Capillary electrophoresis-electrospray ionization mass spectrometry

by

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction of the theory and the literature which provides background and recent progress in this area. Two research papers follow the general introduction with their literature cited. Finally, a general summary presents comments on this work and a list of cited references for the general introduction concludes this dissertation.

Capillary Electrophoresis

The resolving power of capillary electrophoresis (CE) was first demonstrated by Jorgenson and Lukacs[1,2] in 1981. CE is a modern analytical technique which permits rapid and high efficient separations of analytes in very small sample volumes. Separations are based on the differences in effective mobilities of analytes in electrophoretic media inside the capillary. Different separation modes of CE, such as capillary zone electrophoresis (CZE)[1,2], capillary gel electrophoresis (CGE)[3-6], capillary isotachorphoresis (CITP)[7,8], capillary electrochromatography (CEC)[9], capillary isoelectric focusing (CIEF)[10-14], and micellar electrokinetic chromatography (MEKC)[15,16], can be performed using a standard CE instrument. Since the commercial CE instrument became available at the end of 1980s, CE has

increasingly been seen as an alternative or complementary separation method capable of faster analysis and higher efficiency than high performance liquid chromatography (HPLC).

CZE is the most commonly used technique for the separation of ionic compounds in CE. The ionization of surface silanol groups in the fused silica capillary at pH > 2 results in a negatively charged silica surface and an electrostatic diffuse layer of cations adjacent to the negatively charged surface. The migration of cations in the diffuse layer induces the electroosmotic flow in the direction toward the cathode. Ionic compounds with various charge to mass ratios exhibit different electrophoretic mobilities and thus are separated in CZE. Since the electroosmotic flow is usually greater than the electrophoretic mobilities of analytes, both cations and anions can be separated and detected in the same run.

Hjerten and his coworkers were the first to employ polyacrylamide-filled and agarose-filled capillaries for the separation of both small and large molecules[3,4]. Analytes with different size migrate through the pores of the gel matrix at different rates and are thus separated in CGE. Because of the anti-convective media and the minimized solute diffusion, CGE has achieved the highest separation efficiency ever obtained by any analytical separation technique to date. Theoretical plates in the range of 10-20 million can be routinely achieved[5,6] for the separation of proteins and DNA fragments.

CITP is performed in a discontinuous buffer system. Sample components condense between the leading and terminating zones, and produce a steady-state migrating configuration of consecutive sample zones. The leading buffer contains the ions with the highest mobility and the terminating ions are with the lowest mobility[7,8]. Different from other CE techniques,

the migration time of the analyte zones provides no indication of the identity of the analyte and the amount of analyte is proportional to the time between the steps of consecutive analyte zones.

In CEC[9], the capillary is packed with the material which can retain solutes by the partitioning phenomenon similar in HPLC. The mobile phase in CEC is driven by electroosmotic flow. The flow profile in CEC is no longer perfect plug flow as in CZE because of the tortuous nature of the channels resulted from the packing material. CEC, however, still provides a higher efficiency than the pressure-driven system of HPLC.

Capillary Isoelectric Focusing

Capillary isoelectric focusing (CIEF) was first described by Hjerten and Zhu in 1985[10]. The separation of proteins and peptides in CIEF is based on their differences in isoelectric points (pI). In CIEF, the fused silica capillary is usually coated with linear polyacrylamide to eliminate the electroosmotic flow.

The CIEF capillary is initially filled with a solution containing the sample analytes and a mixture of ampholytes. These ampholytes are small amphoteric molecules containing both amino and carboxylic groups with different pIs. Under the influence of applied electric field, the negatively charged acidic ampholytes migrate toward the anode and decrease the pH at the anodic section, while the positively charged ampholytes migrate toward the cathode and increase the pH at the cathodic section. These pH changes will continue until each

ampholyte molecule has come to its pI. Because each ampholyte has its own buffering capacity, a continuous pH gradient is formed in the capillary.

As shown in Figure 1, protein analyte acquires a net negative charge in the region between its pI and cathodic end (where pH > pI) and migrates toward the anode. In contrast, protein analyte acquires a net positive charge in the region between its pI and anodic end (where pH < pI) and migrates toward the cathode. As a result, analyte molecules distributing over the capillary at the beginning of the experiment are focused at the regions where pH = pI. To prevent the ampholytes and analytes from migrating into the inlet and outlet reservoirs by diffusion, the solutions of 20 mM phosphoric acid and 20 mM sodium hydroxide are typically used as the anolyte and the catholyte, respectively.

Due to the focusing effect, CIEF thus permits analysis of very dilute samples with a typical concentration factor of 50-100 times. Furthermore, the analyte molecules leaving the focused zones by diffusion or convection will also migrate back to their pIs due to the same focusing effect. This zone-sharpening effect makes isoelectric focusing a high resolution tool for the analysis of proteins with pI differences as small as 0.02 units[17].

In CIEF, the focused analyte zones must be mobilized and detected at one end of the capillary. Among various mobilization approaches, the hydrodynamic mobilization described by Hjerten and Zhu[12] involves the connection of a pump to the cathodic end of the capillary via a T-tube. The electric field is maintained during the mobilization step to help minimizing the hydrodynamic band broadening. The so called salt mobilization[12,13] involves the replacement of 20 mM phosphoric acid (the anolyte) with 20 mM sodium hydroxide after

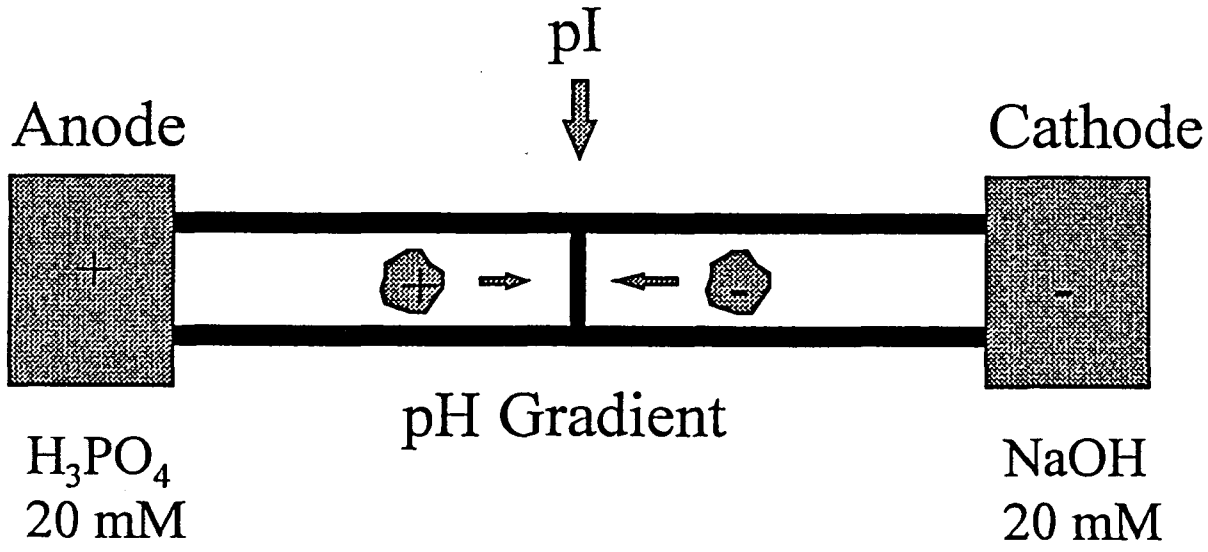

Fig. 1 Separation principle of capillary isoelectric focusing

the focusing is completed. This replacement under the influence of the electric field results in an increase of solution pH inside the CIEF capillary. The focused ampholytes and analytes are no longer neutral and become negatively charged with electrophoretic migration toward the anodic end of the capillary. This is referred to as anodic mobilization. On the contrary, the cathodic mobilization can be achieved by replacing the catholyte (20 mM sodium hydroxide) with a 20 mM phosphoric acid solution after the focusing is completed.

Alternatively, a small amount of buffer additive such as 0.1% methyl cellulose can be added into the sample solution to suppress the electroosmotic flow in the uncoated capillary[14]. The electroosmotic flow is reduced sufficiently to ensure that the focusing is complete before the analytes migrate past the detection window. The analytes are focused and eluted by electroosmotic flow in one step without an extra mobilization procedure. The buffer needs not be changed, nor does the voltage have to be turned off and on. Since a bare silica capillary can be employed, the time consuming coating procedure is eliminated and the potential for instability of a coating, particularly at high pH, is avoided.

Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) was developed by Terabe and his coworkers for the separation of neutral compounds in 1984.[15,16] In MEKC, surfactants are added to the electrophoresis buffer at the concentration above the critical micelle concentration (CMC) to form micelles in equilibrium with the surfactant monomers.

Surfactants are amphiphilic species, comprising both hydrophobic and hydrophilic regions. The micelles are spherical in shape with the hydrophobic tails of the surfactant orientated to the interior of the aggregate and the hydrophilic head groups exposed to the aqueous solution.

A schematic diagram illustrating the separation mechanism of MEKC is shown in Figure 2 for the application of anionic surfactants. The MEKC system contains two phases: an aqueous phase and a micellar phase. Micelles are negatively charged and possess an electrophoretic velocity ($v_{ep,mc}$) that opposes the electroosmotic flow (v_{eo}). In general, the absolute value of v_{eo} is larger than $v_{ep,mc}$, micelles thus migrate toward the cathode with the net velocity, v_{mc}, as the sum of v_{eo} and $v_{ep,mc}$.

Differential partitioning of solutes between the two phases in MEKC results in the separation of neutral and ionic compounds. Similar to reverse phase high performance liquid chromatography (RP-HPLC), more hydrophobic solutes interact more strongly with micelles and thus migrate slower than the more hydrophilic ones. However, the fact that micelles migrate toward the cathode in MEKC leads to the most significant difference between MEKC and conventional RP-HPLC. That is all neutral analytes must migrate out of the capillary between the limited migration window of t_0 and t_{mc}, where t_0 is the migration time of an analyte that has no interaction with the micelles, while t_{mc} is the migration time of an analyte that is totally solubilized by the micelles.

The analyte's migration time in MEKC is given as[15,16]

$$t_r = t_0\{(1+k)/[1+(t_0/t_{mc})k]\} \qquad (1)$$

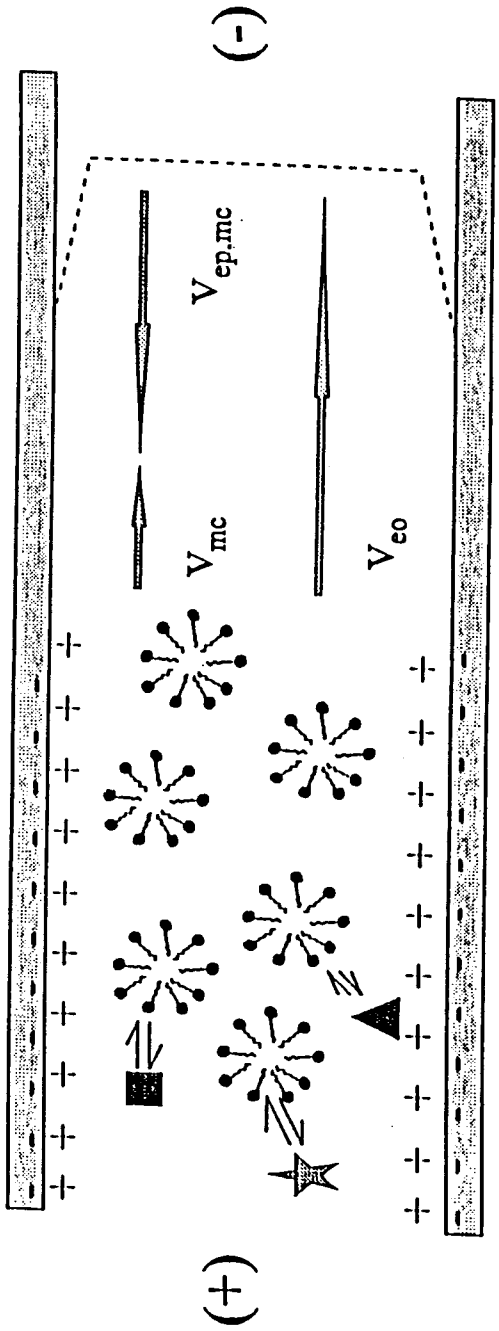

Fig. 2 Separation mechanism of micellar electrokinetic chromatography.

Subsequently, the capacity factor, k, can be calculated from

$$k = (t_r - t_0)/\{t_0[1-(t_0/t_{mc})]\} \quad (2)$$

Resolution in MEKC is given by

$$R = (N^{1/2}/4)[(\alpha-1)/\alpha][k_2/(k_2+1)]\{[1-(t_0/t_{mc})]/[1+(t_0/t_{mc})k_1]\} \quad (3)$$

where α is the separation factor which is equal to k_1/k_2. If the absolute value of v_{eo} is equal to $v_{ep,mc}$, v_{mc} equals zero and the micellar phase becomes stationary in the MEKC capillary. Thus, t_{mc} becomes infinite and the above equations are reduced to

$$t_r = t_0(1+k) \quad (4)$$

$$k = (t_r - t_0)/t_0 \quad (5)$$

$$R = (N^{1/2}/4)[(\alpha-1)/\alpha][k_2/(k_2+1)] \quad (6)$$

which are the same as the ones used in RP-HPLC.

Electrospray Ionization Mass Spectrometry

The researcher who took the first step on the path of electrospray ionization mass spetrometry (ESIMS) is Malcolm Dole[18],who tried to determine molecular weight distribution of some synthetic polymers. After much frustration in trying to develop the so-called "Electrohydrodynamic Ionization" at that time into an useful ion source for mass spectrometry, most investigators abandoned the technique. It took a long way until the first

modern ESIMS was introduced by Yamashita and Fenn in 1984[19,20]. ESIMS soon became one of the most important techniques for the detection and identification of biomolecules.

In ESI, an aqueous solution of analyte is introduced through a capillary at high electric voltage. Under the influence of a positive electric field, the positive ions accumulate on the solution surface. The surface is further drawn out down field such that the so-called Taylor cone[21] forms. At a sufficiently high electric field, the cone is unstable and a liquid filament with a diameter of a few micrometers is emitted from the Taylor cone tip. At some distance downstream, the liquid filament becomes unstable and forms separate droplets. The higher the applied voltage is, the shorter the unbroken filament becomes. Solvent evaporation and droplet disintegration of these charged droplets lead to very small and highly charged droplets capable of producing gas phase ions. The ions formed at atmospheric pressure are then channeled into the high vacuum of the mass spectrometer through a capillary or a set of differentially pumped skimmers.

Two theoretical models have been proposed to account for the formation of gas phase ions from charged droplets. In Dole's Charged Residue Model (CRM)[18], evaporation of solvent molecules from a charged droplet steadily decreases its size, thereby increasing its surface charge density. The droplet continues to shrink until it reaches the Rayleigh limit[22] at which the Coulomb repulsion overcomes the surface tension. The resulting instability breaks up the parent droplet into a hatch of offspring droplets, each of which continues to evaporate until it too reaches the Rayleigh limit. This sequence continues until the offspring droplets ultimately become so small that they contain only one analyte molecule. The analyte

molecule retains some of the droplet charge and becomes an gas phase ion as the last solvent molecules evaporate.

For the Ion Desorption Model (IDM) proposed by Iribarne and Thomson[23,24], the charged droplet commences the same sequence of evaporation and Coulomb fission steps as those in CRM. However, the charged droplets at some intermediate stage are small enough so that the surface charge density is sufficiently intense to overcome solvation forces and to lift an analyte ion from the droplet surface into the ambient bath gas. The exact mechanism for ion formation in ESI remains an active research topic in the literature.[25-28]

Several features of ESIMS have contributed to its great success and significance in various biological and biomedical applications. First, ESIMS can be employed for the direct analysis of liquid effluents from HPLC or CE separations. Furthermore, the multiple charging phenomenon in ESIMS allows the mass determination of macromolecules over 50 kDa using mass spectrometer with limited m/z range. When averaged, these multiple-charged ions provide excellent accuracy and precision in the mass determination of macromolecules.[29-31] Finally, ESI is probably the 'softest' of all ionization techniques yet developed, involving only the combination of high electric fields, moderated by an atmospheric pressure bath gas with typically only mild heating to enhance desolvation. Thus, the preservation of non-covalent association is possible in ESIMS. In fact, the application of ESIMS permits the measurements of certain non-covalent interactions between biological macromolecules, such as the heme-protein complex[32], enzyme-inhibitor complex[33], oligonucleotide duplex[34], and receptor-ligand complex[35-37].

Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry

CE is a high efficiency separation method, while ESIMS allows the formation of multiple-charged ions directly from the electrophoresis eluent and the precise mass determination of high molecular weight ions. The combination of CE with ESIMS is very attractive for obtaining higher selectivity and for structural analysis of analytes in a MS/MS mode.

Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry Interface

The principal requirement of a CE-ESIMS interface is to produce the gas phase ions directly from the CE running buffer and transport the gas ions from the atmospheric pressure region into the mass spectrometer as efficiently as possible. The first CE-ESIMS interface was described by Smith and his coworkers[38]. A stainless steel capillary was employed to ensured immediate electrical contact with the solution eluting out of the CE capillary, hence terminating the CE circuit and initializing the electrospray process. A less satisfactory method for making the electric contact between the electrophoretic buffer and the electrospray interface was made by depositing a thin metal film on the outer surface of the capillary instead of using a stainless steel needle[39].

