DOE/ER/13613--36

DE92 040524

DOE/ER/13613-36

CAPILLARY LIQUID CHROMATOGRAPHY USING LASER-BASED AND MASS SPECTROMETRIC DETECTION

FINAL TECHNICAL PROGRESS REPORT FOR THE PERIOD

SEPTEMBER 1, 1989 - JANUARY 31, 1993

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PREPARED FOR THE

U. S. DEPARTMENT OF ENERGY

ASSISTANT SECRETARY FOR ENERGY TECHNOLOGY

OFFICE OF ENERGY RESEARCH

UNDER GRANT DOE-DE-FG05-86ER13613

DISCLAIMER

FINAL TECHNICAL PROGRESS REPORT OUTLINE

INTRODUCTION

In the decade following the seminal paper describing modern capillary zone electrophoresis (CZE),[1] the prominence of capillary electrokinetic separation techniques has grown dramatically. Numerous symposia dedicated to the techniques have been conducted and the literature is replete with reports concerning CZE, and the related *electrochromatographic* technique, micellar electrokinetic capillary chromatography (MECC). As an illustration, the latest Fundamental Review issue of *Analytical Chemistry* provides over 500 citations for the period 1990 - 1 on capillary electrokinetic techniques and related topics.[2] In the PI's laboratory, Department of Energy-supported research during the past decade has contributed substantially to the fundamental and practical development of CZE/MECC (see Reports and Publications Section of this Document). This report provides a brief synopsis of our research efforts in this area during the current three year (actually 41 month) grant period, which ends January 31, 1993. In addition to a description of our analytical separations-based research, the results of efforts to develop and expand spectrometric detection for the techniques is reviewed. In this regard, we have successfully advanced laser fluorometric detection schemes. Conversely, attempts to develop mass spectrometric detection have been less fruitful, largely due to limits in available personnel (future work in this area is not proposed). An ancillary project, made possible by a convenient overlap in available equipment, resulted in the development of a regenerable fiber optic sensor that can be used to remotely monitor chemical carcinogens and other compounds.

The enormous interest generated in CZE and MECC as separation techniques can be attributed, at least in part, to characteristics that include: [1] very high efficiency, [2] small sample/reagent consumption, [3] facile automation, and [4] rapid modification of separation mode (chromatographic, electrophoretic, or both) and selectivity through adjustment of the solution in the capillary. During early developmental stages, experiments were aimed at demonstrating feasibility and exploring fundamental aspects of the techniques. As an illustration, in 1987 we published the results of the first comprehensive investigation of factors that influence efficiency in MECC.[3] As the techniques have matured (during the current grant period), more practical-minded studies (performed in numerous laboratories including the PI's) have ensued; e.g., [1] applications demonstrate inherent advantages of the techniques for important separations, [2] instrumentation developed, tested, and refined, [3] efforts made to overcome limitations and inherent problems

of the techniques, and [4] modifications of the techniques explored to broaden their scope of application. Fundamental studies performed during this period were also more practical in nature. For example, a consequence of the diminutive sizes of CZE/MECC capillaries is that extremely high voltages can be applied to provide for very rapid separations. Thus, we continued our investigations of factors that influence efficiency in MECC, but constrained by the practical conditions required for rapid-separation.[4] The CZE/MECC research described in this report is arranged based on project topic, but was specifically designed with the practical-minded studies listed above as general aims. Numerous references are made to our DOE registered publications and reports.

