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Analyses of Phthalates and Peptides Using a Gradient μLC/MS System

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Liquid Chromatography/Mass Spectrometry (LC/MS) has been rapidly commercialized and extensively used in analytical applications since it first appeared over twenty years ago.[1-3] The incompatibility between the LC and MS systems had retarded the appearance of a useful LC/MS system before. Fundamentally, the LC system was operated in the liquid phase, and the MS system, in the gas phase. The conventional LC system eluted *ca.* 1 mL liquid corresponding to *ca.* 500-1,000 mL gas per minute, but the conventional MS system could endure 10 mL gas or so in those days.

In the last two decades, ultra-high vacuum systems of high capacity have been developed, and physical concepts of separating solutes from the solvent, mechanical designs, and low-temperature ionization techniques have been devised to construct useful LC/MS systems. Such techniques include thermospray,[4-6] particle beam,[7-9] fast atom bombardment,[10-20] atmospheric pressure ionization,[21] and electrospray[22-25] methods. Some of the commercial systems are known to be directly connected to a conventional LC whose flow rate is 1 mL/min or so. However, reducing the LC flow rate as low as possible is strongly recommended to minimize contamination of the MS system for long term maintenance, thus use of a μLC for μLC/MS is rationalized. Commercial packed silica capillary microcolumns are usually employed for such purposes, but handling the silica capillary columns is inconvenient since they are fragile.

We have been studying to make glass-lined stainless steel microcolumns and recently have constructed a gradient μLC system using such columns.[26-31] In this study, we have constructed a gradient μLC/MS system using the glass-lined stainless steel microcolumns. We believe that this is the first report for a gradient μLC/MS system with a glass-lined stainless steel microcolumn. We have found that this system would be a dependable analytical tool after preliminary analyses of a couple of test samples--a phthalate mixture and a peptide mixture.

Experimental

Two Shimadzu (Tokyo, Japan) 10AD pumps, a Shimadzu DGU-14A membrane degasser, a Tee union with a 1/16 inch I.D. stainless steel frit (as a mixer), a Valco(Houston, USA) CI4W0.05 injector with a 50 nL injection loop, and a 0.5 mm I.D. glass-lined stainless steel C18 microcolumn were combined to construct the micro-flow gradient liquid chromatography part of the system. The Adsorbosphere C18 stationary phase (5 μm) from Alltech (Deerfield, USA) was used as the packing material for the microcolumn. An 1 m × 50 μm I.D. (400 μm O.D.) silica capillary was connected to the column outlet union. A piece of Teflon tubing of 1/16 inch O.D. and 400 μm I.D. was employed to fit the silica capillary in the union. The other end of the silica capillary was introduced into the stainless steel capillary of the electrospray interface of the mass spectrometer. The mass spectrometer used was a VG Biotech (Manchester, UK), Quattro

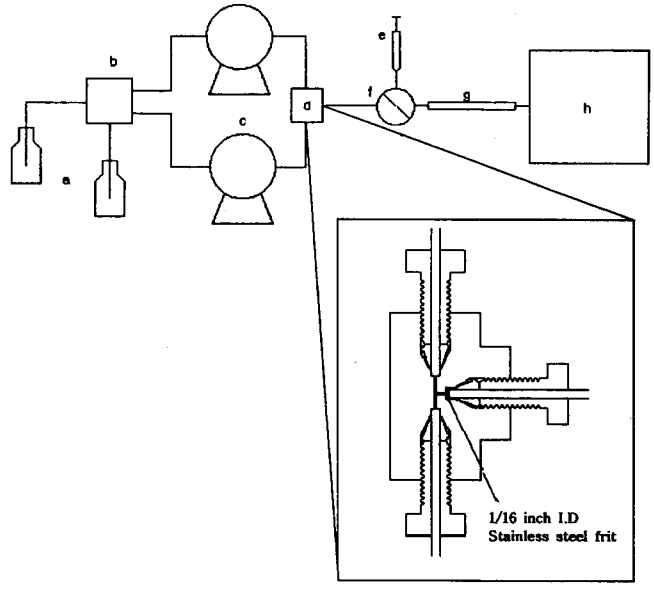

Figure 1. The layout of the LC/MS system. a; mobile phase, b; degasser, c; pump, d; Tee, e; sample syringe, f; injector, g; microcolumn, h; mass spectrometer.

triple quadrupole MS system with a nitrogen-flow assisted electrospray interface. The nitrogen gas flow rate was approximately 0.3 L/min. The electrospray voltage was set at 3.5 kV. The layout of the μLC/MS system is shown in Figure 1.

