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CAPILLARY LIQUID CHROMATOGRAPHY USING LASER-BASED
AND MASS SPECTROMETRIC DETECTION

TECHNICAL PROGRESS REPORT FOR THE PERIOD
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INTRODUCTION

Capillary electrokinetic separation techniques have been very prominent in recent years. Numerous symposia dedicated to these techniques have been conducted and the literature is replete with reports of fundamental studies and practical analyses involving capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). The diminutive sizes of the capillary columns that are utilized with these techniques facilitates efficient dissipation of electrokinetically generated heat, which results in high efficiency (1). With MECC (most commonly employing sodium dodecyl sulfate (SDS) as the micellar phase), efficient electrokinetic separations of neutrals are also possible (DOE/ER/13613-8). Information regarding CZE and MECC can be found in our previous reports and the numerous publications cited herein and included in this submission.

The DOE-supported research performed during the past year has mainly focused on investigating and minimizing three problems that limit the practical utility of these capillary electrokinetic separation techniques in chemical analysis. [1] Analyses are hindered by poor reproducibility. This is largely a result of complicated and irreproducible capillary wall-solute interactions that often result in adsorption and mobility changes (Projects 3 & 4 address this issue.) [2] While the MECC technique permits the separations of neutral solutes, hydrophobic compounds are difficult to separate and manipulation of capacity factors (k 's) is critically important (Projects 1 & 2 address these issues.) [3] The very small solute band volumes require that "on-column" detection be performed (usually optical detection) and this seriously limits detectability (Projects 3, 4, & 7 address this issue.) In addition to these projects, we have begun investigating the electrokinetic equivalent of affinity chromatography (Project 5) and, because of a convenient overlap of equipment needs with the PI's other major area of interest, have included studies into the development of remote fiber-optic sensors to measure chemical carcinogens and other compounds (Project 6).

RESULTS AND DISCUSSION

1. Gradient Elution in Micellar Electrokinetic Capillary Chromatography (MECC) (Personnel - A. C. Powell).

A weakness of the MECC technique is the limited elution window that it exhibits (DOE/ER/13613-6). The fact that the micellar phase elutes from the capillary column results in a departure from conventional LC theory in that resolution (R_s) does not continuously increase with increasing k' . Terabe and coworkers demonstrated that R_s degrades at high k' values and is optimum in the range of 1-5, depending on the breadth of the elution window (2). To aid in the selective "tuning" of solute k' values to this optimum range, we have developed unique solvent gradient delivery systems that are useful in MECC and other electrokinetic methods.

As mentioned in the previous competing proposal, we have demonstrated the ability to generate a variety of solvent gradients (concave, convex, linear) that exhibit profound affects on MECC separations (DOE/ER/13613-20). Since then we have demonstrated the ability, using proven MECC theory, to computationally predict solute retention times for these gradients with good accuracy (DOE/ER/13613-23). Systematic optimization of separation conditions in MECC can be useful in all potential applications. As can be seen from Tables I and II and Figure 5 in DOE/ER/13613-23, the only significant deviation between the experimental and theoretical (predicted) retention times occurred with late eluting components, indicating a need to better define the relationship between the organic modifier gradient and the micellar velocity. Work is presently continuing on employing a double-beam detection scheme to directly measure the micellar phase velocity during the course of the gradients and thereby improve retention predictions. A "second generation" gradient mixing chamber has also been designed and constructed (see accompanying proposal). Applications to the separations of energy related samples will be pursued.

2. The Use of Bile Salt Surfactants in MECC (Personnel - R. O. Cole).

Bile salts (e.g., sodium cholate, NaC, and sodium deoxycholate, NaDC) are naturally occurring surfactants that have a hydroxy-substituted steroid-

like structure. The hydroxyl groups on the monomer result in polar and nonpolar faces and the planar structure of the bile salt-formed micelles aids in the solubilization of rigid planar molecules such as cholesterol and other lipids. Studies of bile salt micelles indicate that they possess a helical structure with the hydrophilic faces of the monomers facing inward (3). These surfactants have low (4-11) aggregation numbers and critical micelle concentrations (CMC) in the 0.006-0.01 M range. We have used bile salts as pseudostationary phases in MECC (DOE/ER/13613-21 and 30). Our first concern in this work was that the low aggregation numbers would lead to very polydisperse micelles, causing unacceptable band broadening (DOE/ER/13613-11). This proved not to be a problem as preliminary data for separations of chiral solutes produced sharp solute bands (DOE/ER/13613-21). Bile salts offer the general advantages in MECC over the commonly used n-alkyl surfactants that are discussed below.