Smith and his coworkers[40] also introduced an improved ESIMS interface equipped with a coaxial sheath liquid as shown in Figure 3. In this design, a fused silica capillary

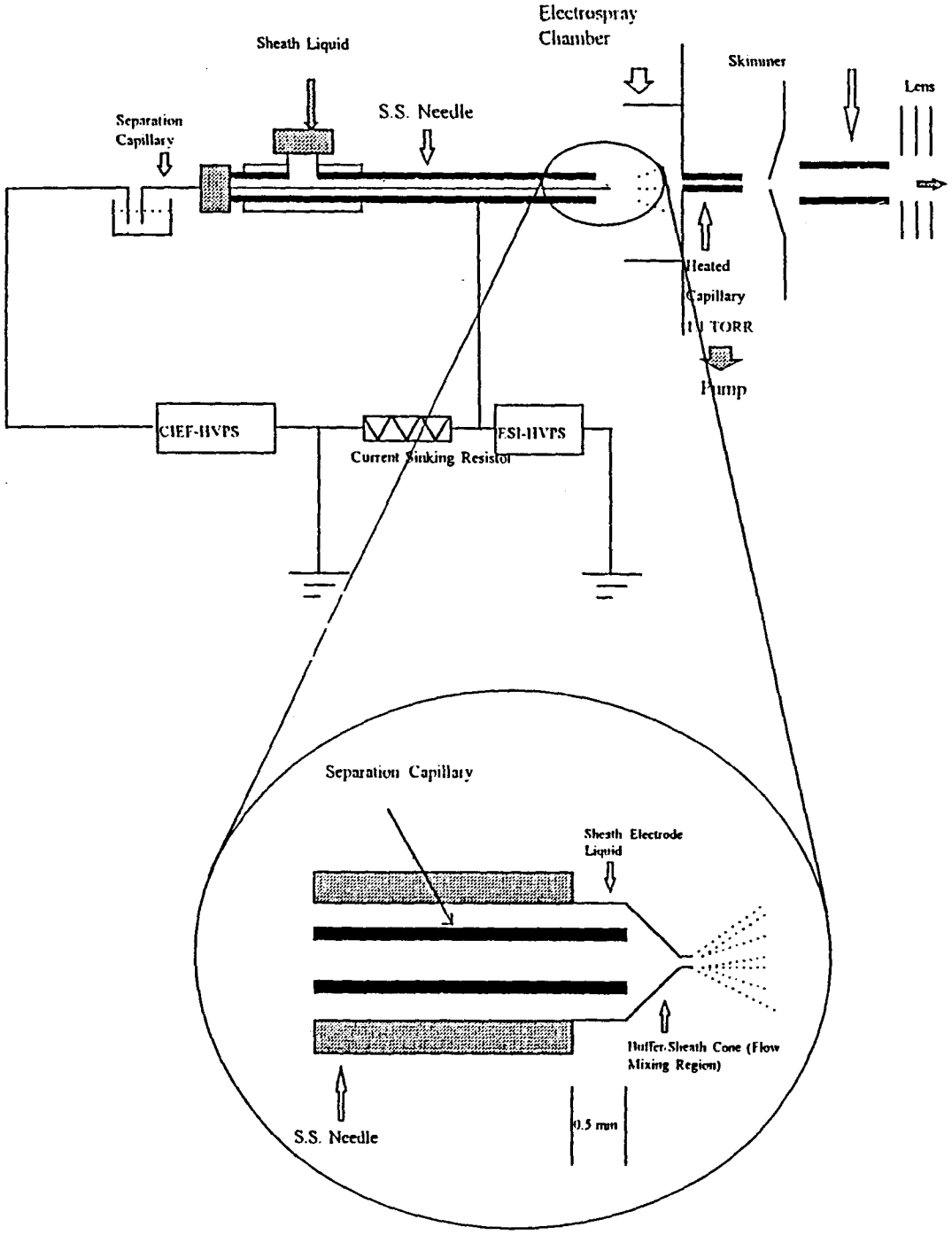

Fig. 3 Schematic illustration of capillary electrophoresis-electrospray ionization mass spectrometry utilizing a coaxial sheath liquid configuration.

protruded about 0.5 mm from a stainless steel needle. The sheath liquid usually consisted of a mixture of water and volatile organic solvent such as methanol. When the CE buffer was mixed with the sheath liquid at the end of the capillary, the surface tension decreased while the volatility increased for enhancing the electrospray efficiency. Furthermore, the sheath liquid established electrical contact at the end of the capillary. The sheath potential was controlled around 5 kV (for positive ion mode), and functioned as both the CE cathodic potential and the electrospray voltage. A counter current flow of warm nitrogen gas (up to 80 °C) between the nozzle and the ESI source was used to aid desolvation, although sufficient heating during transport into the mass spectrometer also accomplished effective desolvation.

Lee et al.[41] developed a liquid junction coupling for CZE-ion spray MS (which is the nebulizing gas assisted ESIMS). The comparison between liquid junction and coaxial interfaces was made by Pleasance et al.[42] and the coaxial sheath flow appeared to have several advantages with regard to ruggedness, ease of use, better sensitivity and electrophoretic performance. Gale and Smith[43] described a sheathless ESI source in which a small diameter etched-tip capillary was incorporated. The ability to electrospray aqueous solutions without the use of an ancillary sheath flow was demonstrated with several biopolymers. Kriger and his coworkers[44] introduced a simple procedure for preparing gold-coated silica capillaries used in ESIMS. The performance characteristics of these durable capillaries as continuous infusion sources were examined, and their utility in on-line CE-ESIMS was demonstrated.

Applications of Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry

One of the advantages of incorporating ESIMS lies in the multiple charging of the analytes under ESI conditions. The multiple charging phenomenon in ESI makes the detection of biomacromolecules possible using a quadrupole mass spectrometer with limited m/z range. Various applications have been reported for the analysis of peptides and proteins by CE-ESIMS.[45-48] A reduced elution speed method was described[45] for the enhancement of detection sensitivity in CZE-ESIMS. Mass spectra for a set of standard proteins were obtained for the injections of 60 femtomole of proteins, while the analysis of an albumin tryptic digestion was achieved with the injections of 40 femtomole of proteins. The use of small i.d. capillaries for the detection of protein analytes at the attomole level was demonstrated by Smith and his coworkers.[46] With the proper selection of running buffers and on-line combination of transient CITP with ESIMS, the concentration detection limits for a full scan was decreased by a factor of 100 times, in comparison with CZE-ESIMS.[48]

In the pharmaceutical area, identification of non-steroidal anti-inflammatory drugs and their metabolites in human urine was demonstrated using CZE-ESIMS.[49] The application of CZE-ESIMS for drug metabolic studies with a particular emphasis on neuroleptic drugs was reported by Tomlinson et al.[50,51] Non-aqueous solution was used as the electrophoretic buffer due to the low solubility of drug analytes in aqueous solution.

Furthermore, CE-ESIMS has been applied for the analysis of compounds of environmental concern, such as agrochemicals, pesticides, inorganic compounds and dyes. [42,

[52-55] For example, Lamoree and his coworkers[54] demonstrated the determination of β-agonists which are used illegally in the cattle industry to increase meat production.

Capillary Isoelectric Focusing-Electrospray Ionization Mass Spectrometry

In addition to CZE-ESIMS, the direct coupling of CIEF with ESIMS is demonstrated for high resolution analysis of bovine serum apotransferrin glycoforms (see first paper of this thesis). On the basis of their differences in isoelectric point (pI), the di-, tri-, and tetrasialotransferrins are separated and resolved in CIEF. The focused protein zones of di-, tri-, and tetrasialotransferrins are eluted by combining gravity with cathodic mobilization. At the end of the CIEF capillary, the mobilized transferrin zones are analyzed by mass spectrometry coupled on-line to an electrospray interface with a coaxial sheath flow configuration. Additional transferrin variants within each of di-, tri-, and tetrasialotransferrins, differing in their molecular weights, are easily distinguished by ESIMS. In combination with sialidase digestion, the distribution of pI and molecular weight of asialo-, mono-, di-, tri-, and tetrasialotransferrin variants is obtained from the CIEF-ESIMS measurements.

Micellar Electrokinetic Chromatography-Electrospray Ionization Mass Spectrometry

Direct coupling of MEKC to ESIMS is hazardous due to the effect of nonvolatile surfactants on the ESIMS performance. The continuous introduction of nonvolatile

surfactants into ESIMS at a relatively high concentration results in a significant loss of electrospray efficiency and mass sensitivity of analytes. In the second paper of this thesis, on-line MEKC-ESIMS is demonstrated for the analysis of chlorotriazine herbicides and barbiturates.

The micellar velocity is directly manipulated by the adjustment of electroosmosis rather than the electrophoretic velocity of the micelle. The elimination of MEKC surfactant introduction into ESIMS is achieved with an anodically migrating micelle, moving away from the electrospray interface. The effects of moving surfactant boundary in the MEKC capillary on separation efficiency and resolution of triazine herbicides and barbiturates are investigated. The detection of herbicides and barbiturates sequentially eluted from the MEKC capillary is acquired by ESIMS using the positive and negative electrospray modes, respectively.

CAPILLARY ISOELECTRIC FOCUSING-ELECTROSPRAY IONIZATION MASS SPECTROMETRY FOR TRANSFERRIN GLYCOFORMS ANALYSIS

A paper accepted by Anal. Biochem.

Liyu Yang, Qing Tang, A. Kamel Harrata, and Cheng S. Lee

ABSTRACT

On-line capillary isoelectric focusing (CIEF)-electrospray ionization mass spectrometry (ESIMS) as a two-dimensional separation system is employed for high resolution analysis of bovine serum apotransferrin glycoforms. On the basis of their differences in isoelectric point (pI), the di-, tri-, and tetrasialotransferrins are separated and resolved in CIEF. The focused protein zones of di-, tri-, and tetrasialotransferrins are eluted by combining gravity with cathodic mobilization. At the end of CIEF capillary, the mobilized transferrin zones are analyzed by mass spectrometry coupled on-line to an electrospray interface with a coaxial sheath flow configuration. Additional transferrin variants within each of di-, tri-, and tetrasialotransferrins, differing in their molecular weights, are easily distinguished by ESIMS. In combination with sialidase digestion, the distribution of pI and molecular weight of asialo-, mono-, di-, tri, and tetrasialotransferrin variants was obtained from the CIEF-ESIMS measurements. Besides the differences in the number of sialic acid, the microheterogeneity of bovine serum apotransferrin glycans might be complicated by the partial fucosylated structure and the a-Gal (1-3)-b-Gal on the a-Man-(1-6) antenna.

INTRODUCTORY STATEMENT

Two-dimensional gel electrophoresis has been the traditional approach for protein characterization.[1] Protein mixtures are separated first by their differences in isoelectric point (pI) and then by size in a two-dimensional gel. The separation by isoelectric focusing is carried out in a column filled with a pH gradient medium. The focused proteins in a pH gradient medium are then applied to the top of a flat gel containing sodium dodecyl sulfate, and the denatured proteins are electrophoretically separated by molecular weight in a second dimension. Despite the selectivity and sensitivity of two-dimensional gel electrophoresis, this technique is the collection of manually intensive procedures and time-consuming tasks prone to irreproducibility and poor quantitative accuracy.

Capillary isoelectric focusing (CIEF) has been introduced and developed by Hjerten and his co-workers[2-4] for the measurement of proteins' pI. In CIEF separations, the capillary contains not only carrier ampholytes for the creation of a pH gradient but also proteins. The proteins are focused into discrete and narrow zones with local pHs corresponding to their pIs. The zone-sharpening effect, the characteristic of isoelectric focusing, makes CIEF a high resolution tool for the analysis of protein variants with a pI difference as small as 0.05 pH unit. Furthermore, the focusing effect of CIEF permits the analysis of very dilute protein samples with a typical concentration factor of 50-100 times.

On-line combination of CIEF with electrospray ionization mass spectrometry (ESIMS) is very attractive for the possibility of selectivity enhancement, for the direct identification of protein analytes, and for structure analysis of protein molecules in a MS-MS mode. In

analogy to two-dimensional gel electrophoresis, proteins are focused and mobilized in the CIEF capillary. At the end of the CIEF capillary, the eluted protein zones are analyzed by mass spectrometry coupled on-line to an electrospray interface with a coaxial sheath flow configuration.[5] ESIMS as the second dimension allows the formation of multiple-charged, high molecular weight ions and the precise mass determination of $\pm$ 0.01% for proteins up to 30 kDa.

On-line direct coupling of CIEF with ESIMS has been demonstrated by Tang et al.[5] for the analysis of model proteins including cytochrome c, myoglobin, and carbonic anhydrase II. The effects of carrier ampholyte concentration on the CIEF separation and the protein electrospray ionization mass spectra were investigated. The ampholyte ions similar to sample electrolyte ions led to higher solution conductivity and contributed to the establishment of the charge excess known to exist in droplets formed during the electrospray process. The concentration detection limit of myoglobin for a full scan CIEF-ESIMS analysis was in the range of 10^{-7} M, two orders of magnitude better than normal capillary zone electrophoresis (CZE)-ESIMS. Initial preconcentration during the CIEF focusing was responsible for improving detection limits.

Concerns about the quality of recombinant glycoproteins arise in connection with the observed macro- and microheterogeneity of glycoforms secreted in the course of a cell culture process. Such variations in glycosylation site occupancy (macroheterogeneity), as well as glycoform antennary structure (microheterogeneity), have been associated with variations in protein properties such as efficacy, clearance rate, antigenicity, and immunogenicity.[6,7]

Thus, the issue of protein quality is not only one of optimizing glycoform composition with respect to the above spectrum of glycoprotein properties but also of maintaining glycoform consistency in spite of cellular and process variation. In this study, the full potential of CIEF-ESIMS in protein characterization is demonstrated for the analysis of bovine serum apotransferrin glycoforms. The integration of CIEF with ESIMS exhibits superior resolving power, speed, and sensitivity for glycoprotein analysis and may have practical utility in the biopharmaceutical industry as the means to demonstrate lot-to-lot consistency.

MATERIALS AND METHODS

Capillary Isoelectric Focusing: UV Measurement

The CIEF apparatus was constructed in-house using a CZE 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY) and a linear UVIS 200 detector (Linear Instruments, Reno, NV). Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with 50 μm i.d. and 192 μm o.d. were coated internally with linear polyacrylamide to eliminate the electroosmotic flow (4).

A 30-cm long capillary was rinsed with deionized water for 2 min and was then filled with a solution containing bovine serum apotransferrin (CalBiochem, San Diego, CA) and carrier ampholyte, pharmalyte 5-8 (Pharmacia, Uppsla, Sweden). Focusing was performed by applying a constant 500 V/cm electric field across the capillary for 10 min. The solutions of 20 mM phosphoric acid and 20 mM sodium hydroxide were used as the anolyte and the catholyte in the inlet and outlet reservoirs, respectively.

When the focusing was completed, the focused protein zones were mobilized by combining gravity with cathodic mobilization. Cathodic mobilization was initiated by replacing the sodium hydroxide catholyte with a solution containing methanol/water/acetic acid in a volume ratio of 50:49:1 at pH 2.6. To induce the gravity mobilization, the inlet reservoir was raised 8 cm above the outlet reservoir. A constant electric field of 500 V/cm was again applied during the mobilization. The protein zones were monitored by UV absorbance (Linear Instruments, Reno, NV) at 280 nm. The distance between the injection point and the UV detector was 23 cm. All chemicals, including phosphoric acid, sodium hydroxide, acetic acid, and methanol, were purchased from Fisher (Fair Lawn, NJ). All solutions were filtered through a 1-μm filter (Whatman, Maidstone, England).

Sialidase Digestion of Bovine Serum Apotransferrin

Bovine serum apotransferrin with a concentration of 20 mg/ml was prepared in the reaction buffer containing 50 mM sodium acetate, 4 mM calcium chloride, and 0.1 mg/ml bovine serum albumin at pH 5.5. A 0.2 unit of sialidase (from *vibrio cholerae*, Oxford Glycosystems, Rosedale, NY) was dissolved in 50 μl of reaction buffer. The sialidase solution was then mixed with the transferrin solution in a volume ratio of 1:1. The reaction mixture was incubated at 37° C for 24 hours.

At the end of incubation, the digestion solution was desalted by using a regenerated cellulose membrane (10,000 molecular weight cutoff) from Millipore (Bedford, MA). The digestion solution was then diluted with the CIEF solution and was analyzed by CIEF with

UV detection at 280 nm. The digestion mixture analyzed by CIEF contained 1 mg/ml bovine transferrin, 0.5% pharmalyte 5-8 (Pharmacia, Uppsla, Sweden), and 0.5% methyl cellulose.

Mass Spectrometer and Electrospray Interface

The mass spectrometer used in this study was a Finnigan MAT TSQ 700 (San Jose, CA) triple quadrupole equipped with an electrospray ionization source. The Finnigan MAT electrospray adapter kit containing both gas and liquid sheath tubes was used to perform the direct infusion experiment and also to couple CIEF with ESIMS without any modifications. A 5 kV electric potential was maintained at the electrospray needle for all direct infusion and CIEF-ESIMS measurements. The first quadrupole was used for the mass scanning of protein ions, while the second and third quadrupoles were operated in the radio frequency only mode. The electron multiplier was set at 1.4 kV with the conversion dynode at -15 kV. Tuning and calibration of the mass spectrometer were established by using an acetic acid solution (methanol/water/acetic acid, 50:49:1 v/v/v) containing myoglobin and a small peptide of MRFA.

Direct Infusion and Capillary Isoelectric Focusing-Electrospray Ionization Mass Spectrometry.

The bovine serum apotransferrin solution with a concentration of 1 mg/ml was directly infused at 5 μl/min in 50% methanol, 49% water, and 1% acetic acid (v/v/v) by using a Harvard Apparatus 22 syringe pump (South Natick, MA). The first quadrupole was scanned from m/z 1600 to 2000 with a scan rate of 2 s/scan for both direct infusion and CIEF-ESIMS measurements. The deconvoluted mass spectra of native and digested

transferrins were obtained by using the BioToolBox analysis software for LC/MS systems from Perkin-Elmer Sciex Instruments (Foster City, CA).

For the combination of CIEF with ESIMS, a 20-cm-long coated capillary with 50 μm i.d. and 192 μm o.d. was mounted within the electrospray probe. The outlet reservoir containing 20 mM sodium hydroxide as the catholyte was located inside the electrospray housing during the focusing step. The inlet reservoir containing 20 mM phosphoric acid as the anolyte was kept at the same height as the outlet reservoir. A constant voltage of 10 kV was applied during the focusing step.