All the dialkyl phthalates and trifluoroacetic acid were purchased from Aldrich (Milwakee, USA) and used without further purification. The peptide standard mixture was obtained from Sigma (St. Louis, USA) and used as received. Acetonitrile and water were of HPLC grade and purchased from Fisher (Pittsburg, USA) and used without further purification.

Results and Discussion

The molecular weights and the masses of molecular ions for the test solutes are given in Table 1. In gradient elution, 10 μL/min was chosen as the total flow rate. The first test sample was a phthalate mixture composed of dimethyl, diethyl, dipropyl, diphenyl, dibutyl, and dicyclohexyl phthalate. The Chromatograms and mass spectra for this sample obtained by μLC/MS are shown in Figure 2 and 3. The solvent A was 0.1% trifluoroacetic acid (TFA) in acetonitrile, and B, 0.1% TFA in water. The eluent composition was initially 30% A+70% B and was linearly changed to 100% A in 25 min. The sequence of elution for the phthalates is as follows: dimethyl (5.97 min), diethyl (9.27 min), dipropyl (15.15 min), diphenyl (18.23 min), dibutyl (21.09 min), and dicyclohexyl phthalate (27.25 min). The molecular ions (MH^+) for all the solutes were observed. Loss of a neutral alcohol molecule from a phthalate molecular ion occured easily, thus the resulting ions formed by loss of an alcohol from the molecular ions were proven to be the base peaks (m/z 163, 177, 191, 225, and 205 for dimethyl, diethyl, dipropyl, diphenyl, and dibutyl phthalates, respectively) except for dicyclohexyl phthalate for which the molecular ion was the base peak. Fragmentations other than loss of an alcohol were, however, very rare since the electrospray ionization mode was applied. It seems that the charge of the fragmented ion formed by loss of an alcohol is more distributed in the aromatic ring and therefore more stabilized than that of the molecular ion.

Table 1. The molecular weights and the masses of the molecular ions (MH^+)

Solute	M.W.	m/z of MH^+
dimethyl phthalate	194	195
diethyl phthalate	222	223
dipropyl phthalate	250	251
dibutyl phthalate	278	279
diphenyl phthalate	318	319
dicyclohexyl phthalate	330	331
Gly-Tyr peptide	238	239
Val-Tyr-Val peptide	379	380
Met-enkephalin	573	574
Leu-enkephalin	556	557
angiotensin	1046	524*

*Doubly charged molecular ion (MH_2^{2+}). The singly charged molecular ion (m/z=1047) was not detected since it is out of the mass scan range.