Because of their relatively polar nature, bile salts exhibit a general reduction in k' values. Because of the low optimum range of k' values discussed above, and the reversed-phase retention characteristics of MECC, hydrophobic compounds are difficult to resolve. Solutes which normally coelute at the micelle retention time, t_M , using SDS are often fully resolved when bile salt pseudostationary phases are employed. Studies with fluorescent probes have shown that the interior of the bile salt micelle is more polar than SDS and this facilitates the separation of hydrophobic compounds (k' values are lower). Moreover, our studies indicate that moderately high concentrations of organic solvents do not effect the bile salt micelles as much as SDS micelles. This permits further improvement in the separation of hydrophobic compounds (DOE/ER/13613-30).

The chiral nature of the bile salts allows enantiomerically-selective interactions. Our work has included investigations of these interactions for chromatographic purposes, with emphasis on experimental parameters influencing the separation of binaphthyl compound enantiomers (DOE/ER/13613-21). It was determined that the use of organic modifiers influence the chiral recognition for certain binaphthyl compounds, and that bile salt micelles allow chiral separation for both rigid and planar neutral solutes. The unique chromatographic and spectroscopic properties of bile salts and other unusual surfactants are being investigated (see accompanying proposal).

3. Metal Ion Separation & Detection by Capillary Zone Electrophoresis (CZE) Via On-Column Chelation (Personnel - D. F. Swaile, T. D. Staller, B. Colburn).

In the past, CZE has not been applied to the separation of metal ions due to inadequate detection schemes and the strong columbic interaction of these ions with the wall of the capillary. By adding chelating agents to the electrophoretic buffer, it is possible to circumvent these problems. Furthermore, by performing the chelation reaction during the separation process (i.e., doping the mobile phase with the chelating agent) it is possible to manipulate the electrophoretic mobility of the band in a predictable fashion based on known complexation chemistry. In our laboratory we have investigated the use of 8-hydroxyquinoline-5-sulfonic acid (HQS) as an on-column chelating agent (DOE/ER/13613-22). HQS forms fluorescent complexes with a large number of metals but exhibits virtually no native fluorescence, so detection of HQS-metal complexes can easily and sensitively be performed using laser(He-Cd)-based fluorimetry.

As an initial study of this new separation/detection scheme we separated Ca, Mg, and Zn ions and achieved low picogram LODs (DOE/ER/13613-22, Table 1). The method had a linear dynamic range of 2 orders of magnitude above the LOD and a RSD of 10% when peak height was used for quantitation. Detectability in this method is limited by a substantial fluorescence background (probably due to metal impurities in the electrophoretic buffer) and probably could be improved if ultra pure water were employed. As well as providing reasonable detectability, this method results in excellent detector selectivity. This is illustrated in DOE/ER/13613-22 - Figure 4, with an electropherogram of untreated blood serum, in which the free calcium and magnesium are easily observed.

Along with producing a fluorescent species, the chelation reaction tends to impart a negative charge to the metal ion which alters its electrophoretic mobility and reduces its interaction with the capillary wall. Since HQS is a bidentate ligand, the reaction proceeds through a stepwise equilibrium. This results in the existence of several species in the eluting band which influences the observed electrophoretic mobility of the band. To explain the effects of this process on the observed mobility, and to optimize selectivity, we derived and tested equations that relate observed mobility to the mobilities of each species in the band and the mole fraction of that species.