RESULTS AND DISCUSSION

I. Micellar Electrokinetic Capillary Chromatography: Technique Advances

1. Aqueous-Organic Mobile Phases and Solvent-Gradients. The observation that elution characteristics in MECC closely mimic reversed-phase HPLC has motivated our extensive studies of the influences of organic solvents on retention.[5, 6(for overview)] The addition of organic solvents to MECC mobile phases reduces solute partition coefficients (as in RP-HPLC), but also alters phase ratio and electroosmotic flow rate. Gradient elution has proven essential in HPLC separations of components in complex mixtures. In the case of MECC, a limited elution range,[7] problems with separating hydrophobic compounds,[8] and complex relationships between mobile phase composition and band migration velocity,[6,9] have rendered gradient elution equally (if not more) important, but theoretically difficult to model. We have developed two instrumental arrangements capable of generating solvent-gradients in MECC and theoretical models for predicting retention during the course of the gradients.[10,11] A variety of continuous-shaped gradients can be generated and it was shown that unusually-shaped gradients can facilitate rapid separations in MECC. Predictions of solute retention, using theoretical models developed in our laboratory, were found to correlate fairly well with the experimentally obtained retention times of test solutes. Preliminary results indicate the feasibility of using this analytical methodology, with a modified simplex procedure, for the computational optimization of solvent gradient separations. References 10 and 11 (the latter is a preliminary manuscript for submission) can be consulted for details of recent work.

2. Exploring Uses of Non-Traditional Organized Media to Extend Applications, Provide Unique Selectivity, and Reduce Analysis Time. Most MECC separations are performed using sodium dodecyl sulfate (SDS)-formed micelles. Unfortunately, moderate to highly hydrophobic compounds tend to completely associate with SDS micelles and co-elute near the end of the elution range. Organic solvents only partially alleviate this problem (at moderate concentrations, organic solvents inhibit micelle formation). During the past few years we have investigated the uses of bile salts (naturally occuring surfactants) as an alternate form of pseudo-stationary phase in MECC. The unique *inverted* aggregates of bile salts are more polar and tolerate higher concentrations of organic solvents, relative to SDS-micelles; both characteristics facilitate the separation of relatively hydrophobic compounds.[8] Bile salts, being chiral, permit the separations of certain enantiomers.[12,13] Our fundamental investigations of efficiency for rapid separations illustrate further advantages of employing bile salts in MECC (high efficiency was attained in sub-minute separations).[4]

We have also investigated the utility of another class of organized media, cyclodextrins (CDs), to selectively modify retention in capillary electokinetic separations. CDs are naturally-occuring macrocyclic sugar molecules that are chiral and possess an axial cavity that is responsible for solute-CD *inclusion complex* formation. The most common forms of cyclodextrins, α-, β-, and γ-CD, differ in number of monomeric sugar units and, hence, cavity size. We have investigated the uses of native and derivatized CDs in both CZE and MECC, with the emphasis on separations of enantiomers.[14] The spatial requirements of inclusion complex formation can also be exploited for highly selective separations of geometric isomers (see information below). [15]

3. Evaluating and Improving Qualitative Capabilities. The facile manner in which retention can be manipulated in MECC, by changing the composition of the mobile phase (without altering instrumental configuration), can be exploited to enhance the qualitative characteristics of the technique. Using a test sample of 10 mycotoxins (naturally-occuring environmental toxins) we were able to acquire two distinctive, fully-resolved chromatograms (using two sets of *optimized* mobile phase conditions) in a period of only 45 minutes, including capillary rinsing and equilibration.[16] By using both sets of conditions, and adjusting retention times using indigenous or added components in samples as normalization standards (normalized retention time CVs ~ 1%), we were able to identify, based on sample/standard co-elution, all of the mycotoxins in 5

randomly generated mycotoxin/interferent samples, without any mis-identifications (see Reference 16 for details).

II. Micellar Electrokinetic Capillary Chromatography: Separations of Energy/Environmentally Significant Toxins & Pollutants.