Once the focusing was completed, the electric potential was turned off and the outlet reservoir was removed. The capillary tip was fixed about 0.5 mm outside the electrospray needle. The sheath liquid consisted of 50% methanol, 49% water, and 1% acetic acid (v/v/v) and was delivered at a flow rate of 5 μl/min with the use of a Harvard Apparatus 22 syringe pump (South Natick, MA). During the mobilization step, two high-voltage power supplies (Spellman High-Voltage Electronics, Plainview, NY) were used for delivering the electric potentials of 15 kV and 5 kV at the inlet electrode and the electrospray needle, respectively. Because most high-voltage power supplies are not designed to operate as current sinks, a resistor ladder, parallel with the high-voltage electrode connecting with the electrospray needle, was incorporated.

To speed up protein mobilization and minimize the moving ionic boundary inside the CIEF capillary, a combination of cathodic mobilization with a gravity-induced hydrodynamic flow was employed. Formation of a moving ionic boundary inside the CIEF capillary,[8]

similar to those observed in CZE-ESIMS, was the result of the migration of liquid sheath acetate ions into the polyacrylamide coated capillary. The moving ionic boundary led to delays or inversions in migration order and resulted in loss of separation resolution of protein analytes in CIEF-ESIMS. To combine gravity with cathodic mobilizations, the inlet reservoir was raised 8 cm above the electrospray needle. The induced hydrodynamic flow counterbalanced the negative electrophoretic velocity of acetate ions. No sheath gas was employed during the CIEF-ESIMS measurements.

RESULTS AND DISCUSSION

Capillary Isoelectric Focusing with UV Detection

To investigate the effect of carrier ampholyte concentration on transferrin separation in CIEF, the polyacrylamide-coated capillary was filled with a solution containing pharmalyte 5-8 and bovine serum apotransferrin. The concentration of pharmalyte 5-8 was varied between 2% and 0.5%. The total transferrin concentration in the solution was 1 mg/ml before the focusing. The focused transferrin zones were mobilized by introducing a gravity-induced hydrodynamic flow and by replacing the sodium hydroxide catholyte with a solution containing methanol/water/acetic acid (50:49:1 v/v/v) at pH 2.6. The catholyte used in cathodic mobilization was the same as the sheath liquid later employed in the electrospray interface.

The electropherograms of transferrin separation under the influence of various pharmalyte concentrations are shown in Fig. 1. No significant change in separation resolution of transferrin variants was observed at various pharmalyte concentrations. The migration time

of transferrin zones during the cathodic mobilization step increased with increasing pharmalyte concentration. The increase in solution viscosity with increasing pharmalyte concentration accounted for the longer migration time. On the basis of rapid CIEF separation, the pharmalyte 5-8 solution with a concentration of 0.5% was selected and employed for the rest of CIEF separations in this study.

The transferrins are constituted of a single polypeptide chain organized in two lobes, each of which contains one iron-binding site. The transferrins transport iron into cells and bone marrow. It is known that all transferrins from various species are glycosylated via posttranslational modification.[9,10] Three major bovine serum apotransferrin components were observed in Fig. 1 with the corresponding pI values of 6.10, 5.97, and 5.82. Their pIs were measured by comparing their migration times with those of standard proteins including carbonic anhydrase I (pI 6.6), carbonic anhydrase II (pI 5.9), and b-lactoglobin A (pI 5.1) under the same CIEF separation conditions (data not shown). The migration time of transferrin variants increased with decreasing pI value.

The CIEF separation results of bovine serum apotransferrin agreed fairly well with the earlier gel electrophoresis studies,[11,12] which have shown two major bands and one minor band. These three electrophoresis bands were resolved on the basis of their differences in the content of sialic acids. The two major components shown in Fig. 1 contained two and three sialic acids and were designated as di- and trisialotransferrins. The minor component with the most acidic pI exhibited four sialic acids. The only sialic acid found in serum transferrin

glycans is N-acetylneuraminic acid, except in horse serum transferrin glycans which contain N-acetyl, O_5-acetylneuraminic acid in addition to N-acetylneuraminic acid.[13]

It has been shown previously that the bovine serum transferrin sample underwent a continuous decrease in electrophoresis mobility under the treatment with sialidase.[11] As shown in Fig. 2C, the CIEF separation of digested product at 24 hours exhibited two major transferrin components. In comparison with the results shown in Fig. 2A, the digested product eluted earlier than the di-, tri-, and tetrasialotransferrins under the same CIEF separation conditions. The CIEF separation of solution mixture containing the native and the digested apotransferrins, shown in Fig. 2B, clearly presented the distribution of four major and one minor transferrin components. The two major components resolved from the digestion mixture were designated as asialo- and monosialotransferrins with the measured pI values of 6.33 and 6.23. The removal of sialic acids from the transferrin molecules accounted for the increase in pI and the decrease in the migration time.

Capillary Isoelectric Focusing-Electrospray Ionization Mass Spectrometry

The positive electrospray ionization mass spectrum of bovine serum apotransferrin obtained from the direct infusion experiment is shown in Fig. 3A. The mass spectrum displayed two major components with the molecular weights of 77,979 and 78,276, respectively. The molecular weight difference of 297 between the two major apotransferrin components was probably due to the sialic microheterogeneity and seemed to suggest one additional sialic acid in the second component. A third minor component, whose molecular weight was about 300 higher than the second major component, is also visible in the Fig. 3B.

By comparing the results shown in Figs. 1 and 3, the pI and molecular weight differences among three transferrin variants could all be attributed to the distribution of di-, tri-, and tetrasialotransferrins. The variation in the UV intensity (at 280 nm) of transferrin variants was in good qualitative agreement with a two-step decrease in the mass intensity. Even though the charge state of apotransferrin was lower in our study than those reported in the literature, our mass spectrometry results, in the mass determination and the distribution of transferrin variants, agreed fairly well with the earlier ESIMS studies.[14,15]

The pharmalyte ions similar to simple electrolyte ions led to higher solution conductivity and contributed to the establishment of the charge excess known to exist in droplets formed during the electrospray process. Considering the effect of pharmalyte concentration on the CIEF separation and the protein electrospray ionization mass spectra (5), a solution containing 0.5% pharmalyte 5-8 and bovine serum apotransferrin was used in the CIEF-ESIMS measurements. The reconstructed total ion electropherogram of transferrin variants with a total concentration of 1 mg/ml is shown in Fig. 4. In comparison with the reported CZE-ESIMS separation of bovine serum apotransferrin,[14] significant enhancement in separation resolution of transferrin variants was obtained by CIEF-ESIMS. Only one broad transferrin peak was displayed in the reconstructed total ion electropherogram of CZE-ESIMS.

All transferrin variants were directly identified on the basis of mass spectra of transferrins taken from the average scans under the peaks. An example of the mass spectra obtained from the average scans under the peaks of Fig. 4 is shown in Fig. 5 for

trisialotransferrin. The mass spectrum displayed two major components with the molecular weight of 77,985 and 78,255. Similarly, the mass spectra obtained from the average scans under the peaks of disialo- and tetrasialotransferrin (data not shown) yielded two and three mass components, respectively. All three transferrin variants contained two mass components with the molecular weight around 77,973 $\pm$ 12 and 78,259 $\pm$ 4. The tetrasialotransferrin exhibited the third mass component with the molecular weight of 78,571.

In contrast to human serum transferrin, bovine serum transferrin is known to possess the most complex heterogeneity among all transferrins.[10] By combining ion exchange chromatography with gel electrophoresis, the bovine serum apotransferrin has been resolved into two pairs of main bands and one pair of minor bands.[11,12] The differences in the electrophoresis mobility among these pairs were again attributed to the presence of two and three sialic acids in the two pairs of major bands and four sialic acids in the third pair of minor components. It has been suggested that the scission occurred between the amino acids of 54 and 55 from the C-terminus and contributed to additional heterogeneity within each of major and minor bands.[10]

The cleaved portion of transferrin chain has a molecular weight around 6,000. On the basis of our mass spectrometry results, the molecular weight differences within the di-, tri-, and tetrasialotransferrins were too small to support the occurrence of peptide cleavage in transferrin variants. Besides the differences in the number of sialic acid, the microheterogeneity of bovine lactotransferrin glycans were complicated by the partial fucosylated structure and the a-Gal (1-3)-b-Gal on the a-Man-(1-6) antenna.[16] Thus, the

molecular weight differences within the bovine serum di-, tri-, and tetrasialotransferrins could be caused by other forms of carbohydrates, such as additional fucoses and galactoses in the biantennary complex structure.

The reconstructed total ion electropherogram of sialidase treated bovine serum apotransferrin with a total concentration of 1 mg/ml is shown in Fig. 6. Both asialo- and monosialotransferrin variants were directly identified on the basis of mass spectra of transferrins taken from the average scans under the peaks. An example of the mass spectra obtained from the average scans under the peaks of Fig. 6 is shown in Fig. 7 for asialotransferrin. The mass spectrum displayed two major mass components with the molecular weight of 77,369 and 77,674. Additionally, one minor mass component was observed with the molecular weight of 76,976. Furthermore, the mass spectrum obtained from the average scans under the peak of monosialotransferrin (data not shown) yielded three mass components with similar molecular weights and mass intensities to those of asialotransferrin.

The pI values and the molecular weights of bovine serum apotransferrin variants measured by CIEF-ESIMS are summarized and shown in Fig. 8. The separation of asialo-, mono-, di-, tri-, and tetrasialotransferrin variants with similar molecular weights, however, was established by their elution order in the CIEF separation. On the other hand, the transferrin variants containing the same number of sialic acids, differing in their molecular weights, were easily distinguished by ESIMS. The results shown in Fig. 8 clearly illustrate the two-dimensional separation power of CIEF-ESIMS for the analyses of complex

glycoforms in bovine serum apotransferrin. The integration of CIEF with ESIMS exhibits superior resolving power, speed, and sensitivity for glycoprotein characterization in various biological and biomedical studies.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

Fig. 1 CIEF separation of bovine serum apotransferrin at various pharmalyte concentrations. Capillary, 30 cm total length/23 cm to detector, 50 μm i.d. and 192 μm o.d.; voltage, 15 kV for focusing and mobilization; UV detection at 280 nm. Pharmalyte 5-8 concentrations: (A) 0.5%, (B) 1.0%, and (C) 2%. The di-, tri-, and tetrasialotransferrin variants are marked as 2, 3, and 4, respectively.

Fig. 2 CIEF separation of bovine serum apotransferrin using 0.5% pharmalyte 5-8. (A) native apotransferrin, (B) mixture of native and sialidase treated apotransferrins, (C) sialidase treated apotransferrin. The asialo-, mono-, di-, tri-, and tetrasialotransferrin variants are marked as 0, 1, 2, 3, and 4, respectively. Other conditions are the same as in Fig. 1.

Fig. 3 (A) Positive electrospray ionization mass spectrum of bovine serum apotransferrin. The transferrin solution with a concentration of 1 mg/ml is infused at 5 μl/min in 50% methanol, 49% water, and 1% acetic acid (v/v/v). (B) The deconvoluted mass spectrum of transferrin.

Fig. 4 CIEF-ESIMS reconstructed total ion electropherogram of bovine serum apotransferrin. Capillary, 20 cm total length, , 50 μm i.d. and 192 μm o.d.; voltages: 10 kV for focusing and mobilization, 5 kV for electrospray; sheath liquid, methanol/water/acetic acid (50:49:1 v/v/v) at pH 2.6, 5 μl/min; mass scan: m/z 1600 to 2000 at 2 s/scan. The di-, tri-, and tetrasialotransferrin variants are marked as 2, 3, and 4, respectively.

Fig. 5 (A) Positive ion electrospray mass spectrum of trisialotransferrin taken from the average scans under the peak 3 of Fig. 4. (B) The deconvoluted mass spectrum of trisialotransferrin.

Fig. 6 CIEF-ESIMS reconstructed total ion electropherogram of sialidase treated bovine serum apotransferrin. The asialo- and monosialotransferrin variants are marked as 0 and 1, respectively. Other conditions are the same as in Fig. 4.

Fig. 7 (A) Positive ion electrospray mass spectrum of asialotransferrin taken from the average scans under the peak 0 of Fig. 6. (B) The deconvoluted mass spectrum of asialotransferrin.

Fig. 8 The distribution of pI and molecular weight of bovine serum apotransferrin variants measured by CIEF-ESIMS. The mass intensity of transferrin variants is represented by the dot intensity. The pI order: asialo- (pI 6.33), mono- (pI 6.23), di- (pI 6.10), tri- (pI 5.97), and tetrasialotransferrin (pI 5.82).

