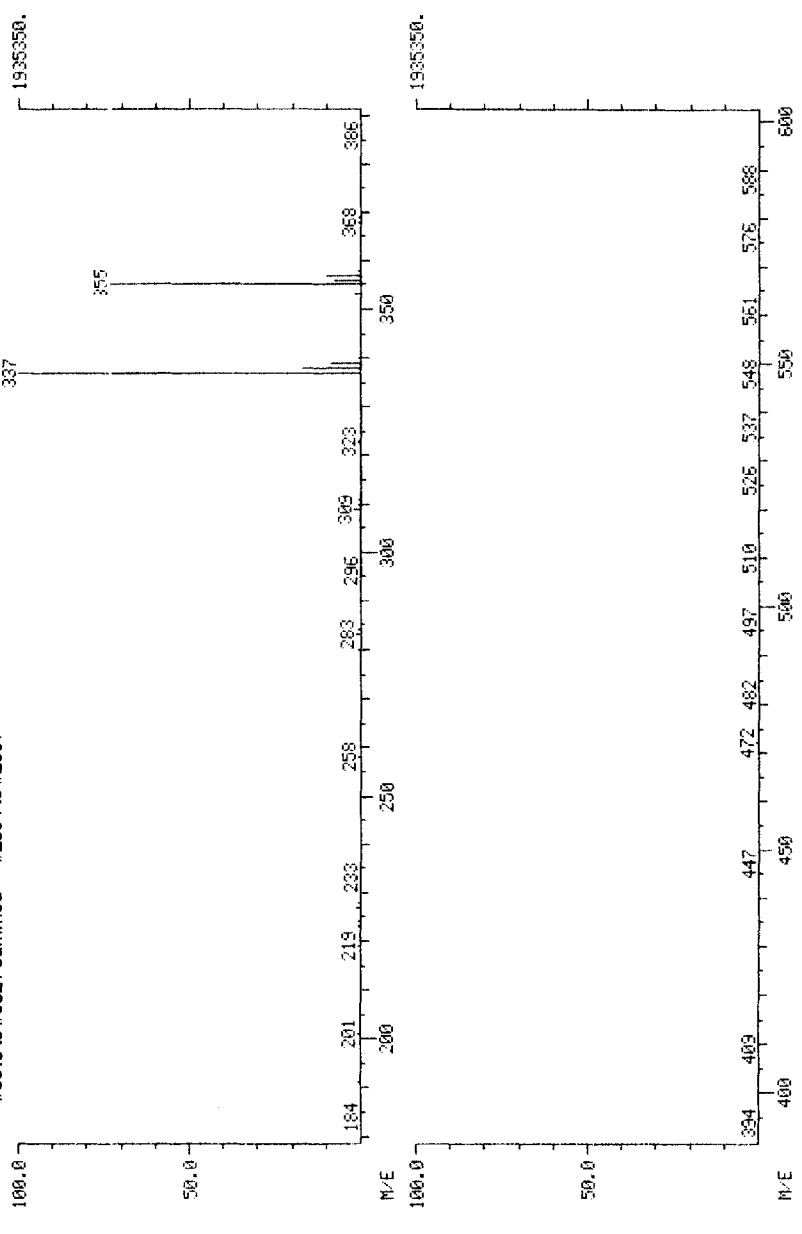

Figure 3. Thermospray MS spectrum of vincamine (mw 354).