The utility of CZE/MECC in bioanalysis can be attributed to the ubiquity of ionic and water soluble neutral compounds in samples of biological origin. Indeed, much of our prior research was illustrated with- or applied to- biosamples. During the current grant period we have endeavored to extend the scope of MECC to include moderately hydrophobic compounds (see above), such as those commonly found in samples of environmental or energy origin. We have focused our attention on two classes of compounds, mycotoxins and polycyclic aromatic hydrocarbons (PAHs). Mycotoxins are metabolites of various fungi and can be found in many foodstuffs. They are considered among the most toxic of naturally-occuring compounds. By studying the influences of various mobile phase additives on retention and selectivity, we were able to enhance the qualitative capabilities of MECC for mycotoxin analysis (see above). Moreover, predictable changes in mycotoxin retention could be implemented to minimize problems with matrix interferences.[16] Since high sample throughput is important in mycotoxin analysis, our studies of efficiency under rapid separation conditions is of particular practical significance (e.g., see efficient 20 sec separation of aflatoxins, Figure 4, in Reference 4).

During the past year, we have made a substantial effort to establish MECC as a viable separation method for PAH mixtures, including those isolated from complex fossil fuel samples.[15] The uses of different micellar systems and mobile phase additives, such as organic solvents to effect retention and CDs to exploit topological selectivity, were investigated. Cyclodextrin mobile phase additives proved to be particular useful when separating isomeric mixtures of 3-, 4-, or 5-ring PAHs. For example, despite similar structures and hydrophobic character, benzo(a & e)pyrene could be easily separated with the proper conditions (see Figure 2 in Reference 15). Compared to HPLC columns, MECC capillaries were shown to be more robust in handling complex "dirty" samples. The small sample volume requirements of MECC permitted coupling with HPLC fractionation procedures. The B(a)P concentration of a DOE-supplied shale oil sample was rapidly and accurately determined using this separation methodology with laser fluorometric detection (see last section of Reference 15).

III. Investigating On-Column Chemistries to Facilitate Laser Fluorometric Detection and Manipulate Retention

1. Metal Ion Separations Using On-Column Chelation. Although CZE exhibits exceptional resolving power for metal ion analysis, it has received relatively little attention, partly due to the scarcity of suitable detectors. As an alternative to indirect fluorometric detection, we have demonstrated and characterized a detection scheme based on on-column chelation with ligands that form fluorescent complexes.[17] Most of our work involved adding 8-hydroxyquinoline-5-sulfonic acid (HQS) to the electrophoretic running buffer. Free HQS is nonfluorescent, while many HQS-metal complexes fluoresce when excited with the near UV outputs of argon ion or He-Cd lasers. As an initial study of this detection scheme we separated Ca, Mg, and Zn ions and achieved low picogram LODs and a linear dynamic range extending 2 orders of magnitude above the LOD (see Table 1 in Reference 17). The effects of chelation on electrophoretic mobility and separation efficiency were also studied with this system. Preliminary efforts to extend this methodology to the separation and detection of rare earth metals (see Progress Report DOE/ER13613-31) have been shelved for the time being in favor of developing generalized approaches to indirect fluorometric detection (see Section VI of this report).

2. Association of Hydrophobic Probes for Detection of Proteins and Surfactant Systems. The application of CZE to the separation of proteins has become quite common due to the exceptional efficiency that is possible, particular if capillary wall adsorption is reduced or eliminated.[18, 19] We have compared various laser fluorimetry-based approaches (native fluorescence, pre-column labeling, and on-column labeling using hydophobic probes) to detect proteins separated by CZE.[18] On-column labeling with probes such as 2-p-toulidnylnapthalene-6-sulfate (TNS) is based on the unusually large increase in fluorescence quantum efficiency of the probe when it interculates into the hydrophobic regions of proteins. This approach offered advantages that include: [1] no loss of protein peak integrity (unlike pre-column labeling), [2] no sample preparation required, [3] easily accessed laser line used for excitation, and [4] results in LODs that are substantially lower than observed for absorbance detection (see Figure 4 in Reference 18 for a sample electropherogram).