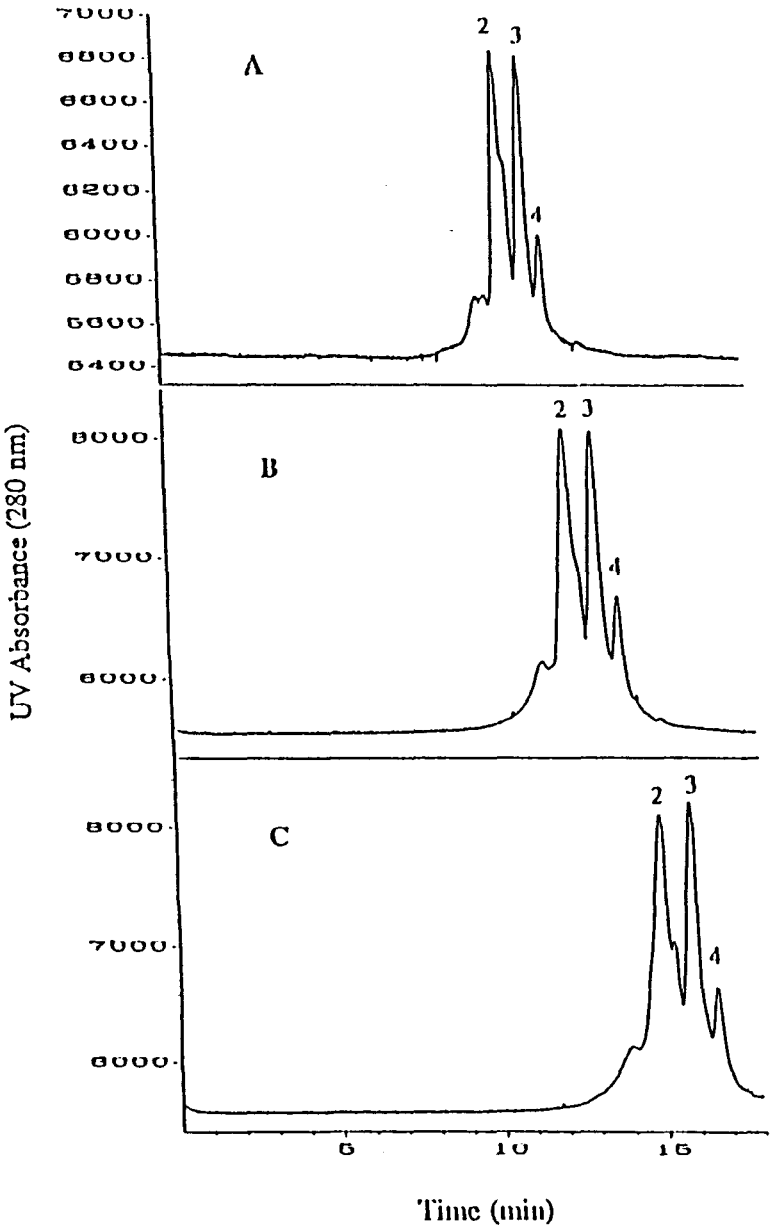


Fig. 1

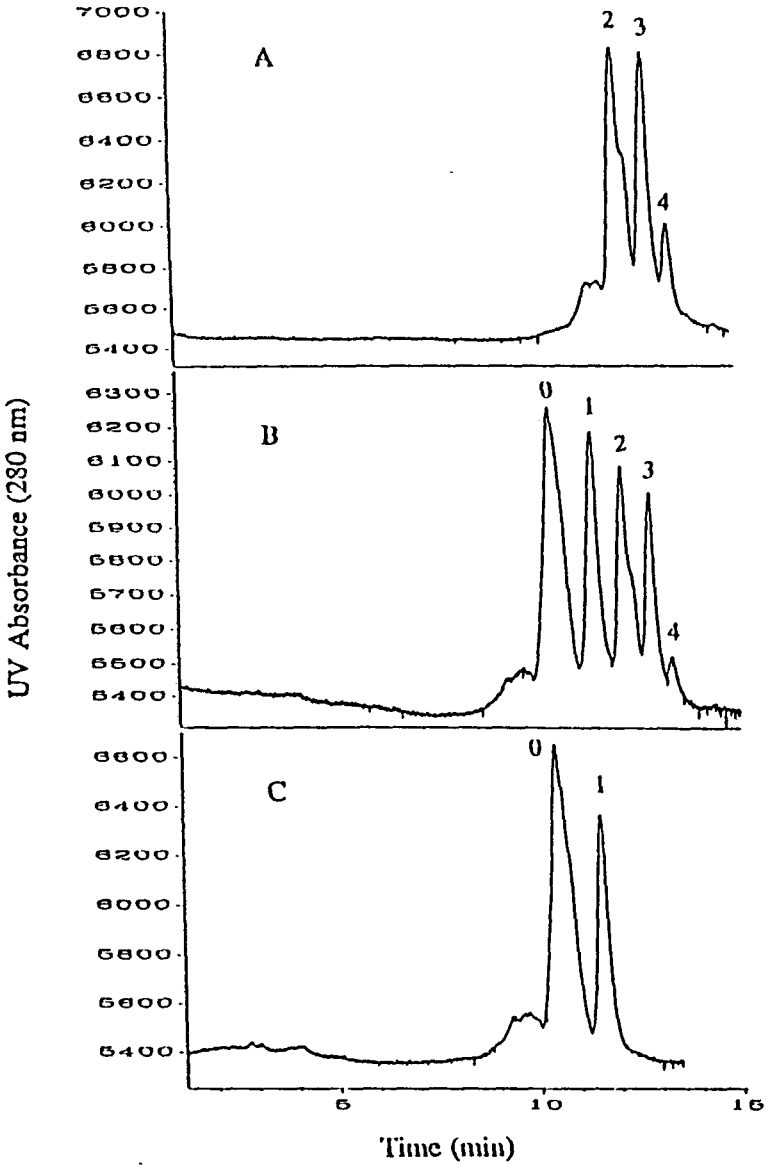


Fig. 2

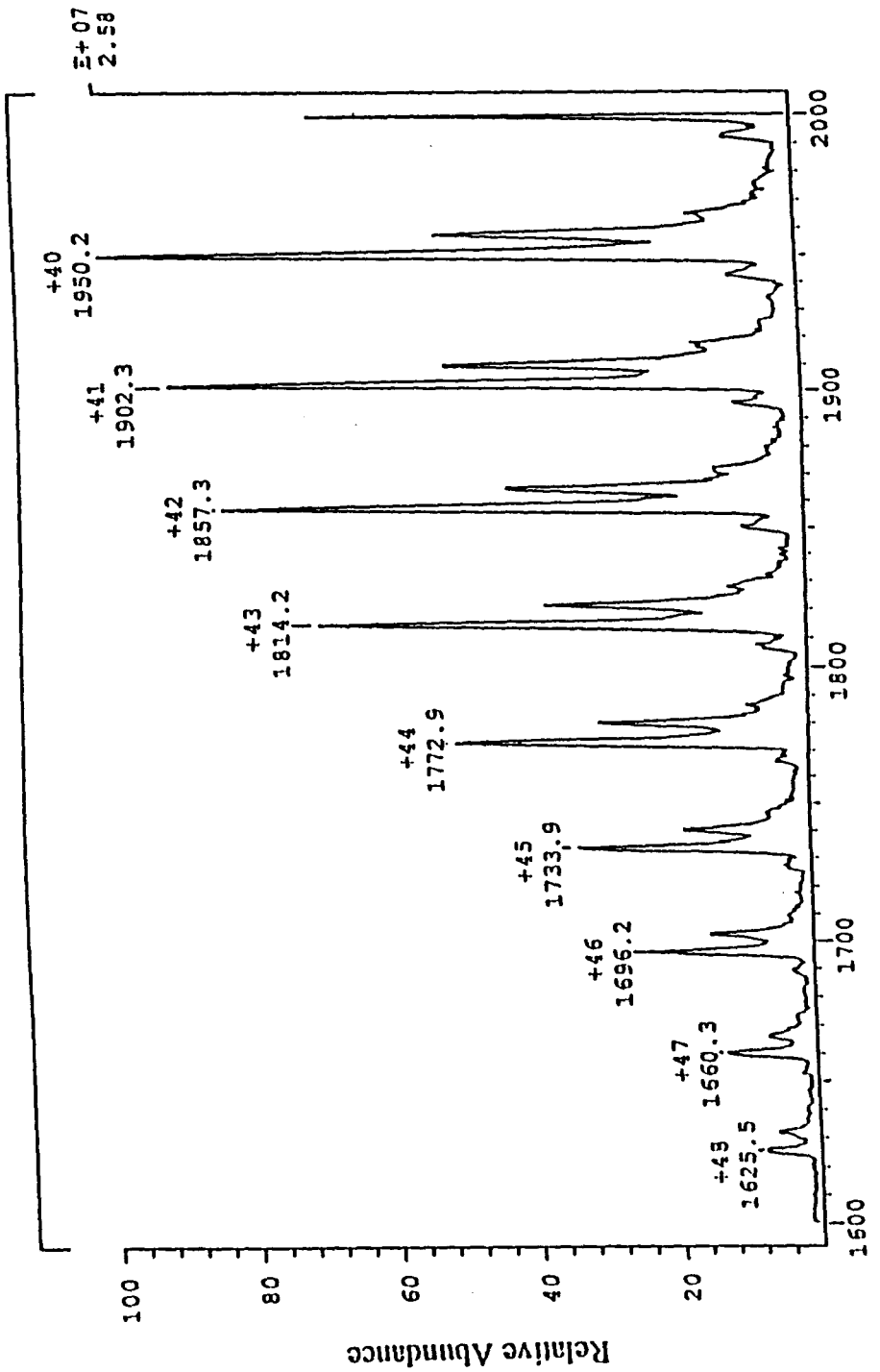
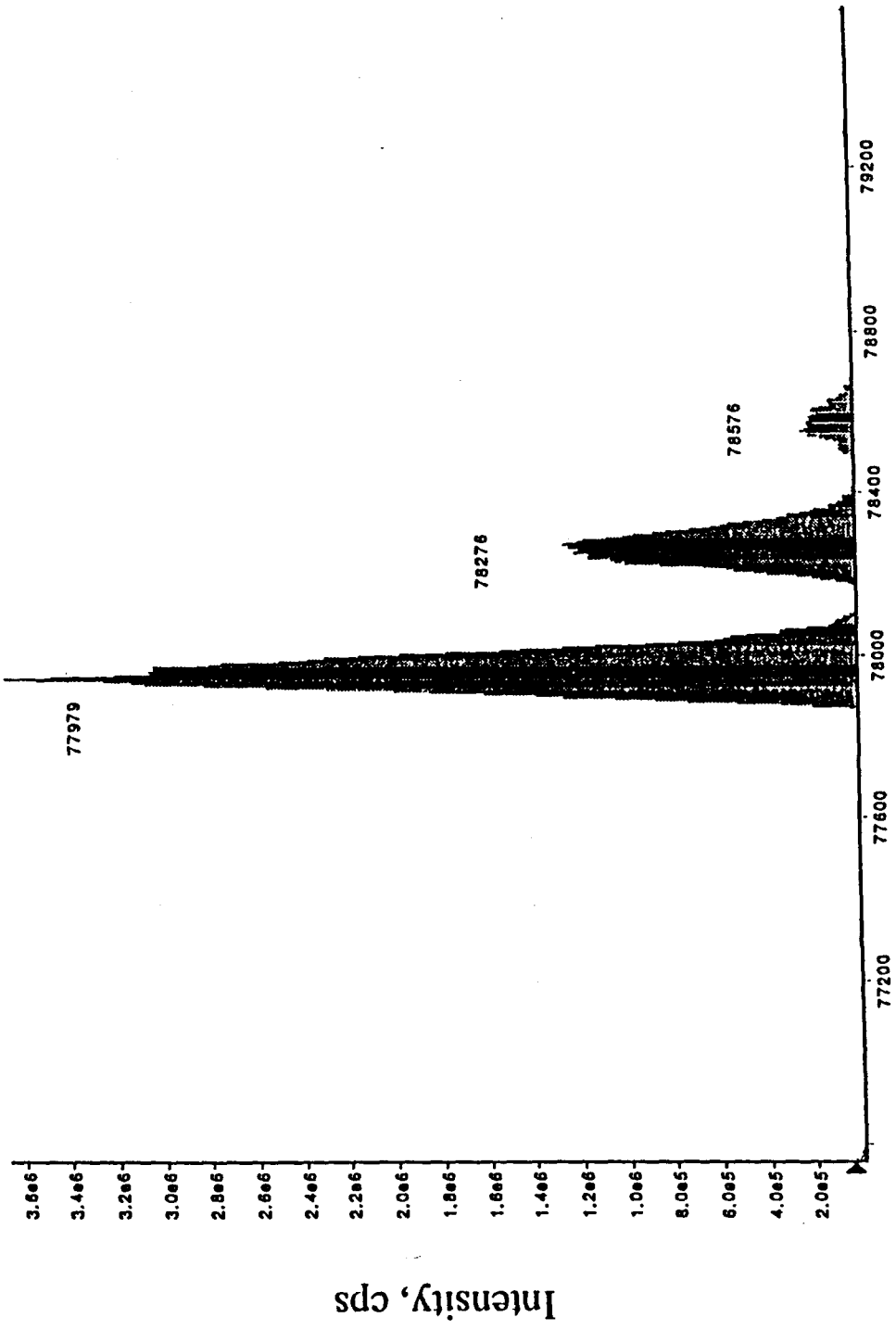

Relative Abundance
100
80
60
40
20
1600
1700
1800
1900
2000
m/z
+48
1625.5
+47
1660.3
+46
1696.2
+45
1733.9
+44
1772.9
+43
1814.2
+42
1857.3
+41
1902.3
+40
1950.2
E+07
2.58

Fig. 3 A


Intensity, cps
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3.4e6
3.2e6
3.0e6
2.8e6
2.6e6
2.4e6
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2.0e6
1.8e6
1.6e6
1.4e6
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
77200
77600
78000
78400
78800
79200
77979
78276
78576
Mass, amu

Fig. 3 B

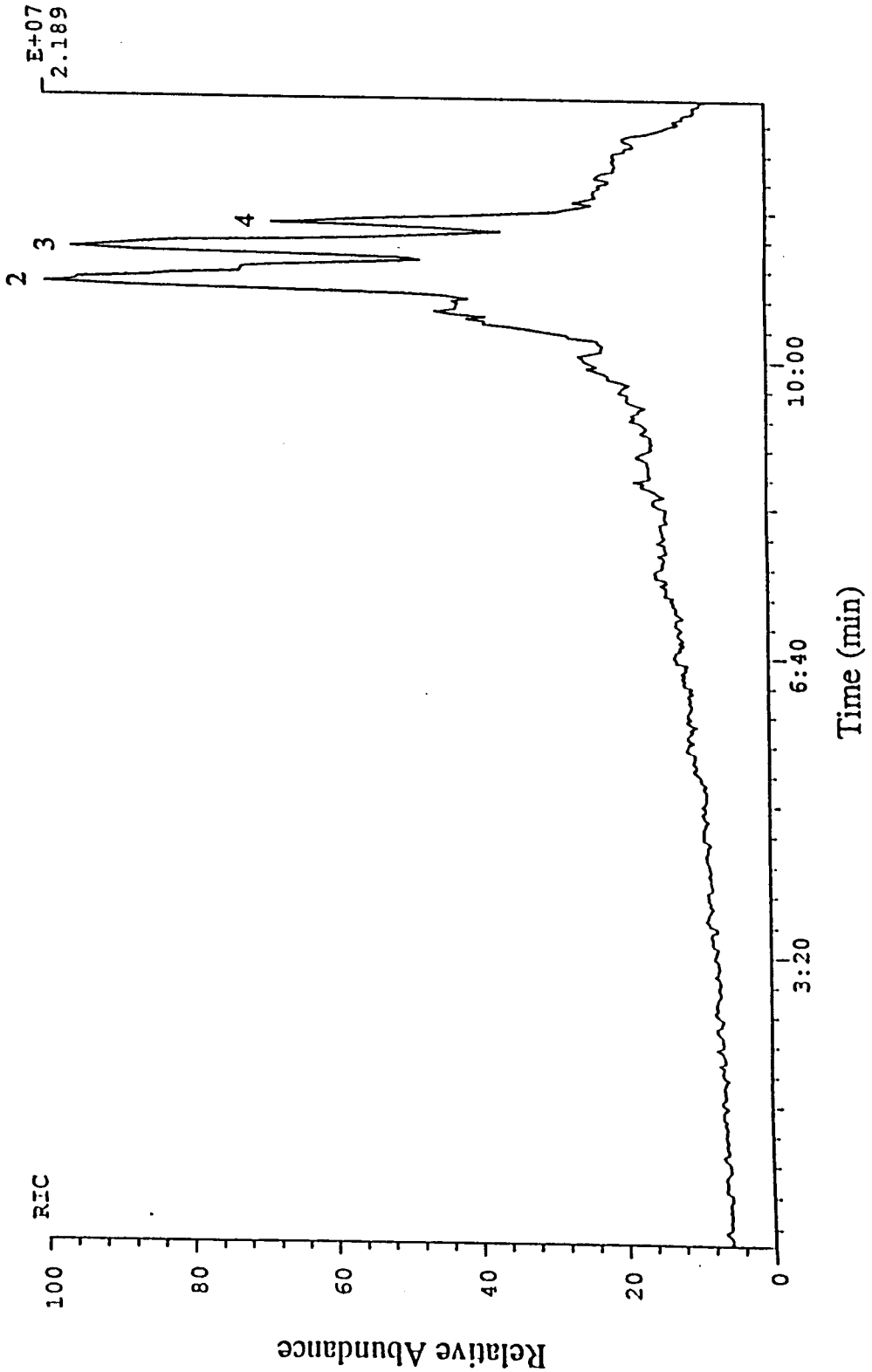
Relative Abundance
Time (min)
RIC
E+07
2.189
100
80
60
40
20
0
3:20
6:40
10:00
2
3
4

Fig. 4

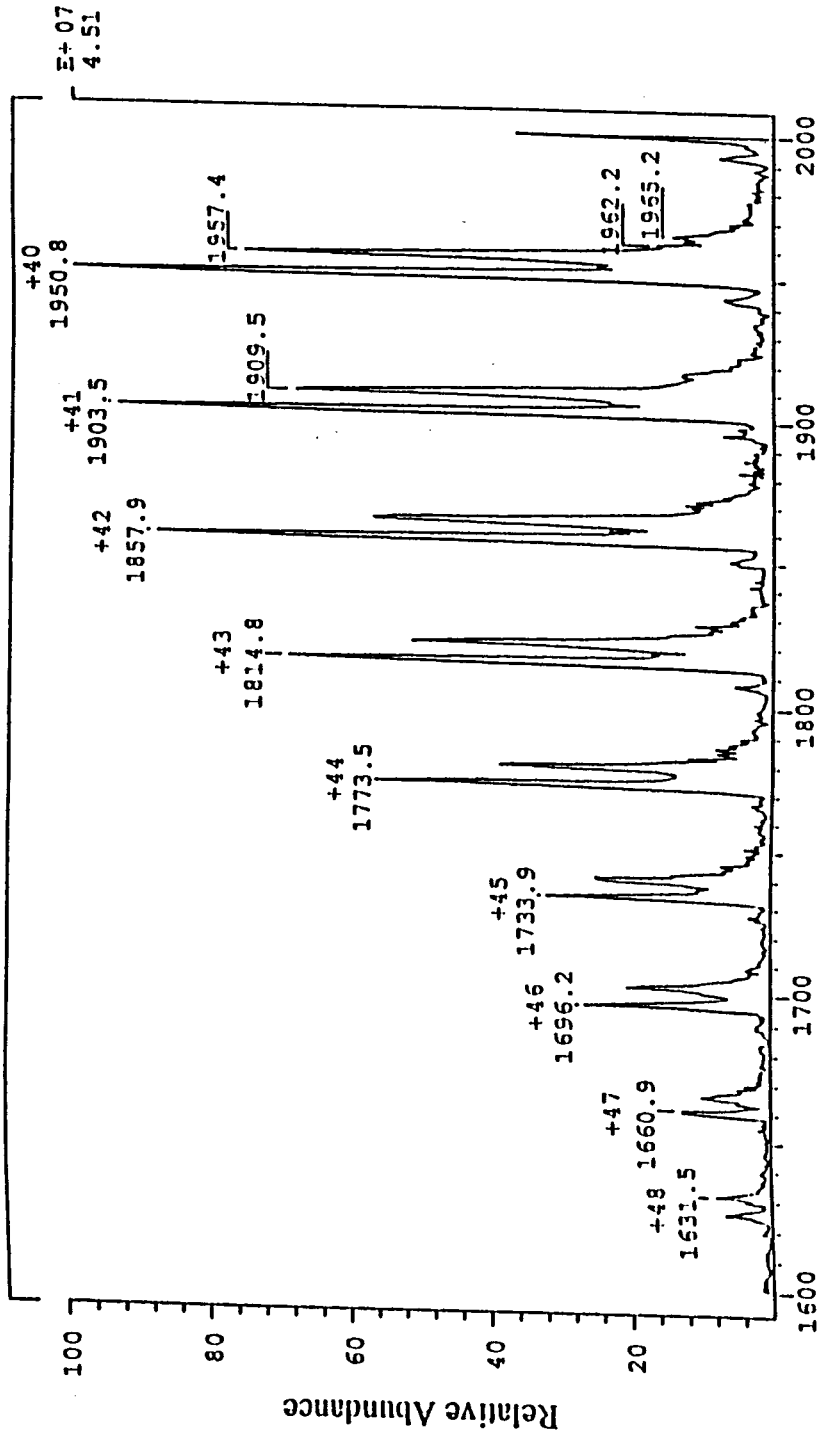
E+07
4.51
+40
1950.8
1957.4
962.2
1955.2
+41
1903.5
1909.5
+42
1857.9
+43
1814.8
+44
1773.5
+45
1733.9
+46
1696.2
+47
1660.9
+48
1631.5
Relative Abundance
m/z
100
80
60
40
20
1600
1700
1800
1900
2000

Fig. 5 A


Intensity, cps
6.5e6
6.0e6
5.5e6
5.0e6
4.5e6
4.0e6
3.5e6
3.0e6
2.5e6
2.0e6
1.5e6
1.0e6
5.0e5
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78000
78400
78800
79200
Mass, amu