The effects of pH and [HQS] on selectivity and efficiency were investigated. In the future we plan to apply this method to the determination of rare earth metal ions by employing β -diketone derivatives as the chelating agent. Since the fluorescence of the rare earth complexes vary greatly we plan to employ photodiode array detection (an instrument has been purchased and software developed) to provide both retention and spectroscopic information and to employ time resolved detection to improve sensitivity (see accompanying proposal).

4. On-column labeling of proteins for laser-based fluorometric detection (Personnel - D. F. Swaile, B. McCubbin).

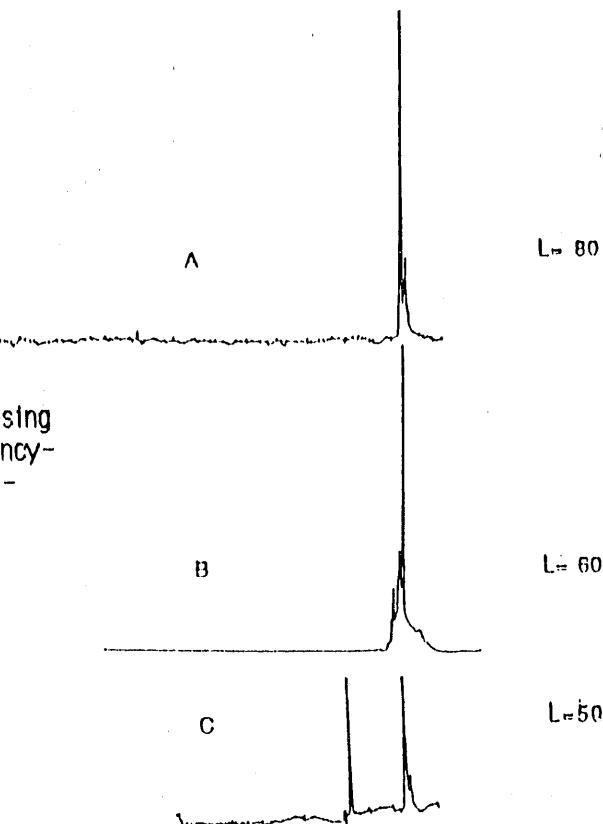
The effects of environmental, energy, and industrial substances on biological systems often is manifested in cellular or fluid protein levels. The application of CZE to the separation of proteins has become quite common due to its ability to separate proteins that differ by only a few amino acids. However, detection techniques are generally inadequate for practical utilization of CZE in protein analysis. The most common detection scheme for proteins, spectrophotometry, is pathlength limited and not useful for on-column detection in very small diameter capillaries (25 μm id or less). The high sensitivity possible with laser-based fluorimetry can overcome the short pathlength disadvantage of on-column detection. In our laboratory we have been working on ways to extend the application of this detection method to the analysis of proteins.

The detection of the native fluorescence of proteins requires the use of UV excitation (to excite the aromatic amino acid residues). Using a frequency double argon ion laser (9 W at 514 nm doubled to produce 9 mW at 257 nm), we were able to achieve good detectability (minimum detectable concentration of 25 nM for conalbumin) but the expense and complexity of this system reduces its general utility. To circumvent the need for UV excitation, proteins can be derivatized with fluorophores prior to the separation process. Although this method is commonly performed in HPLC, it is not applicable to CZE because the protein molecules are not uniformly labeled with fluorophore. Uneven labeling results in a range of electrophoretic mobilities in the sample, which results in either multiple peaks or band broadening. The adverse effects of precolumn labeling with fluorescein isothiocyanate (excitation using the 488 nm

line of an argon ion laser) can be seen in Figure 1B (compare with the peak profile for native fluorescence detection, Figure 1A).