We have also explored the use of CZE with this mode of detection to investigate surfactant/micelle systems.[20] An increase in TNS fluorescence is observed when the probe is in the hydrophobic

environment of micelles, allowing visualization of the aggregates. The quantitative and qualitative capabilities of this approach were investigated via injections of various [SDS] solutions using mobile phases containing different [TNS]. Calibration plots were constructed to evaluate LODs and linearity, and relationships between [TNS] and mobility were established. Injection of more complicated surfactant systems (technical grade SDS, commercial soaps, etc.) provided interesting information concerning the stabilities of mixed micelles under electrophoretic field conditions.[20]

Kuhr first reported the use of mobile phases containing a fluorophor (quinine sulfate) for indirect fluorescence detection in MECC.[21] In a similar manner, we are exploiting the unique fluorescence characteristics of these hydrophobic probes for detection of non-chromophoric, neutral solutes separated by MECC. The analytical characteristics of this mode of detection are currently being evaluated and will be incorporated in Reference 20 before submission of the manuscript later this summer.

3. Immuno-Affinity Capillary Electrophoresis. The on-column labeling procedures described above have the additional effect of influencing solute mobility. Similarly, preliminary experiments were conducted to investigate the effects of adding affinity reagents to the electrophoretic buffer in CZE to bioselectively bind to specific ligands and alter, in a controlled fashion, their mobility. It should also be possible to obtain basic information (e.g., affinity constants) from these experiments. Theoretical details concerning this electrokinetic equivalent of *affinity chromatography* appear in the competing proposal for this grant period. Three affinity reagent-ligand systems were investigated in our work. Murphy's Law was evident with two of the systems (immunological-based ones), in that the affinity complex and free ligand (analyte) exhibited similar mobilities. The third experiment, injection of biotin into a CZE system containing avidin, yielded predictable results, but was limited as a test system due to the enormous avidin-biotin formation constant ($K_a = 10^{15}$). As a consequence of the large K_a, the affinity reaction proceeded to completion under all experimental conditions and it was not possible to continuously adjust retention by changing [avidin] or the pH of the mobile phase(see Progress Report DOE/ER13613-19, p. 7 for sample electropherograms and details). With improvements in surfaced modified capillaries (to minimize affinity reagent adsorption) and detection, this project will continue in conjunction with proposed work involving the uses of *highly organized assemblies* as additives in CZE/MECC. As a next step, we plan to further develop this methodology by using anti-B(a)P antibody to selectively

manipulate B(a)P retention within the elution profiles of complex chromatograms (e.g., the MECC chromatograms of shale oil appearing in Reference 15).

IV. Immuno-Affinity Fiber Optic Sensors

Remote sensing of chemicals can be performed using fiber optic chemical sensors (FOCS) that use affinity reagent phases.[22] We have pioneered the development of FOCS that exploit the specificity of antibody-antigen interactions and the sensitivity of laser fluorimetry. Initially, simple sensors were designed; signals resulted from passive diffusion of analyte into a chamber, that contained antibody to the analyte and was positioned at the fiber terminus.[23] While attomole quantities of benzo(a)pyrene tetraol could be detected in the presence of interferences, this particular sensor was limited to small, naturally-fluorescent analytes, and could not sense over extended periods of time. During the current grant period, a much more versatile, regenerable design of FOCS was developed.[24, 25] The reagent phase of this *microscale regenerable biosensor* is solid phase antibody (immunobeads) that can be delivered to the sensing chamber and held in the field of view of the fiber while the procedures of various immunoassay protocols are performed, then removed from the chamber. Details concerning the design and operation of this regenerable sensor can be found in Reference 25. Most recently, these sensors were employed to measure DNA adducts in real biomatrices (human placenta samples).[26]

V. Mass Spectrometric Detection

The coupling of CZE with mass spectrometry is motivated by the desire to obtain information on the identity of separated solutes. The topic has received much attention in recent years.[2 (p. 395R)] Most commonly, electrospray ionization is employed, although atmospheric pressure ionization and fast atom bombardment have also been used. We originally proposed experiments to characterize the impact of chromatography-enforced chemical parameters on mass spectrometric sensitivity (see competing proposal). Modest progress was made in designing and constructing electrospray interfaces for the Co-PI's UTI-100C quadruple MS and the UTK departmental VG ZAB-EQ hybrid mass spectrometer (see Progress Report DOE/ER13613-19). Unfortunately, our inability to identify a suitable student, or other researcher, to devote full time to this effort has substantially impeded research progress. As a

result of this difficulty, and the success of other facets of our proposed research, we have not emphasized this project.