Fig. 5 B

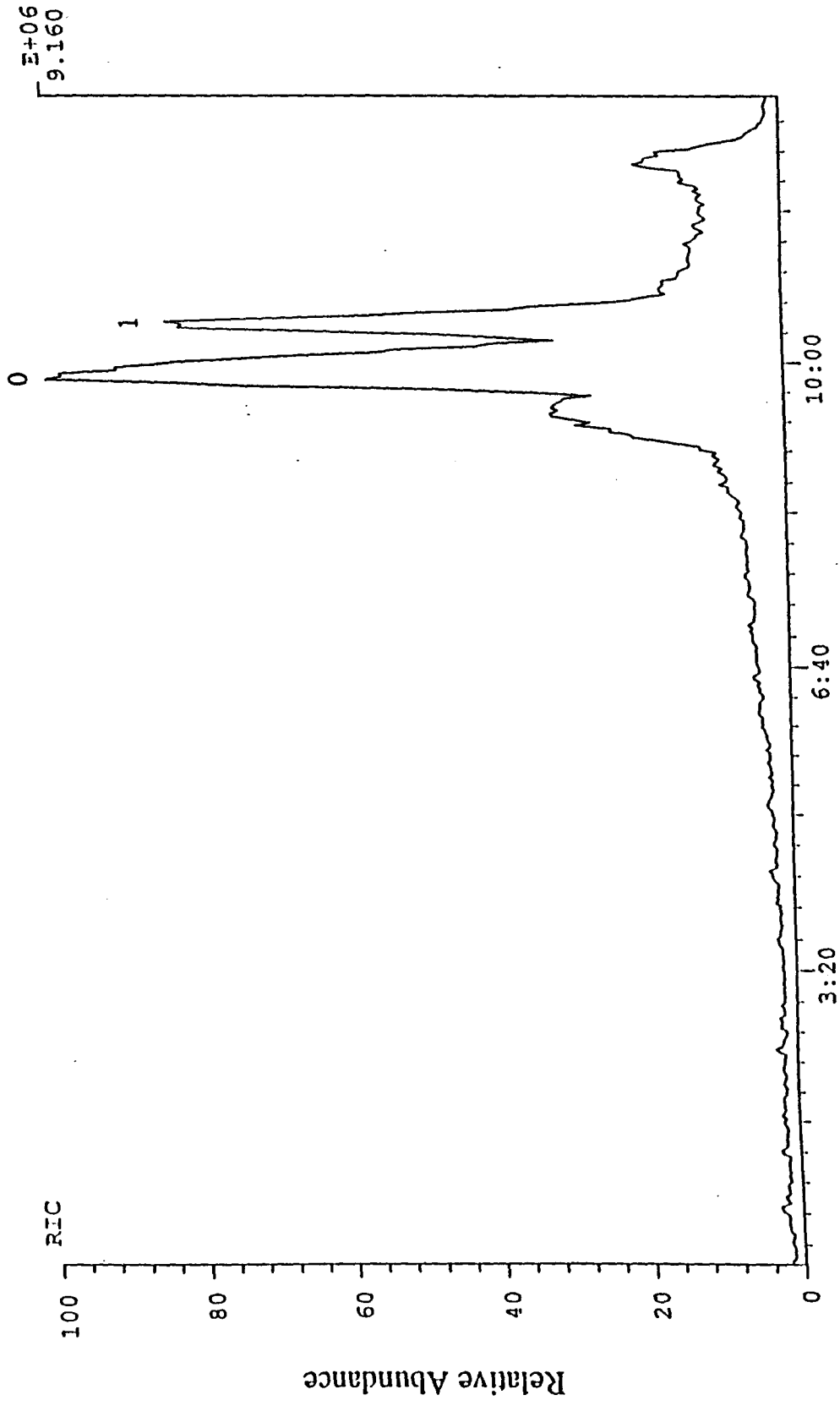

RIC
E+06
9.160
100
80
60
40
20
0
0
1
3:20
6:40
10:00
Relative Abundance
Time (min)

Fig. 6

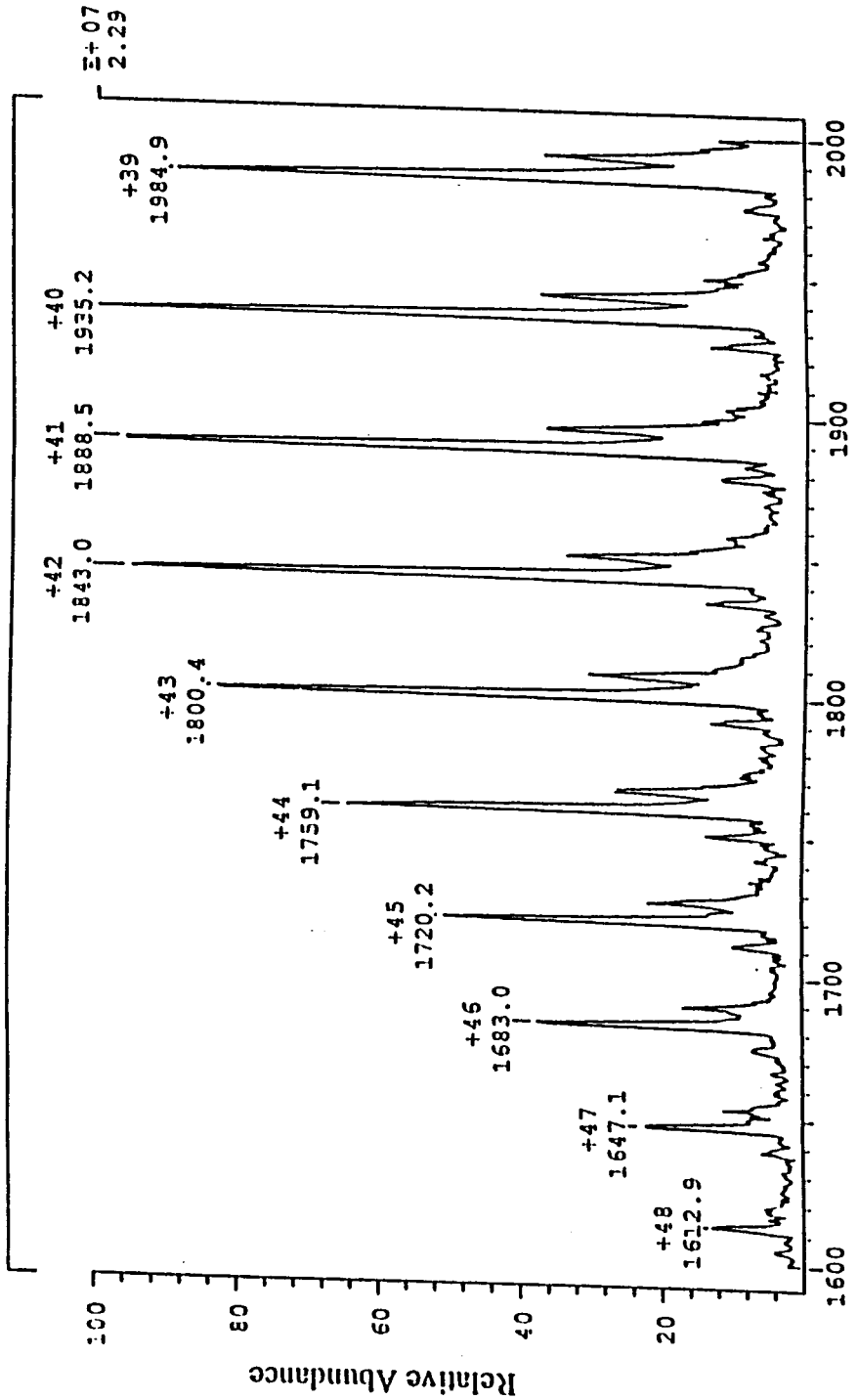
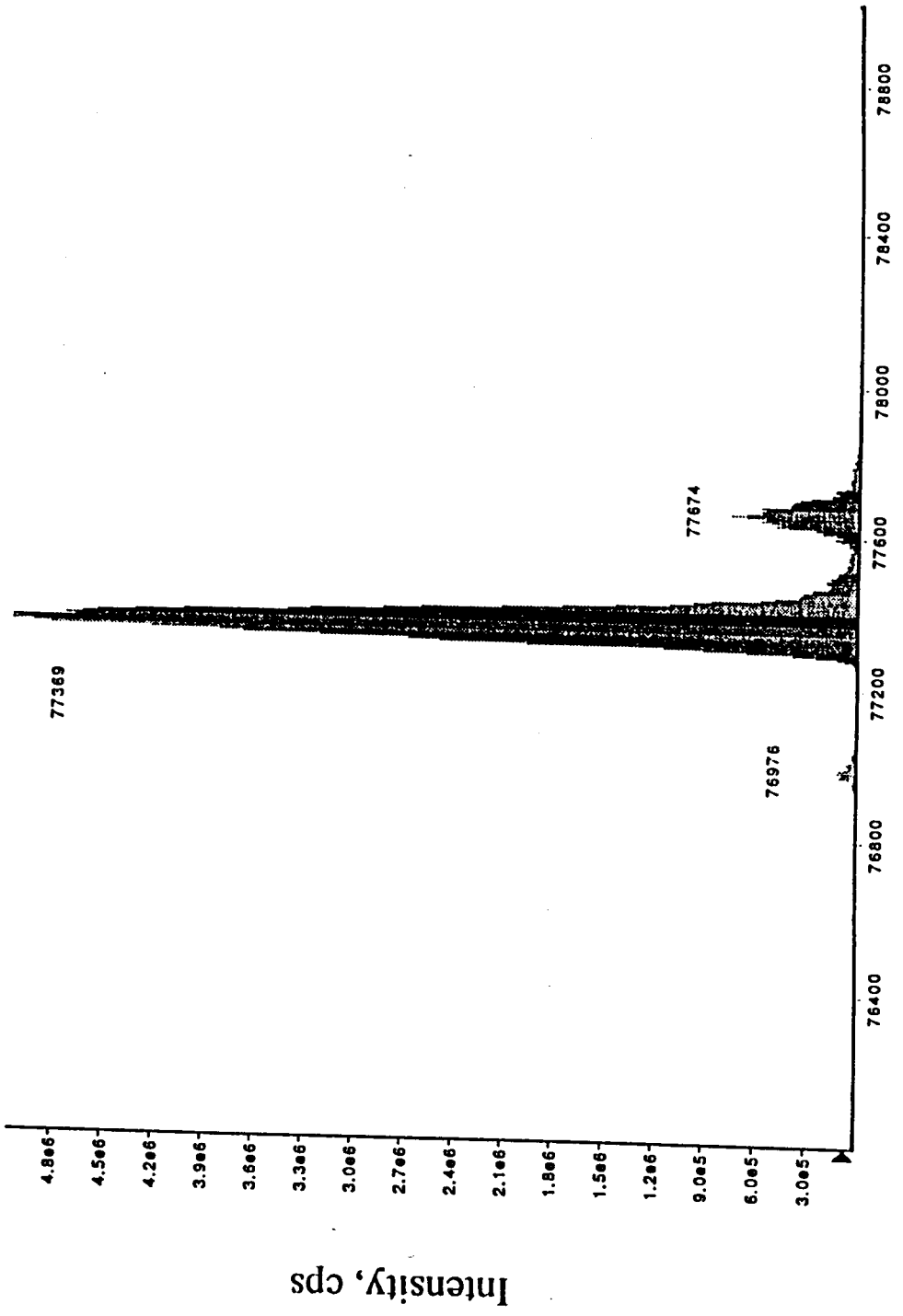

Relative Abundance
m/z
100
80
60
40
20
1600
1700
1800
1900
2000
+48
1612.9
+47
1647.1
+46
1683.0
+45
1720.2
+44
1759.1
+43
1800.4
+42
1843.0
+41
1888.5
+40
1935.2
+39
1984.9
Ξ÷07
2.29

Fig. 7 A


Intensity, cps
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4.5e6
4.2e6
3.9e6
3.6e6
3.3e6
3.0e6
2.7e6
2.4e6
2.1e6
1.8e6
1.5e6
1.2e6
9.0e5
6.0e5
3.0e5
77369
76976
77674
76400
76800
77200
77600
78000
78400
78800
Mass, amu

Fig. 7 B

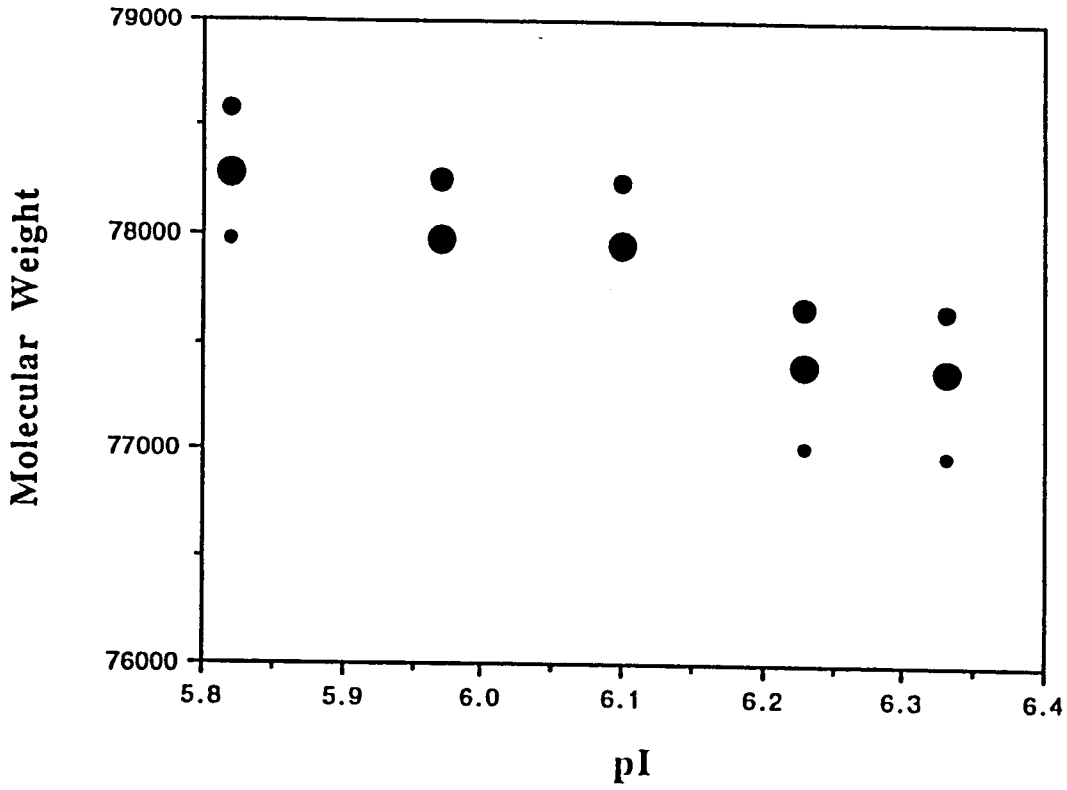
79000
78000
77000
76000
Molecular Weight
5.8
5.9
6.0
6.1
6.2
6.3
6.4
pI

Fig. 8

ON-LINE MICELLAR ELECTROKINETIC CHROMATOGRAPHY- ELECTROSPRAY IONIZATION MASS SPECTROMETRY USING ANODICALLY MIGRATING MICELLES

a paper submitted to Anal. Chem.

Liyu Yang, A. Kamel Harrata, and Cheng S. Lee

ABSTRACT

On-line micellar electrokinetic chromatography (MEKC)-electrospray ionization mass spectrometry (ESIMS) is demonstrated for the analysis of chlorotriazine herbicides and barbiturates. In this study, the micellar velocity is directly manipulated by the adjustment of electroosmosis rather than the electrophoretic velocity of the micelle. The elimination of MEKC surfactant introduction into ESIMS is achieved with an anodically migrating micelle, moving away from the electrospray interface. The effects of moving surfactant boundary in the MEKC capillary on separation efficiency and resolution of triazine herbicides and barbiturates are investigated. The mass detection of herbicides and barbiturates sequentially eluted from the MEKC capillary is acquired using the positive and negative electrospray modes, respectively.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC) was first introduced by Terabe et al.[1,2] for the separation of neutral and charged analytes in capillary electrophoresis (CE). In MEKC, surfactants are added to the electrophoresis buffer to form micelles at a concentration

above the critical micelle concentration (CMC). The separation is based on the differential partitioning of neutral and charged compounds between the micellar phase and the surrounding aqueous solution. Since the micellar phase is similar to a chromatographic stationary phase, MEKC is considered as an interface between electrophoretic separations and liquid chromatography[3].

On-line coupling of CE with electrospray ionization mass spectrometry (ESIMS) is very attractive for the direct identification of analytes and for the possibility of selectivity enhancement, and for the structure confirmation of analyte molecules in a MS-MS mode. Various interface designs for the integration of CE with ESIMS have been developed, including the use of metallized capillary terminus, sheath (coaxial) interface, liquid junction, and electrical contact through use of a gold wire[4-9]. The comparison between sheath interface and liquid junction was made by Pleasance et al.[10] and the coaxial sheath flow appeared to have several advantages.

However, the applications of CE-ESIMS for various biological and biomedical studies are limited by the use of nonvolatile buffer components and organic additives for achieving a wide variety of CE separations. For example, surfactant additives including cetyltrimethylammonium chloride (CTAC) and sodium dodecyl sulfate (SDS) were employed in CE for resolving closely related tripeptides and tamoxifen metabolites, respectively[11,12]. Although the concentrations of added CTAC and SDS were below their CMCs, the ESIMS signal responses for tripeptides and tamoxifens were significantly reduced.

The possibility of on-line integration of MEKC with ESIMS was suggested because both positive and negative ESI mass spectra of SDS were observed by Smith et al.[5] In

practice, the direct coupling of MEKC to ESIMS is hazardous due to the effect of nonvolatile MEKC surfactants on ESIMS performance. The continuous introduction of nonvolatile surfactants into ESIMS at a relatively high concentration results in a significant loss of electrospray efficiency and ion source contamination.

The need for coupling MEKC with ESIMS has led to several new approaches[13-18]. Ozaki et al.[13] demonstrated the use of high molecular weight surfactants, butyl acrylate-butyl methacrylate-methacrylic acid copolymer sodium salt, to avoid the production of high levels of low-mass background ions in the mass spectrum and the potential interference with the mass detection of small analytes. An electrospray-chemical ionization interface was introduced by Takada et al.[14] for the analysis of organic amines in MEKC. Their results indicated that the chemical ionization process was not greatly affected by the presence of phosphate salts and SDS surfactants in the electrophoresis buffers.