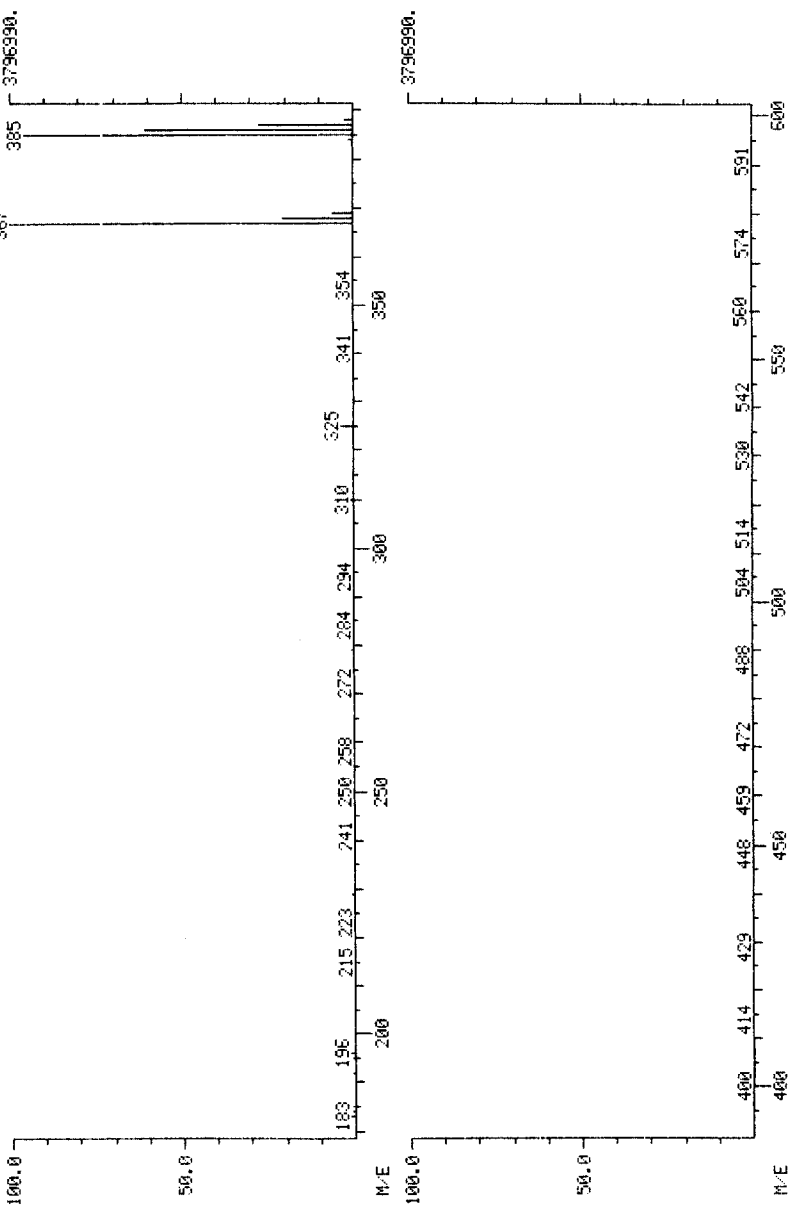

Figure 4. Thermospray MS spectrum of vincine (mw 384).

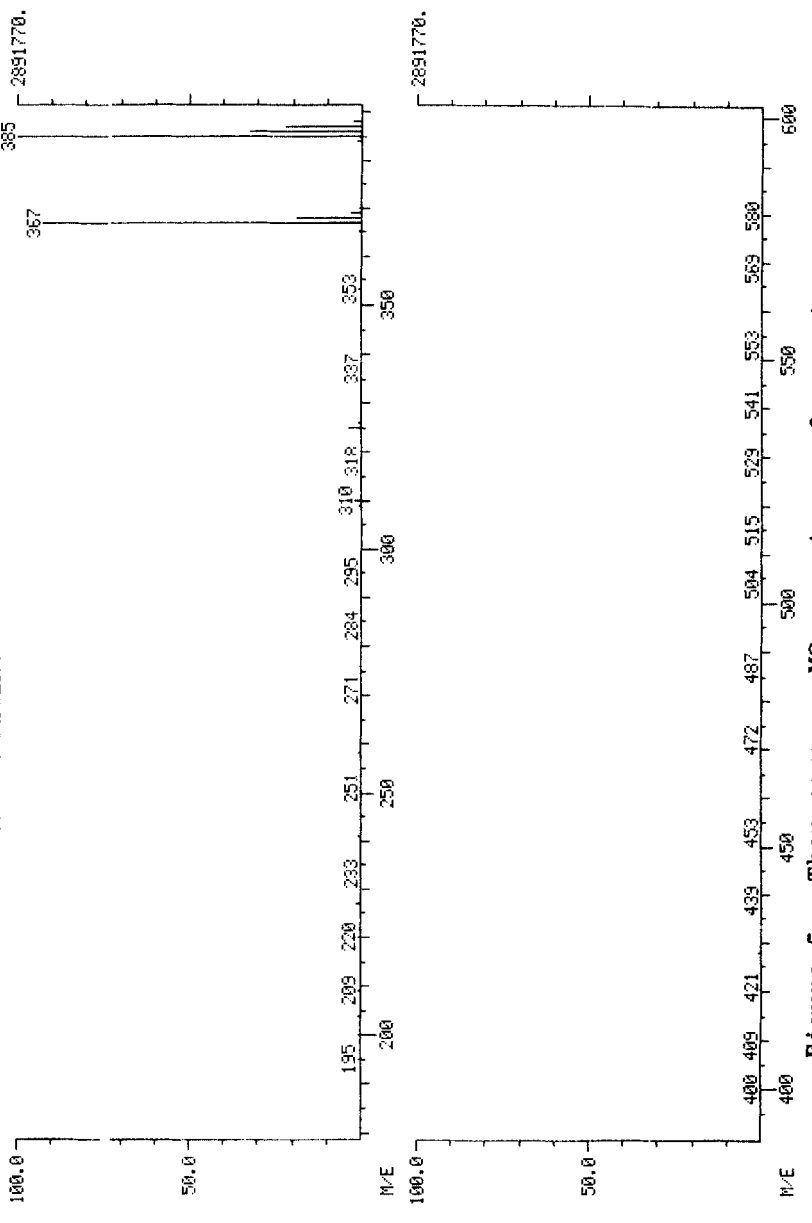

Figure 5. Thermospray MS spectrum of an impurity, identified as an isomer of vincine.