In our laboratory we have devised an on-column labeling method using electrophoretic buffers doped with either 2-p-touidnylnaphthalene 6-sulfonate (TNS) or 1-anilinonaphthalene-6-sulfonate (ANS) which combines the advantages of the above two methods. By performing the derivatization reaction during the separation process we [1] achieved good peak profiles, [2] require no sample preparation, and [3] can employ easily accessed laser line. These compounds fluoresce poorly in aqueous solution but fluoresce strongly when solubilized in the hydrophobic region of proteins, where they are shielded from possible quenchers. The solubilization of these compounds into proteins is a dynamic equilibrium process, which has a forward rate constant of between 10^4 and 10^6 depending on the compound and protein (4). Since the labeling mechanism is a dynamic process the peak profile is not distorted, as can be seen in Figure 1C. We are currently investigating the parameters which effect this equilibrium to maximize detectability (a manuscript is in preparation).

Figure 1. Electropherograms of conalbumin using (A) direct fluorescence detection (exc. - frequency-doubled argon ion laser), (B) pre-column labeling with FITC (exc. - argon ion laser), and (C) on-column labeling using ANS in mobile phase (exc. - He-Cd laser). The small second peak in A is a protein impurity and the first peak in C is an internal standard. L is capillary length in cm.

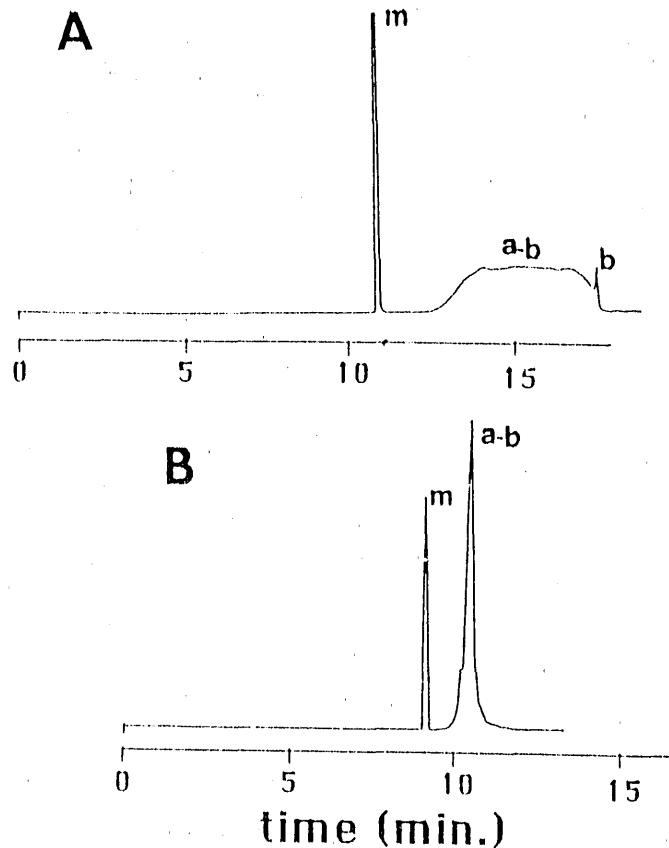


5. Affinity CZE (Personnel - S. S. Finniss).

Inorganic complexation chemistry was exploited in Project 3 to manipulate mobility in the separation of metal ions. Project 5 involves adding affinity reagents to the electrophoretic buffer in CZE to bioselectively bind to a specific ligands and alter, in a controlled fashion, their mobility. Theoretical details concerning this electrokinetic equivalent of "affinity chromatography" appear in the previously submitted competing proposal. Because the affinity reagents are usually proteins, this project has suffered (progress has been slowed) by the adsorption problems mentioned above. During the past few months three capillary column suppliers have approached us to test developmental capillaries designed to minimize adsorption. Early results have been encouraging (some of the data obtained for Project 4 and reported at the most recent FACSS meeting in Cleveland utilized these capillaries).