Additionally, the combination of MEKC with ESIMS has been achieved by eliminating surfactant introduction into ESIMS[15-18]. A coupled-capillary setup with the possibilities of voltage switching and buffer renewal was developed by Lamoree et al.[15], allowing on-line heart-cutting of analyte zones in the MEKC capillary with subsequent transfer via a second capillary zone electrophoresis (CZE) capillary to the mass spectrometer. Foley and Masucci[16] utilized a semipermeable membrane that selectively permeated small analytes to the mass spectrometer while retaining the relatively larger surfactants. Nelson et al.[17,18] studied the separation mechanism of partial filling-MEKC and successfully combined partial filling MEKC with ESIMS. Partial filling MEKC involved filling a small portion of the capillary with a micellar solution to achieve the separation. The analytes first migrated into

the micellar plug where the separation occurred, and then into the electrophoresis buffer, which was free of surfactants. The analytes in the electrophoresis buffer sequentially eluted out of the MEKC capillary and were subsequently introduced into ESIMS, while the surfactant plug remained behind in the capillary.

As shown in Fig. 1, the micellar velocity in MEKC is determined by the sum of its electrophoretic velocity and the opposing electroosmotic flow. Thus, the micellar velocity can be directly manipulated by the adjustment of electroosmosis rather than the electrophoretic velocity of the micelle. Reported efforts for affecting the electroosmotic flow included the use of surface active agents, buffer pH, buffer composition, temperature, chemical derivatization of the surface, and a radial electric potential gradient across the capillary[19-23]. In this study, the electroosmotic flow is adjusted against the electrophoretic velocity of the SDS micelle by changing the solution pH in MEKC. The elimination of SDS surfactant introduction into ESIMS is achieved with a slightly negative micellar velocity, moving away from ESIMS. Successful demonstration of on-line coupling of MEKC to ESIMS is presented using triazine herbicides and barbiturates as two model systems. Positive and negative ESI are employed for the mass analysis of herbicides and barbiturates, respectively.

EXPERIMENTAL SECTION

Micellar Electrokinetic Chromatography: UV measurement

MEKC separations were carried out using fused silica capillary from Polymicro Technologies (Phoenix, AZ) with capillary dimensions of 50 μm i.d. and 192 μm o.d. UV absorbance wavelength was set at 226 nm using a linear UVIS 200 detector (Linear

Instruments, Reno, NV). A CZE 1000R high-voltage (HV) power supply (Spellman High-Voltage Electronics, Plainview, NY) was employed to deliver a negative electric potential to the detector end of the capillary for electrokinetic injection and for electrophoretic separation. Data collection was performed by a HP 35900D analog-to-digital interface board with the HP G1250C General Purpose Chemstation Software (Hewlett Packard, Fullerton, CA).

SDS of protein research grade was purchased from Boehringer Mannheim (Mannheim, Germany) and used as received. The pH of the electrophoresis buffer containing ammonium acetate and SDS was adjusted using an ammonium hydroxide solution or an acetic acid solution all obtained from Fisher Scientific (Pittsburgh, PA). Dimethyl sulfoxide and quinine hydrochloride (Aldrich, Milwaukee, WI) were used as the electroosmotic flow marker and the micellar marker, respectively. Atrazine, desethylatrazine, desisopropylatrazine, and simazine were purchased from ChemService (West Chester, PA). The herbicide stock solutions of each individual triazine, other than simazine, in a concentration of 5×10^{-3} M were prepared by dissolving in methanol (Fisher Scientific). Simazine was dissolved with a greater solubility in acetone than in methanol. A standard triazine herbicide solution with an individual concentration of 10^{-4} M was then obtained by adding 40 µl of each stock solution in 20 mM ammonium acetate buffer with a final volume of 2 ml.

Barbiturates including amobarbital, barbital, butalbital, pentobarbital, and secobarbital were obtained from Sigma (St. Louis, MO) and were dissolved in methanol at the concentration of 1 mg/ml. Methanol was evaporated using dry nitrogen gas and a standard barbiturate solution with an individual concentration of 10^{-3} M was prepared in 20 mM ammonium acetate buffer.

Direct Infusion and Micellar Electrokinetic Chromatography-Electrospray Ionization Mass Spectrometry

The mass spectrometer used in this study was a Finnigan MAT TSQ 700 (San Jose, CA) triple quadrupole equipped with an electrospray ionization source. The Finnigan MAT electrospray adapter kit containing both gas and liquid sheath tubes was used to perform the direct infusion experiment and also to couple MEKC with ESIMS without any modification. The first quadrupole was used for the mass scanning or selected ion monitoring (SIM), while the second and third quadrupoles were operated in the radio frequency only mode.

An acetic acid solution (methanol/water/acetic acid, 50:49:1 v/v/v) containing 10^{-5} M each of triazine herbicides was directly infused at 5 μl/min using a Harvard Apparatus 22 syringe pump (South Natick, MA). By applying a 5 kV electric potential at the electrospray needle, the first quadrupole was scanned from m/z 50 to 300 with a scan rate of 1.2 sec/scan for the detection of herbicide ions. In contrast, the negative electrospray mode was employed by applying a -4.5 kV electric potential at the electrospray capillary for the mass detection of barbiturates with an individual concentration of 10^{-4} M in an ammonium hydroxide solution containing 50% methanol, 49.75% water, and 0.25% ammonium hydroxide (v/v/v). Tuning and calibration of the mass spectrometer were established using an acetic acid solution containing triazine herbicides or an ammonium hydroxide solution of barbiturates.

For the integration of MEKC with ESIMS, a fused silica capillary with 50 μm i.d. and 192 μm o.d. was mounted within the electrospray probe. The capillary tip was fixed about 0.5 mm outside the electrospray needle. The inlet reservoir containing SDS and ammonium acetate buffer was maintained at the same height as the electrospray needle. The sheath liquid

consisted of either an acetic acid solution (methanol/water/acetic acid, 50:49:1 v/v/v) or an ammonium hydroxide solution (50% methanol, 49.75% water, and 0.25% ammonium hydroxide) for the MEKC-ESIMS analysis of triazine herbicides or barbiturates, respectively. The sheath liquid was delivered at a flow rate of 5 μl/min using a Harvard Apparatus 22 syringe pump.

During the MEKC-ESIMS analysis of triazine herbicides, two HV power supplies (Spellman) were used for delivering the electric potentials of 12 kV and 5 kV at the inlet electrode and the electrospray needle, respectively. Because most HV power supplies are not designed to operate as current sinks, a resistor ladder, parallel with the HV electrode connecting with the electrospray needle, was incorporated. In contrast, one HV power supply was used to apply a -4.5 kV electric potential at the electrospray needle during the MEKC-ESIMS analysis of barbiturates, while the inlet reservoir was grounded. The first quadrupole was used in the SIM mode for the mass detection of herbicide and barbiturate ions sequentially eluted from the MEKC capillary. No sheath gas was employed during the MEKC-ESIMS measurement.

RESULTS AND DISCUSSION

Micellar Electrokinetic Chromatography-Electrospray Ionization Mass Spectrometry of Triazine Herbicides

Micellar electrokinetic chromatography: UV measurement. Chlorotriazine herbicides with their corresponding structures and pK_a values are summarized in Table 1. For MEKC-UV separation of chlorotriazines, a fused silica capillary was filled with electrophoresis buffer containing 10 mM SDS and 10 mM ammonium acetate buffer at pH

7.0. The standard herbicide solution with the addition of 0.05% dimethyl sulfoxide and 10^{-4} M quinine hydrochloride was electrokinetically injected at the anodic end of the capillary by applying -10.6 kV for 1 sec to the cathodic end (the UV detector end). The same -10.6 kV was applied for maintaining a constant electric field of 280 V/cm during the electrokinetic separation of triazine herbicides shown in Fig. 2. The elution order and capacity factor of triazine herbicides are summarized in Table 2, with the more hydrophobic herbicides eluting latter.

The electroosmotic and micellar electrophoretic mobilities measured from the markers of dimethyl sulfoxide and quinine hydrochloride were 6.12×10^{-4} cm^2/V-sec and -4.64×10^{-4} cm^2/V-sec, respectively. The mobility was assigned as positive when the direction of migration was toward the cathodic end. It has been reported that the electrophoretic mobility of SDS micelle remained constant between pH 5 and 9[28]. This observation seems reasonable because the amount of charge on the micelle should not change in these pH regions. However, the electroosmotic flow was shown to be strongly dependent on the solution pH[20]. For example, an approximately twofold increase of electroosmotic flow has been reported by Lukacs and Jorgenson[20] for the fused silica capillary at pH from 6 to 7 in the phosphate buffer.

Successful demonstration of on-line coupling of MEKC to ESIMS requires the elimination of surfactant introduction into the mass spectrometer. In this study, the electroosmotic flow is adjusted against the electrophoretic velocity of the SDS micelle by reducing the solution pH in MEKC. The electroosmotic flow in the fused silica capillary was further reduced by pretreating the capillary with 1 N hydrochloric acid solution (instead of 1

N sodium hydroxide solution), followed by water and electrophoresis buffer. As shown in Fig. 3, MEKC separation of triazine herbicides was performed in the electrophoresis buffer of 10 mM SDS and 10 mM ammonium acetate at pH 5.9. The electroosmotic mobility measured from the migration of dimethyl sulfoxide was 3.64 x 10^{-4} cm^2/V-sec. Thus, the migration direction of SDS micelles was toward the anodic end and the migration mobility was given as -1.00 x10^{-4} cm^2/V-sec. Furthermore, the increase in the range of the migration-time window contributed to an enhancement in separation resolution of triazine herbicides in MEKC.

The increase in MEKC migration time of triazine herbicides at pH 5.9 was contributed by the reduction in the electroosmotic flow. The migration velocity of herbicide analytes at pH 5.9 was given as

$$v = [m_{eo} E/(1+k)] + [m_{mc} E k/(1+k)] \quad (1)$$

where k was the capacity factor (see Table 2), E was the electric field, and m_{eo} (3.64 x 10^{-4} cm^2/V-sec) and m_{mc} (-1.00 x 10^{-4} cm^2/V-sec) were the electroosmotic and the micellar migration mobilities, respectively. The migration time of herbicides at pH 5.9 was then estimated as

$$t_R = l/v \quad (2)$$

where l was the separation distance between the injection point and the UV detector. For example, the predicted migration time of 10.5 min for atrazine was in good agreement with 10.8 min observed in Fig. 3. However, the analytes with the capacity factor greater than 3.64 would either be stationary in the MEKC capillary (see eq. (1)) or migrate toward the anodic end away from the UV detector. Even though it is possible to raise the electrophoresis pH and the electroosmotic flow for increasing the range of the capacity factor to be analyzed, the ability to couple MEKC with ESIMS is obtained at the expense of analysis time.

Direct infusion of triazine herbicides in positive electrospray ionization. The pK_a value of chlorotriazines ranges between 1.58 and 1.85 (see Table 1). Thus, the mixture of triazine herbicides was electrosprayed in an acetic acid solution by applying a 5 kV electric potential at the electrospray needle. The positive electrospray ionization mass spectrum of 10^{-5} M each of desisopropylatrazine, desethylatrazine, simazine, and atrazine is shown in Fig. 4. The ^{37}Cl isotope ions of chlorotriazines were observed with the intensity ratios corresponding to the natural abundance of 32.5% of ^{37}Cl isotope. The strongest ionization intensity of atrazine could be in part contributed by its greatest pK_a value. Molecular ions with m/z ratios of 174, 188, 202, and 216 were chosen for the SIM mode of desisopropylatrazine, desethylatrazine, simazine, and atrazine in MEKC-ESIMS, respectively.

On-line coupling of micellar electrokinetic chromatography with electrospray ionization mass spectrometry. In the MEKC-ESIMS experiments, the MEKC separation conditions including capillary dimensions, effective separation distance, electrophoresis buffer, and electric field across the capillary were identical to those employed in MEKC-UV

studies at pH 5.9. To combine MEKC with ESIMS, a sheath liquid containing 50% methanol, 49% water, and 1% acetic acid was introduced to the electrospray probe at the cathodic end. On the basis of volatility, the ammonium acetate buffer was chosen over phosphate buffer. As shown in Fig. 5, the triazine herbicides including desisopropylatrazine, desethylatrazine, simazine, and atrazine were sequentially eluted and monitored by ESIMS. At pH 5.9, the SDS micelles migrated toward the inlet reservoir and avoided the introduction of nonvolatile surfactants into the mass spectrometer for the direct and on-line coupling of MEKC with ESIMS.

As shown in Fig. 5, an additional m/z 174 ion was observed together with the molecular ion of atrazine at m/z 216 in MEKC-ESIMS. Under the same electrospray conditions, an additional m/z 174 ion was also measured in the direct infusion of atrazine (data not shown). Furthermore, the MS/MS daughter ion mass spectrum of m/z 216 atrazine ion again revealed the presence of m/z 174 ion (data not shown). All the results seemed to indicate the loss of isopropyl group of atrazine under our electrospray conditions for the production of additional m/z 174 ion.

In comparison to MEKC-UV studies at pH 5.9 (see Fig. 3), the migration time of triazine herbicides in MEKC-ESIMS was slightly shorter under the same separation distance. For example, the migration times of atrazine in MEKC-UV and MEKC-ESIMS were 10.8 min and 9.30 min, respectively. The decrease in the migration time of triazine herbicides in MEKC-ESIMS was attributed to the migration of SDS micelles toward the inlet reservoir, the anodic end. In MEKC-ESIMS, the cathodic end of the electrospray probe contained a sheath liquid of acetic acid solution without any SDS surfactant. Thus, the continuous elution of

SDS micelles into the inlet reservoir resulted in a moving boundary of SDS surfactants in MEKC-ESIMS. Once the analytes migrated across the moving SDS boundary, the neutral analytes were transported by the electroosmotic flow to ESIMS at the end of the MEKC capillary. The extent of decrease in migration time of triazine herbicides contributed by the moving SDS boundary was greater for late eluting compounds than for early migrating analytes.

The mixing between the capillary eluent and the sheath liquid at the capillary tip might result in the decrease of separation efficiency and resolution of herbicides in MEKC-ESIMS. The presence of an additional band broadening phenomenon at the interface between the moving SDS phase and the electrophoresis buffer further contributed to generally lower separation efficiency of triazine herbicides. Briefly, the analyte band in the SDS phase migrated slower due to its partitioning with the micelle and the presence of a lower electric field. Thus, all analyte molecules that diffused past the micellar phase encountered a higher electric field in the electrophoresis buffer, and therefore, migration speed driven by electroosmosis increased. Further detail on band broadening of analyte solutes across the interface can be found in our recent work of partial filling MEKC[17].

Micellar Electrokinetic Chromatography-Electrospray Ionization Mass Spectrometry of Barbiturates

The barbiturates, as summarized in Table 3, are a class of compounds with a 6-membered heterocycle ring containing nitrogen. At or near neutral pH, the barbiturates possess no net charge. Thus, MEKC is the method of choice for the separation of barbiturates as demonstrated by Thormann et al.[29] As shown in Fig. 6, the barbiturate

analytes including barbital, butalbital, amobarbital, pentobarbital, and secobarbital were successfully resolved in MEKC at pH 5.9. Even though the SDS concentration was increased to 15 mM, the electroosmotic mobility and the electrophoretic mobility of SDS micelle remained relatively constant for the values of 3.69×10^{-4} $cm^2/V\text{-}sec$ and -4.56×10^{-4} $cm^2/V\text{-}sec$, respectively.

The oxygen attached at the 4 position of the 6-membered heterocycle of barbiturates carries a negative charge at basic pH values of 9-10[30]. Thus, the direct infusion of barbiturates in an ammonium hydroxide solution (50% methanol, 49.75% water, and 0.25% ammonium hydroxide) at pH 10.5 was carried out in the negative electrospray mode by applying a -4.5 kV electric potential at the electrospray needle. The negative electrospray ionization mass spectrum of 10^{-4} M each of barbital, butalbital, amobarbital, pentobarbital, and secobarbital is shown in Fig. 7. The m/z 225 ion was contributed by both amobarbital and pentobarbital. Molecular ions with m/z ratios of 183, 223, 225, and 237 were chosen for the SIM mode of barbital, butalbital, amobarbital and pentobarbital, and secobarbital in MEKC-ESIMS, respectively.

In the MEKC-ESIMS experiments, the MEKC separation conditions including capillary dimensions, effective separation distance, electrophoresis buffer, and electric field across the capillary were identical to those employed in MEKC-UV studies at pH 5.9. To combine MEKC with ESIMS, a sheath liquid containing 50% methanol, 49.75% water, and 0.25% ammonium hydroxide at pH 10.5 was introduced to the electrospray probe at the cathodic end. As shown in Fig. 8, the barbiturate analytes including barbital, butalbital, amobarbital, pentobarbital, and secobarbital were sequentially eluted and detected by the SIM

mode of ESIMS. The identification of amobarbital and pentobarbital with the identical m/z ion of 225, however, was established by their elution order in MEKC separation.

In comparison to MEKC-UV studies at pH 5.9 (see Fig. 6), the migration time of barbiturates in MEKC-ESIMS was again slightly shorter under the same separation distance. The decrease in separation efficiency and migration time of barbiturates in MEKC-ESIMS was also attributed to the anodic migration of SDS micelles in the MEKC capillary. To reduce or eliminate the moving SDS boundary, it is possible to raise the electrophoresis pH and the electroosmotic flow against the negative electrophoresis of SDS micelles. In this study, the electroosmotic mobility was adjusted to be slightly smaller than the absolute value of micellar electrophoretic mobility. The resulted migration of SDS micelles toward the anodic end not only ensured the absence of SDS surfactant in ESIMS, but also illustrated the negative effects of moving SDS boundary on the separation performance of MEKC-ESIMS.

As demonstrated in this study, the application of either stationary or moving SDS micellar phase (toward the anodic end) provides an alternative approach for the direct and on-line coupling of MEKC separation with ESIMS. There is no need for any instrument modification. Due to the absence of surfactant in ESIMS, long-term and stable operation of MEKC-ESIMS is ensured. Future applications of MEKC-ESIMS involve the structure analysis of analyte molecules in a MS-MS mode. The utilization of MS-MS would be particularly important for the determination of unknown analytes.