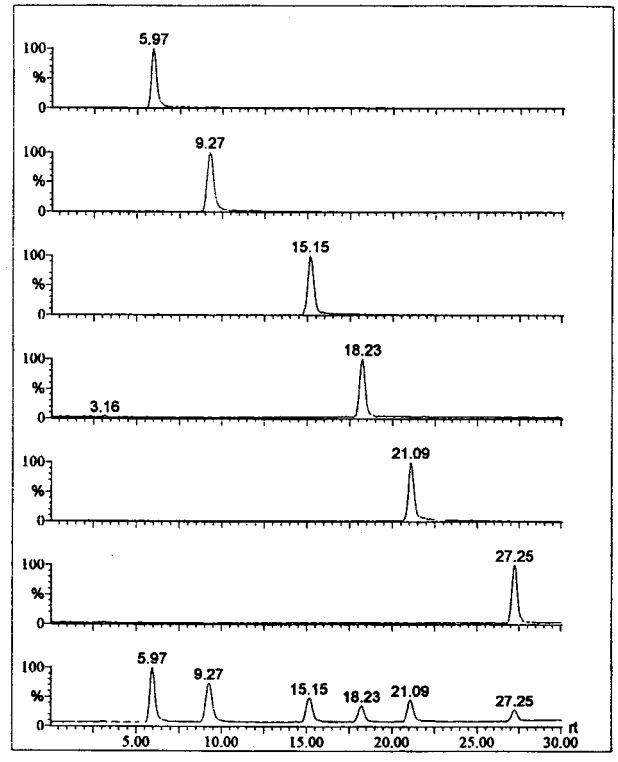

Figure 2. The chromatograms of phthalates obtained by LC/MS. The upper six plots are single ion mass chromatograms for individual molecular ions of dimethyl, diethyl, dipropyl, diphenyl, dibutyl, and dicyclohexyl phthalate, respectively. The bottom plot is the total ion chromatogram. See the text for operational conditions.

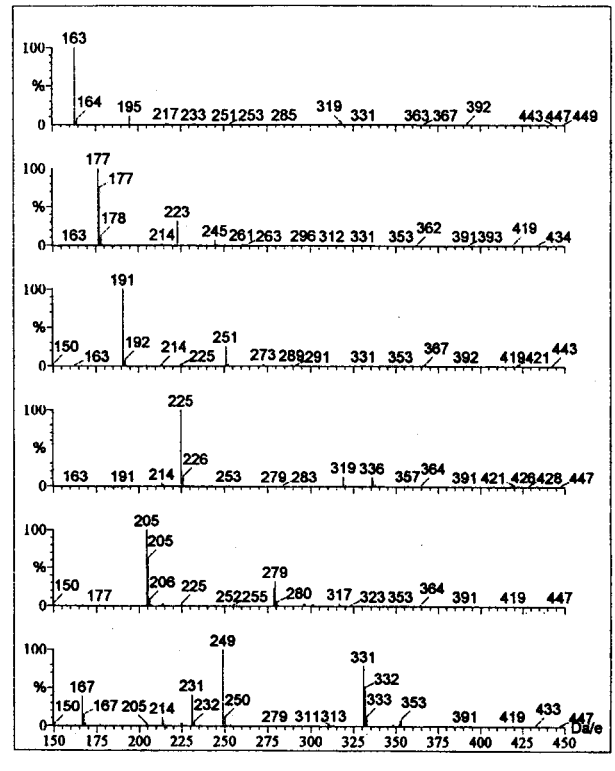

Figure 3. The mass spectra of individual phthalates separated and detected by the LC/MS system. From the top, dimethyl, diethyl, dipropyl, diphenyl, dibutyl, and dicyclohexyl phthalate.

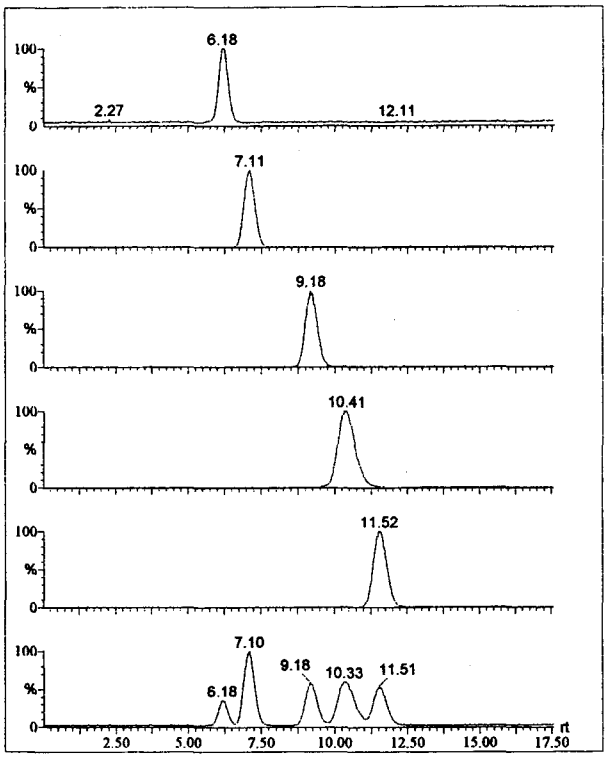

Figure 4. The chromatograms of peptides obtained by LC/MS. The upper five plots are single ion mass chromatograms for individual molecular ions of Gly-Tyr peptide, Val-Tyr-Val peptide, Met-enkephalin, angiotensin II, and Leu-enkephalin. The bottom plot is the total ion chromatogram. See the text for operational conditions.