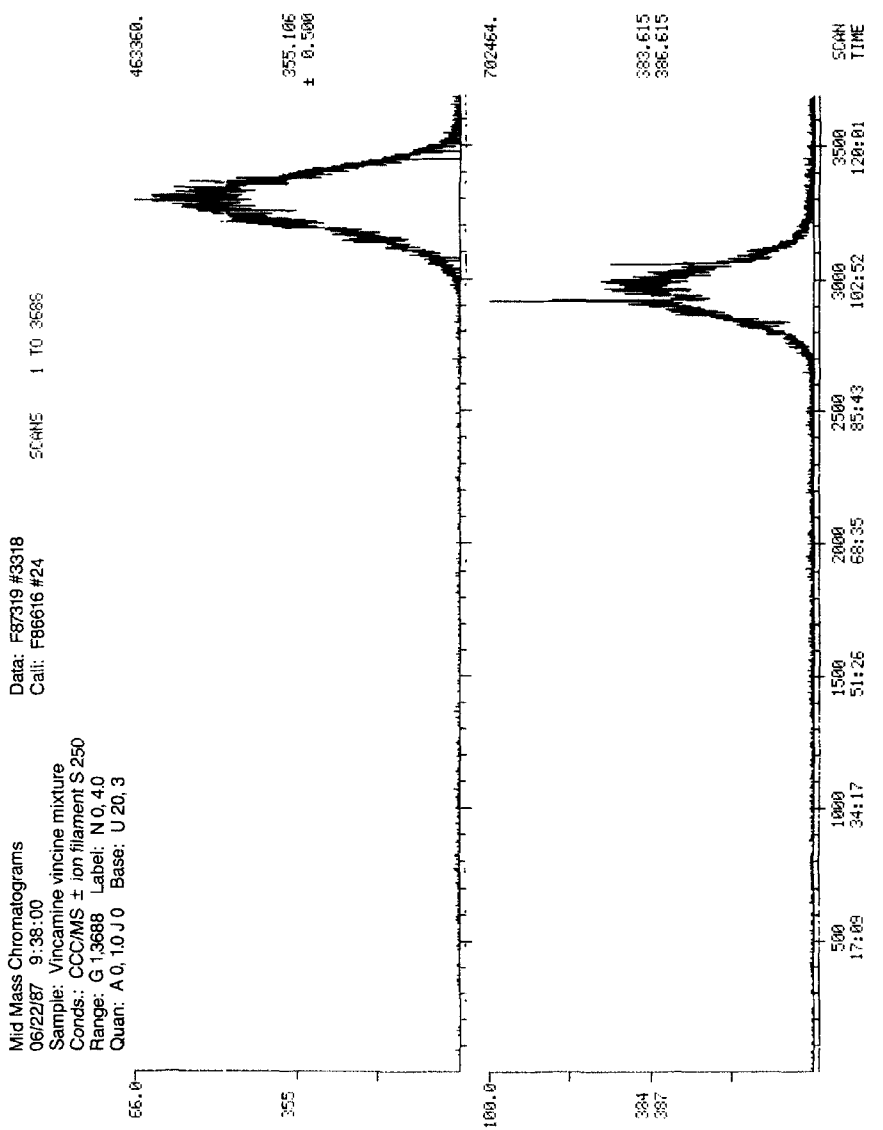

Figure 6. Thermospray CCC/MS selected ion chromatogram for the $[M+H]^+$ ion of vincamine 1 at m/z 355 (top chromatogram) and for the $[M+H]^+$ ion of vincine 2 m/z 385 (bottom chromatogram.

$[M+H]^+$ together with a fragment ion at m/z 367 $[M+H-H_2O]^+$. The similarities between Figures 4 and 5 lead us to postulate that the minor peak in Figure 1 represents an isomer of vincine. HPLC analysis failed to provide adequate chromatographic resolution to separate this isomer from vincine.

The thermospray CCC/MS analysis proved sensitive for the detection of the alkaloids. The analysis of 10-40 µg of the mixture resulted in spectra exhibiting little or no noise. The noise observed in the total ion current (Figure 2) is mostly due to the presence of solvent cluster ions found from water and ammonium acetate. This background disappears when the ion chromatogram for the $[M+H]^+$ ion of each alkaloid is plotted (Figure 6). Direct injection of dilute solution of the alkaloid mix, under full scan MS operation, showed quantities down to 100 ng could be detected by MS. This demonstrated capability of the CCC/MS encourages our current interest in its application to the analysis of macromolecules and biotechnology products as well as thermally unstable molecules.

CONCLUSIONS

A newly developed analytical high speed planet centrifuge is used to interface with mass spectrometer to provide a new analytical methodology (CCC/MS) which integrates the versatility and high resolution of CCC with the identification capability and low detection limit of the mass spectrometer. The two-phase solvent system employed, particularly with the aqueous phase as the mobile phase, offers distinct advantages for thermospray ionization. Our preliminary study with plant alkaloids demonstrates that thermospray CCC/MS can become a complementary technique to HPLC/MS for the analysis of high molecular weight and/or thermally unstable molecules.

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