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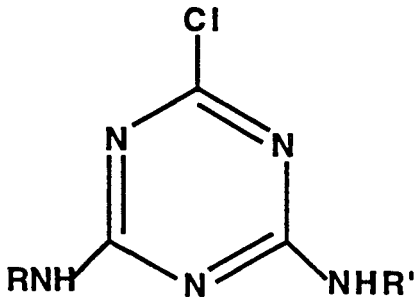
TABLE 1

Triazine Herbicides with Corresponding Structures, pK_a Values, and Molecular Weights[a]



Triazines	R	R'	pK_a	MW
Atrazine	Ethyl	Isopropyl	1.68-1.85	216
Desethylatrazine	H	Isopropyl	1.65	188
Desisopropylatrazine	Ethyl	H	1.58	174
Simazine	Ethyl	Ethyl	1.65-1.80	202

a) Taken from references (24-27).

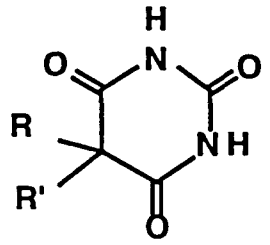
TABLE 2

Elution Order and Capacity Factor for Chlorotriazine Herbicides

Analyte Peak	Elution Order	Capacity Factor
Dimethyl sulfoxide	1	
Desisopropylatrazine	2	0.09
Desethylatrazine	3	0.15
Simazine	4	0.45
Atrazine	5	0.92
Quinine hydrochloride	6	

TABLE 3

Barbiturates with Corresponding Structures and Molecular Weights[a]



Barbiturates	R	R'	MW
Amobarbital	$CH_2CH_2CH(CH_3)_2$	CH_2CH_3	226
Barbital	CH_2CH_3	CH_2CH_3	184
Butalbital	$CH_2CH(CH_3)_2$	$CH_2CH{=}CH_2$	224
Pentobarbital	$CH(CH_3)CH_2CH_2CH_3$	CH_2CH_3	226
Secobarbital	$CH(CH_3)CH_2CH_2CH_3$	$CH_2CH{=}CH_2$	238

a) Taken from references (29).

FIGURE LEGENDS

Fig. 1 Schematic diagram of various velocity components in MEKC. v_{eo}, electroosmotic flow; $v_{ep,mc}$, micellar electrophoretic velocity; v_{mc}, micellar migration velocity as the sum of micellar electrophoretic velocity and the opposing electroosmotic flow.

Fig. 2 MEKC separation of 10^{-4} M each of (2) desisopropylatrazine, (3) desethylatrazine, (4) simazine, and (5) atrazine. Dimethyl sulfoxide (1) and quinine hydrochloride (6) were used as the electroosmotic flow marker and the micelle marker, respectively. Buffer: 10 mM SDS/10 mM ammonium acetate, pH 7.0; capillary, 38-cm total length, 50 μm i.d. and 192 μm o.d., length to detector 25 cm; voltage, -10.6 kV and 1 sec for injection, -10.6 kV for electrophoresis; UV detection at 226 nm.

Fig. 3 MEKC separation of 10^{-4} M each of (2) desisopropylatrazine, (3) desethylatrazine, (4) simazine, and (5) atrazine. Dimethyl sulfoxide (1) was used as the electroosmotic flow marker. The electrophoresis buffer contained 10 mM SDS and 10 mM ammonium acetate at pH 5.9. Other conditions were the same as in Fig. 2.

Fig. 4 Positive ion mode electrospray mass spectrum of chlorotriazine herbicides. The herbicide mixture with an individual concentration of 10^{-5} M was infused at 5 μl/min in an acetic acid solution (methanol/water/acetic acid, 50:49:1 v/v/v) at pH 2.6. The first quadrupole was scanned from m/z 50 to 300 with a scan rate of 1.2 sec/scan.

Fig. 5 Reconstructed ion electropherogram of chlorotriazine herbicides in MEKC-ESIMS using positive ion mode. Capillary: 25-cm total length; sheath liquid, methanol/water/acetic acid (50:49:1 v/v/v) at pH 2.6, 5 μl/min; voltage, 12 kV at the inlet reservoir and 5 kV at the electrospray needle; selected ion monitoring at m/z

174 (desisopropylatrazine), 188 (desethylatrazine), 202 (simazine), and 216 (atrazine). Other conditions were the same as in Fig. 3.

Fig. 6 MEKC separation of 10^{-3} M each of (2) barbital, (3) butalbital, (4) amobarbital, (5) pentobarbital, and (6) secobarbital. Dimethyl sulfoxide (1) was used as the electroosmotic flow marker. Buffer: 15 mM SDS/10 mM ammonium acetate, pH 5.9; capillary, 33-cm total length, 50 μm i.d. and 192 μm o.d., length to detector 20 cm; voltage, -7.4 kV and 1 sec for injection, -7.4 kV for electrophoresis; UV detection at 226 nm.

Fig. 7 Negative ion mode electrospray mass spectrum of barbiturates. The barbiturate mixture with an individual concentration of 10^{-4} M was infused at 5 μl/min in an ammonium hydroxide solution (methanol/water/ammonium hydroxide, 50:49.75:0.25 v/v/v) at pH 10.5. The first quadrupole was scanned from m/z 50 to 300 with a scan rate of 1.2 sec/scan.

Fig. 8 Reconstructed ion electropherogram of barbiturates in MEKC-ESIMS using negative ion mode. Capillary: 20-cm total length; sheath liquid, methanol/water/ammonium hydroxide (50:49.75:0.25 v/v/v) at pH 10.5, 5 μl/min; voltage, -4.5 kV at the electrospray needle and grounding at the inlet reservoir; selected ion monitoring at m/z 183 (barbital), 223 (butalbital), 225 (amobarbital and pentobarbital), and 237 (secobarbital). Other conditions were the same as in Fig. 6.

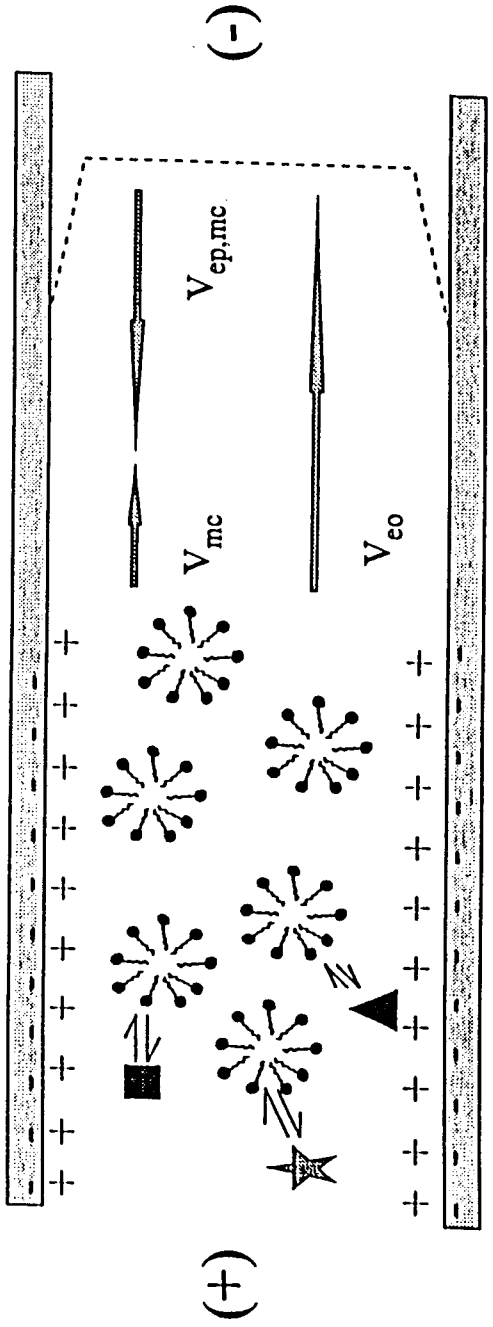
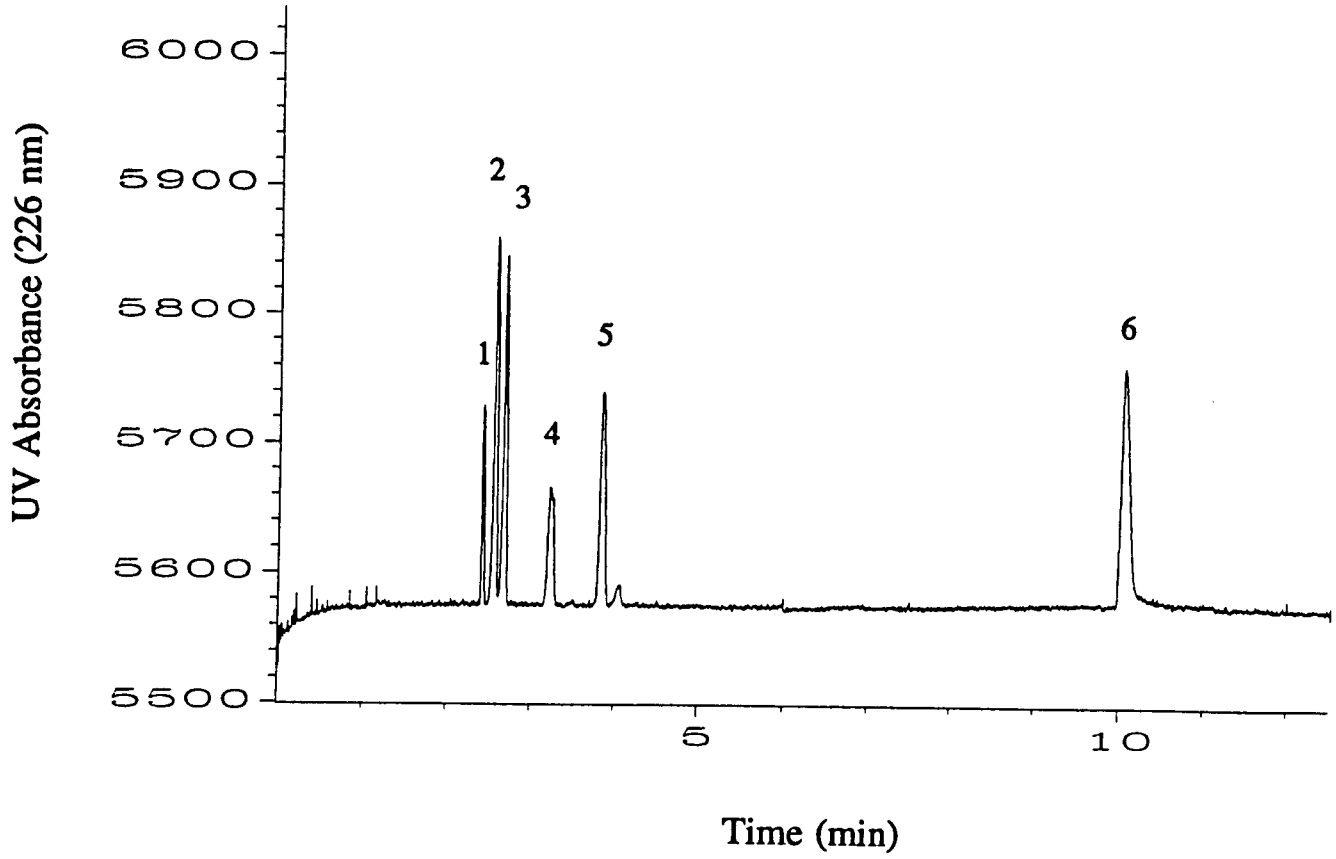


Fig. 1


UV Absorbance (226 nm)
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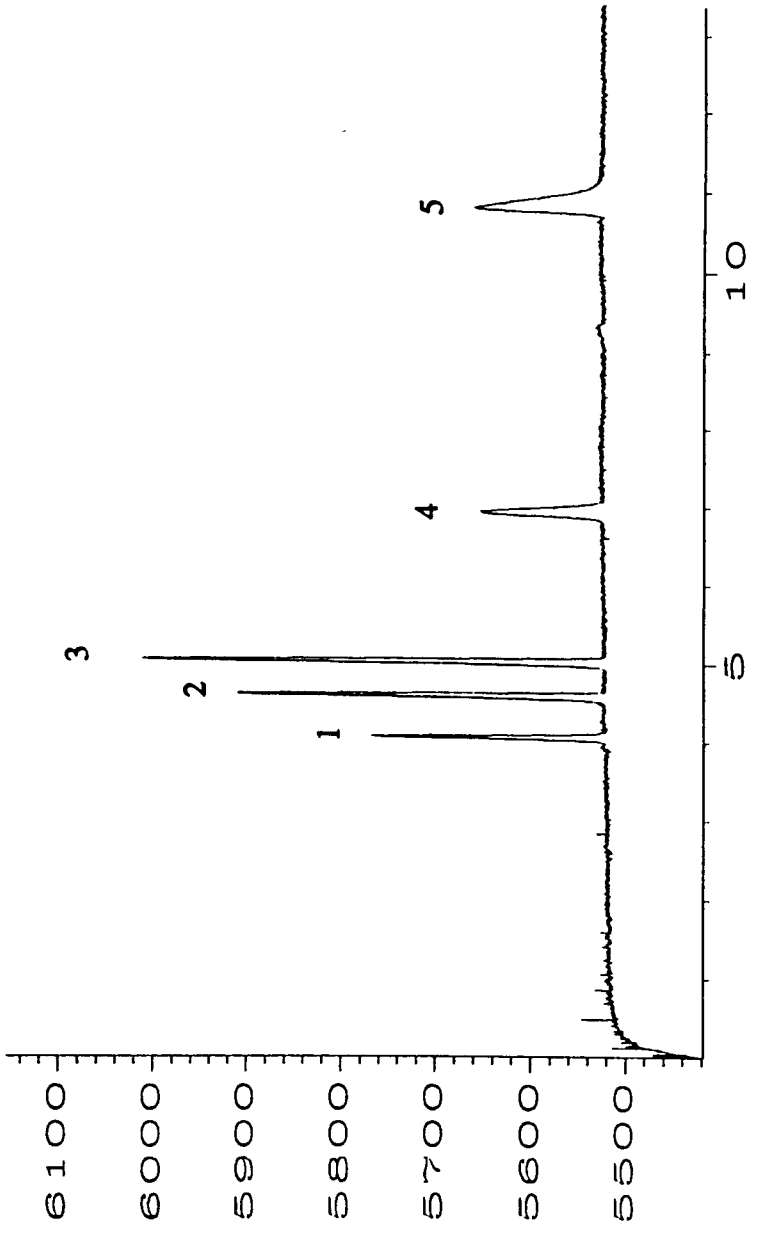
5700
5600
5500
1
2
3
4
5
6
5
10
Time (min)

Fig. 2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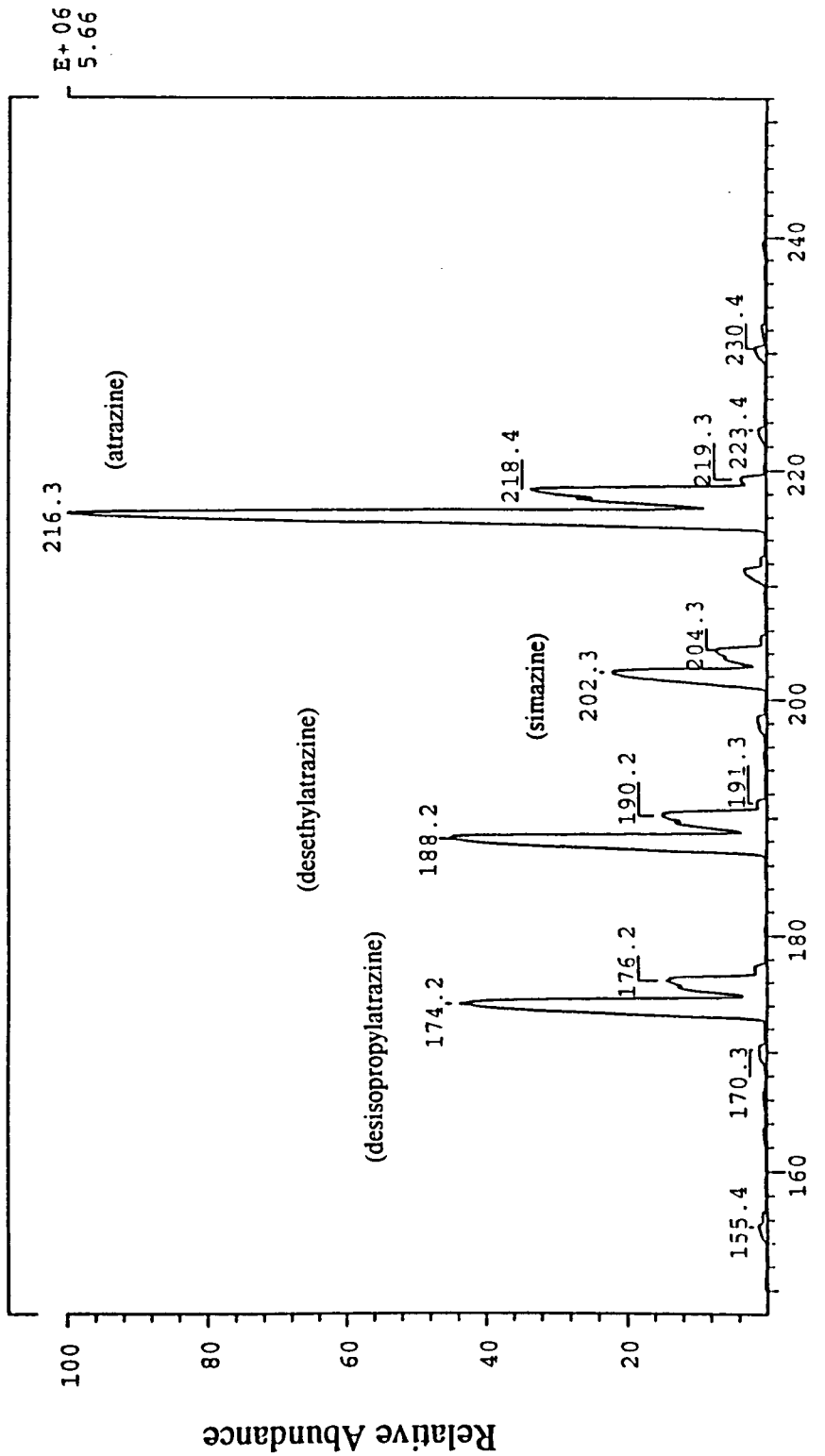
10
Time (min)