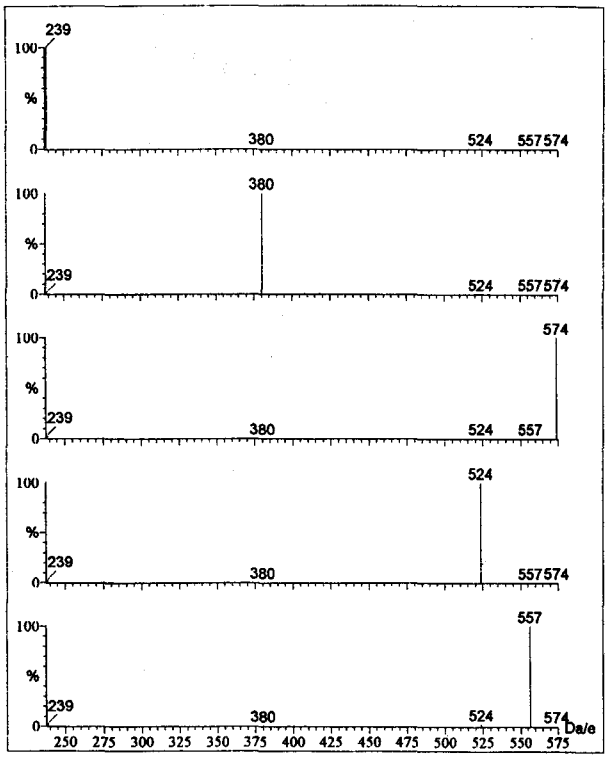

Figure 5. The mass spectra of individual peptides separated and detected by the LC/MS system. From the top, Gly-Tyr peptide, Val-Tyr-Val peptide, Met-enkephalin, angiotensin II, and Leu-enkephalin.

The second test sample was a HPLC peptide standard composed of Gly-Tyr peptide, Val-Tyr-Val peptide, Met-enkephalin (Tyr-Gly-Gly-Phe-Met), angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). The chromatograms and mass spectra for this sample obtained by μLC/MS are shown in Figure 4 and 5. The eluent composition was initially 30% A (0.1% TFA in acetonitrile)+70% B (0.1% TFA in water) and was linearly changed to 100% A in 25 min. The elution sequence for the peptides is as follows: Gly-Tyr (6.20 min), Val-Tyr-Val (7.10 min), Met-enkephalin (9.15 min), angiotensin II (10.41 min), and Leu-enkephalin (11.58 min). The molecular ions (MH^+) for all the peptides were the only peaks observed. The doubly charged molecular ion (m/z=524) was detected for angiotensin II, and the singly charged molecular ion (m/z=1047) was not detected because it is out of the mass scan range. There are few fragmented ions in the mass spectra obtained by the electrospray ionization under normal operational conditions especially for peptides. Simplicity of mass spectra is useful for identification and quantitative determination of a component in the sample since its SIM (selective ion monitoring) chromatogram is likely to be free of interferences from other components.

Nevertheless, the chromatographic separation efficiency shown in Figure 4 was far behind what we had expected. We suspect that the 1 m silica capillary tubing between the column outlet and the electrospray interface could contribute to the degeneration of column efficiency. Its void volume, of course, impose a negative effect. In addition, residual silanol groups on the inside wall of the capillary could cause adsorption of peptide molecules resulting in band broadening of the chromatographic peaks. Furthermore, the 5 μm Adsorbosphere C18 stationary phase is not very effective for separation of peptides.

We believe that the chromatographic separation efficiency will be much improved if a deactivated silica capillary of smaller I.D. (25 μm or less) is used to couple the μLC part to the MS part, and other powerful stationary phase is employed to make a microcolumn, which will be the goal of our future study.

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