Despite the adsorption problems, three affinity reagent-ligand systems have been investigated. Murphy's Law was evident with two of the systems in that the affinity complex and ligand (analyte) exhibited similar mobilities. The third experiment, injection of biotin into a CZE system containing avidin, yielded predictable results (see Figure 2). The avidin and biotin reagents are readily available but their affinity formation constant is so large ($K_a = 10^{15}$) that it was not possible to manipulate retention by adjusting [avidin] or pH (i.e., with such an enormous K_a the reaction proceeded to completion under all experimental conditions). Figure 2A & B represent low and high [avidin]. The beginning of the broad "hump" in Figure 2A is the avidin-biotin complex retention time and the small glitch at the end is unreacted biotin. The conditions used for Figure 2B were such that sufficient avidin was available to completely react with the injected biotin. Future systems to be investigated will include immune complexes (K_a values are considerably smaller) and separations will be performed using gel-filled capillaries (the size discrimination characteristic of these capillaries should assure that the complex and ligand have different mobilities). Once the analytical characteristics of the technique are established, separation of analytes of environmental/energy interest will be investigated.

Figure 2. Electropherograms of biotin using mobile phases containing (A) 1×10^{-8} M and (B) 4×10^{-7} M avidin. The M is a retention marker and a-b is the avidin-biotin complex.



6. Development of Fiber Optic Sensors (Personnel - J. R. Bowyer).

Remote spectroscopic measurements of chemicals have been made possible with the advent of small-diameter optical fibers that are capable of providing an optical link between the sample, located in its native environment (perhaps in a hostile, e.g., radioactive, or inaccessible, e.g., living systems, process streams, or underground chemical deposit sites), and spectroscopic instrumentation located in the laboratory. Recently, "fiber optic chemical sensors" (FOCS) have been the focus of much research activity. Spectroscopic signals with FOCSs result from the interaction of the analyte with an immobilized reagent. Our FOCS work has involved the use of immunochemical reagents to perform remote fluoroimmunoassays (5). Reference 5 is included in this submission and provides considerable information on fiber optic sensing.

While these devices can be termed biosensors they can be used to monitor virtually any analyte, including those of environmental/energy concern. For example, we have developed sensors to monitor trace levels of benzo(a)pyrene tetraol (BPT), a hydrolysis byproduct resulting from the interaction of B(a)P with DNA (DOE/ER/13613-26 TO 29). Our recent emphasis has been the utilization of the capillaries and laser detection instrumentation used in our separations research to develop microscale regenerable sensors. Because of the overlap in required instrumentation between these separate research efforts, and obvious energy-related significance, we have included the development of regenerable fiber optic sensors in our DOE research. Technical details can be found in the aforementioned references.

7. Mass spectrometric detection (Personnel - K. D. Cook, J. Cosgrove).

There has been encouraging progress in this area, despite our inability to locate a suitable student or other researcher to devote full time to the project. The most significant headway was the design of an electrospray interface for the UTI-100C quadruple mass spectrometer dedicated to this project. This design draws heavily on successful interfaces described in the literature. When construction is completed, it should allow us to begin the proposed experiments characterizing the impact of chromatography-enforced chemical parameters on mass spectrometric sensitivity. The interface should be transferable to a Hewlett-Packard quadruple mass spectrometer which is being resurrected because its mass range and tuning flexibility exceed those of the UTI instrument.

Progress has also been made toward the goal of achieving an LC/MS interface with the Departmental VG ZAB-EQ hybrid mass spectrometer (B-E-q-Q geometry), with an eventual aim of enabling LC/MS/MS experiments. The flow-FAB source is being modified to accommodate a new Cs^+ ion gun (Phrasor Scientific) capable of operation at a wide range of primary ion energies and fluxes. This should enhance our high mass capabilities. A new ion source (also from Phrasor Scientific) with electrohydrodynamic and/or electrospray capabilities is scheduled for delivery by the end of 1990. This research source will help bridge the difficult gap of high-energy electrospray, a goal that has been achieved only to a limited extent elsewhere. Parallel efforts at developing an LC spray interface for a sectored mass spectrometer are

getting underway in collaboration with Dr. P. J. Todd of Oak Ridge National Laboratory, using a near-new Kratos MS-25 spectrometer. Debbie Ailey, a full-time ORNL employee and part-time UT graduate student, has expressed interest in working with Dr. Todd on this project. Although her research efforts will only be part-time, it is hoped that assignment even of a part-time student to this project will result in steadier progress than has been realized to date.

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See Publications and Reports for other references.

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