Fig. 3


E+06
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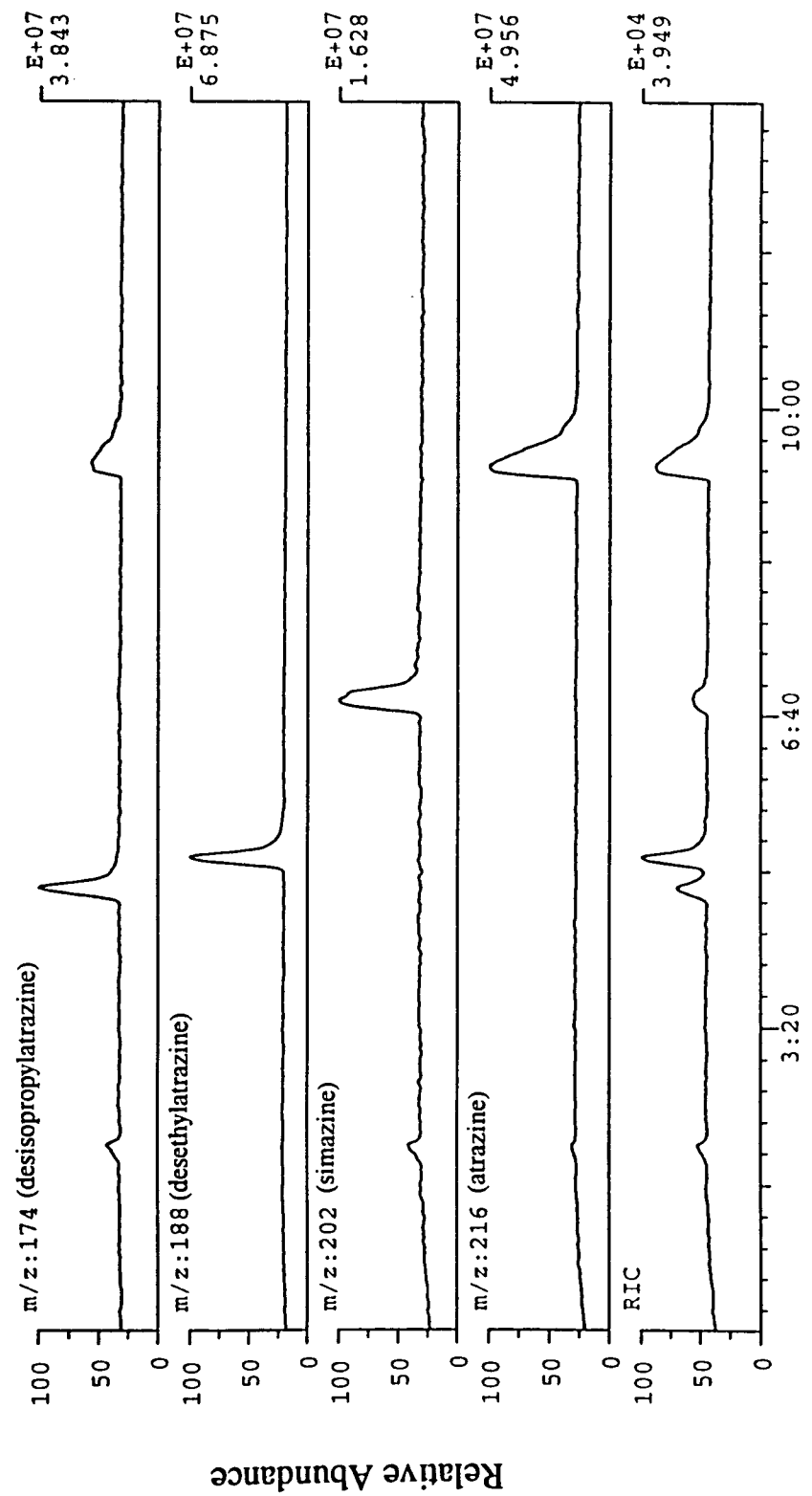
Relative Abundance
100
80
60
40
20
m/z
160
180
200
220
240
216.3
(atrazine)
218.4
219.3
223.4
230.4
(desethylatrazine)
188.2
190.2
191.3
(simazine)
202.3
204.3
(desisopropylatrazine)
174.2
176.2
170.3
155.4

Fig. 4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1.628
m/z:216 (atrazine)
E+07
4.956
RIC
E+04
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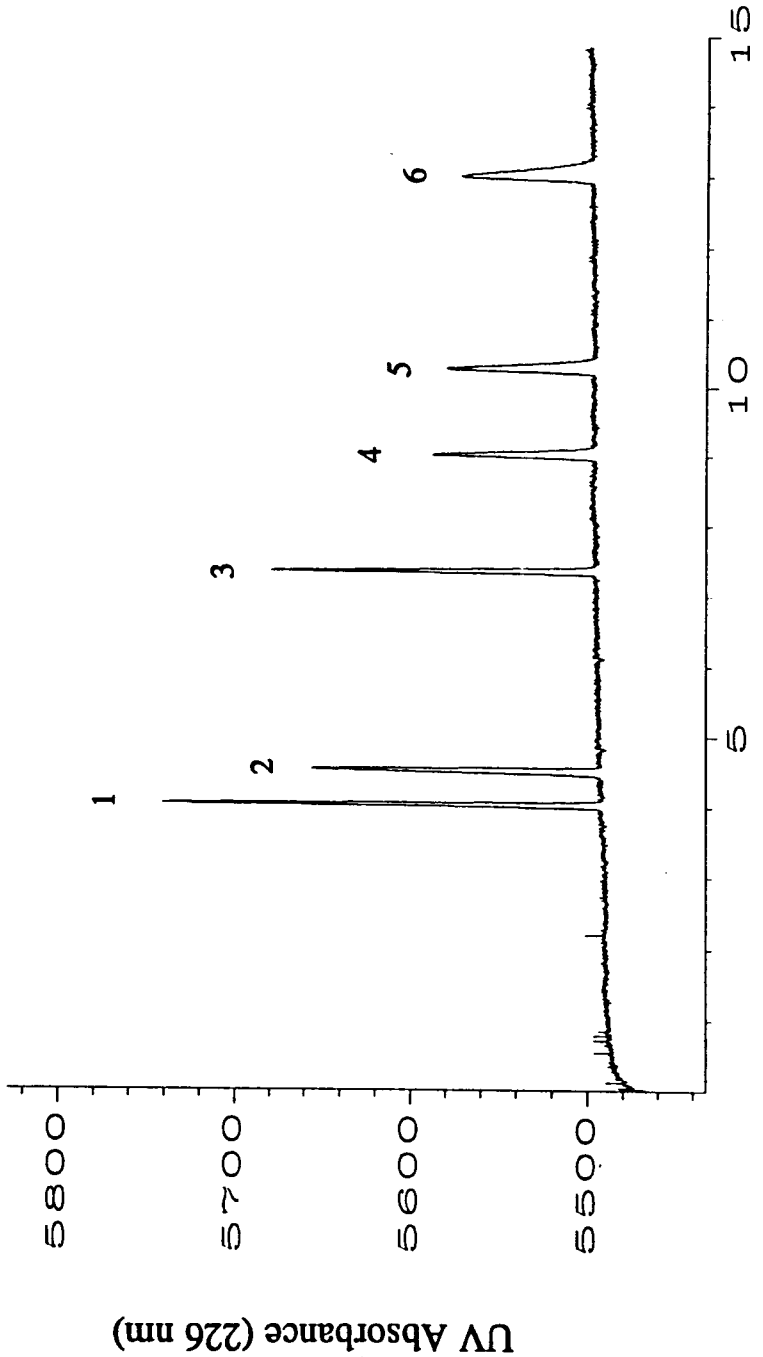
100
50
0
3:20
6:40
10:00
Time (min)
Relative Abundance

Fig. 5


UV Absorbance (226 nm)
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5600
5500
1
2
3
4
5
6
5
10
15
Time (min)

Fig. 6

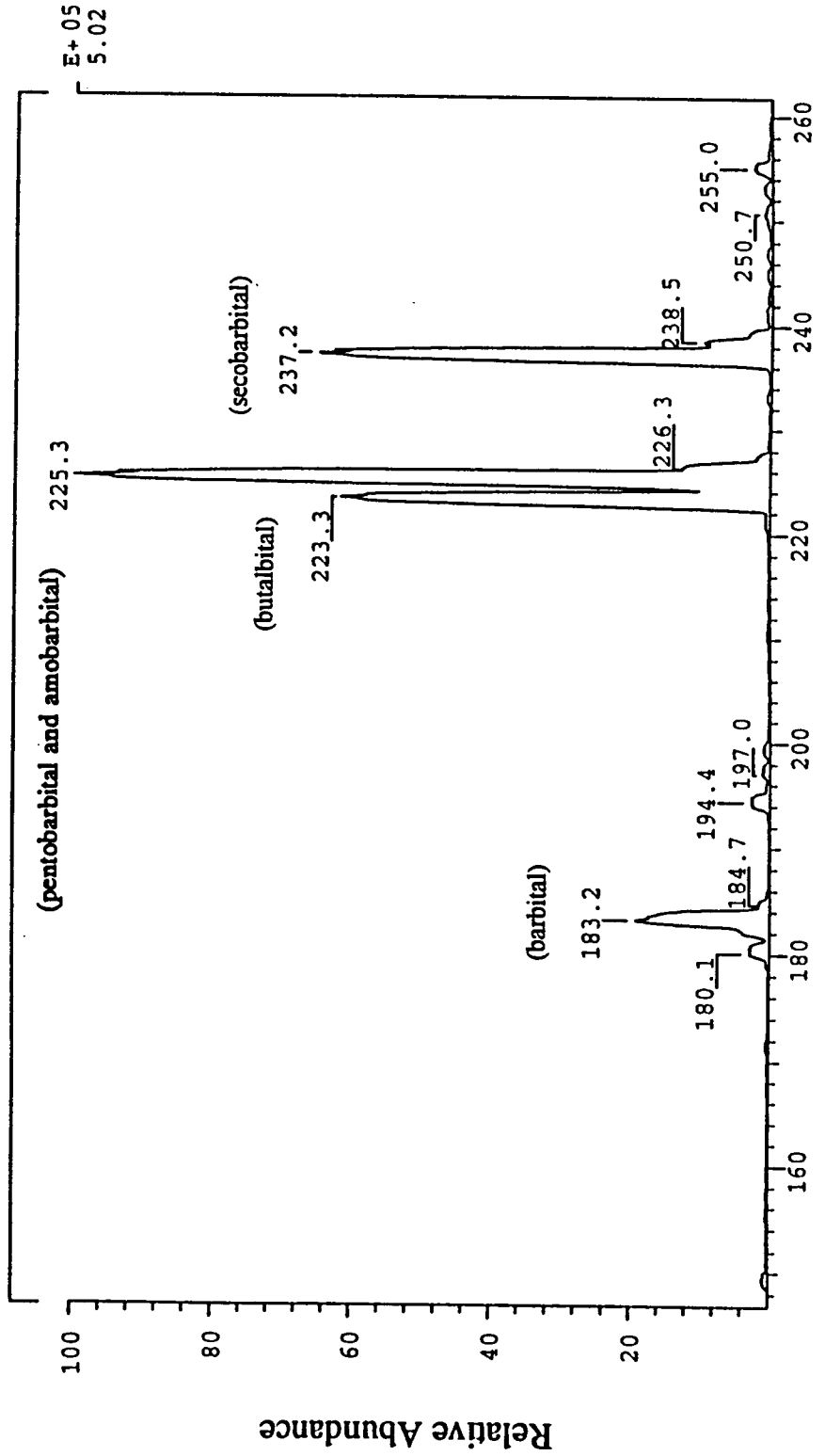
E+05
5.02
Relative Abundance
100
80
60
40
20
160
180
200
220
240
260
m/z
(pentobarbital and amobarbital)
225.3
(butalbital)
223.3
(secobarbital)
237.2
(barbital)
183.2
180.1
184.7
194.4
197.0
226.3
238.5
250.7
255.0

Fig. 7

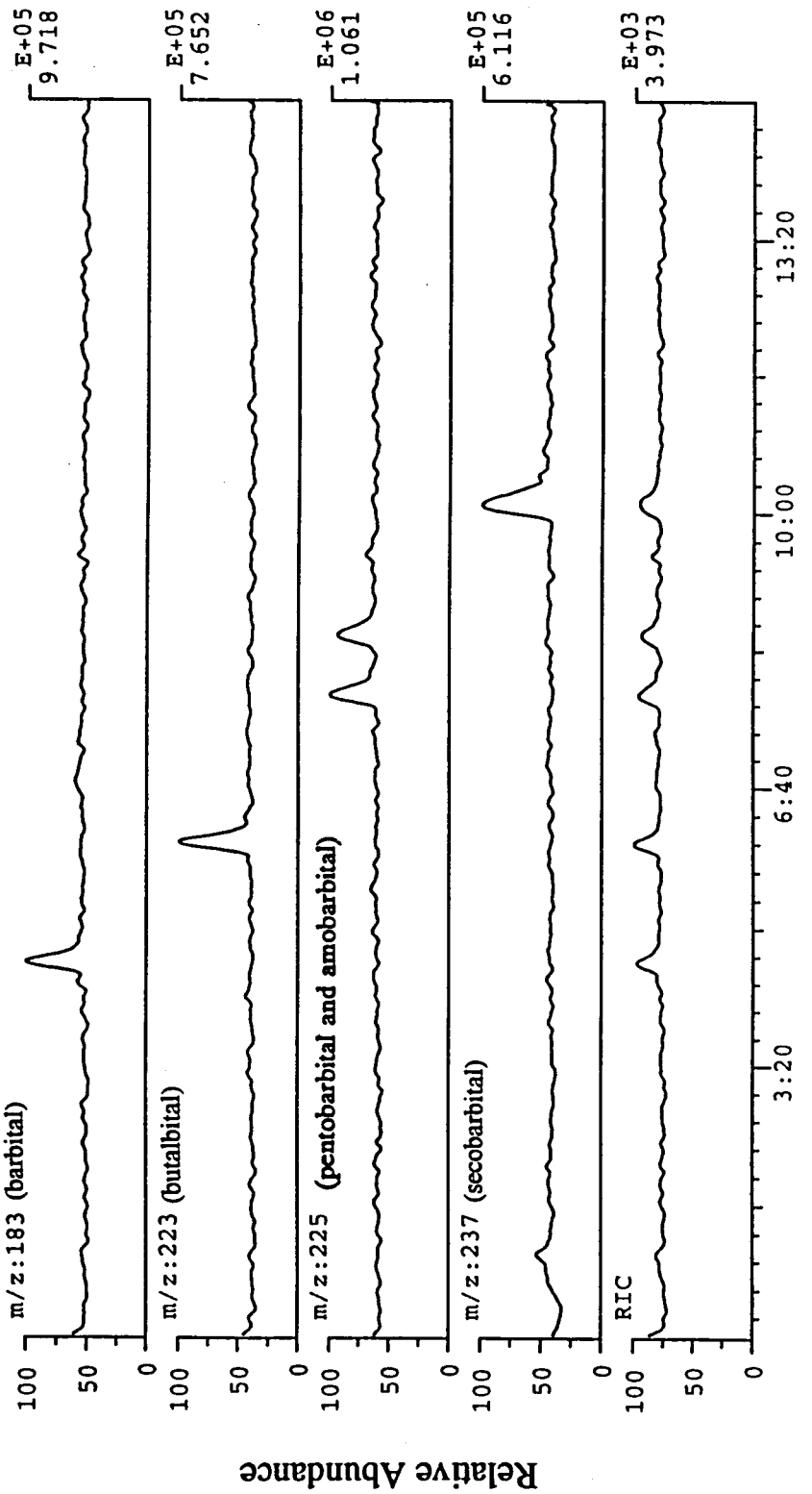
m/z:183 (barbital)
E+05
9.718
m/z:223 (butalbital)
E+05
7.652
m/z:225 (pentobarbital and amobarbital)
E+06
1.061
m/z:237 (secobarbital)
E+05
6.116
RIC
E+03
3.973
100
50
0
3:20
6:40
10:00
13:20
Relative Abundance
Time (min)

Fig. 8

GENERAL SUMMARY

The area of CE-ESIMS has experienced tremendous advances over the last few years in theoretical understanding, instrumentation design, and application. However the limitations of detection sensitivity and buffer compatibility in CE-ESIMS restrict the application of CE-ESIMS for routine analysis. Thus, both the issues of detection sensitivity and buffer compatibility are addressed in this thesis, and alternative solutions for solving the limitations are presented.

In our first paper, detection sensitivity is enhanced using on-line CIEF-ESIMS for high resolution analysis of bovine serum apotransferrin glycoforms. In comparison to CZE, the focusing effect of CIEF permits the analysis of very dilute protein samples with a typical concentration factor of 50-100 times. On the basis of their differences in pI, the di-, tri-, and tetrasialotransferrins are focused and resolved in CIEF. Additional transferrin variants within each of di-, tri-, and tetrasialotransferrins, differing in their molecular weights, are easily distinguished by ESIMS. In combination with sialidase digestion, the distribution of pI and molecular weight of asialo-, mono-, di-, tri-, and tetrasialotransferrin variants is obtained from the CIEF-ESIMS measurements.

Buffer incompatibility is the main hurdle in the combination of MEKC with ESIMS. Because of the nonvolatile surfactants used in MEKC, direct coupling of MEKC to ESIMS is hazardous. In our second paper, on-line MEKC-ESIMS is demonstrated in both positive and negative ion modes for the analysis of chlorotriazines and barbiturates. The successful

combination of MEKC to ESIMS is achieved by the elimination of micelle introduction from the MEKC capillary into the ESIMS. Successful demonstration of MEKC-ESIMS offers great potentials in high efficiency separation and mass identification of neutral and ionic compounds that cannot be resolved in CZE-ESIMS.

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