VI. Preliminary Investigations (Details in Proposal)

The accompanying proposal contains project descriptions that represent an on-going research program dedicated to the development of capillary electrokinetic separation techniques and suitable methods of optical detection. In some cases, the proposed projects are direct extensions of the research described in this report and the cited publications. However, we have also embarked on certain new initiatives. Below is a very brief description of preliminary work, mostly performed during the first half of 1992, in these new areas. The proposal can be consulted for additional information and preliminary results.

1. Detection (Chemiluminescence & Indirect Fluorimetry). Peroxyoxalate chemiluminescence (CL) has proven useful in the detection of PAHs separated by RP-HPLC; in some cases yielding selectivity that differed from fluorimetry.[27] Utilizing skills acquired through the fabrication of regenerable fiber optic sensors (see above), we have construct acrylic-molded, post-capillary, CL reactors for use in CZE/MECC. The reactors have been shown to maintain electroosmotic flow and contribute only slightly to band dispersion. We are currently exploring the use of different acrylic resins in order to minimize sweiling (and in some cases occulsion of the very narrow (25 μm) reactor channels) which has been problematic in early work when organic solvents are employed. The reactor and post-mixing capillary (flow cell) are placed in a specially constructed holder that permits efficient collection of the CL emission. The holder can be mounted directly to a PMT housing and consists of an adjustable block with a semicircular mirrored channel in which the detection capillary is axially placed. The holder can be fitted with an optical filter and/or a mini-collection lens. By adjusting the position of the observation zone of the assembly with respect to the post-mixing capillary, it should be possible to study the kinetics of the CL reagent mixing and reaction processes. Diagrams of the post-capillary reactor and holder, preliminary results, and plans for evaluating and using this detection scheme are presented in the proposal.

In order to better accommodate our interest in the separation of rare earth ions by CZE, we are investigating methods of improving and simplifying indirect fluorescence detection. By employing a photodiode array (PDA) detector, we can independently monitor the fluorescence of two dyes incorporated in the electrophoretic buffer. By choosing an

ionic dye (to be displaced by ionic solutes) and a neutral dye (for normalization purposes), we can correct for many sources of noise in indirect fluorescence detection. We have employed He-Cd laser excitation of laser dyes for initial studies and obtained dynamic reserves of 10^3 (with hydrostatic flow), when this simple normalization procedure is employed. This compares with values of 10^3 - 10^4 reported by Yeung and coworkers using more sophisticated optical configurations.[28] We are currently investigating the effects of operational parameters on detector performance and plan to test this mode of detection on actual separations shortly.

2. Capillary Electrokinetic Separations Using "Highly-Ordered" Mobile Phase Additives. We propose to investigate the use of two distinct, highly-ordered, organized assemblies, [1] selected soluble polymers and [2] carbon clusters (buckyballs), as running buffer additives in CZE (see proposal). Some preliminary experiments have been conducted using both systems. Size selective separations of DNA fragments by CZE using soluble polymer additives have been report.[29] We plan to conduct fundamental studies of the separation process. However, our initial work has involved on-column labeling with ethidium bromide (and other interculating dyes) for detection of DNA fragments. We are currently evaluating the effects of different laser sources, methods of signal recovery, and running buffer composition on detector performance. A simple He-Ne "greenie" laser yielded minimum detectable concentrations in a 75 μm i.d. capillary of 80 ng herring-sperm DNA/mL, approximately 100-fold lower than previously reported using a commercial fluorescence detector modified for use with CZE.[30]

We are exploring the potential of performing non-aqueous capillary electrokinetic separations using *modified* buckyballs (C_{60}) as a secondary phase. Ultimately, we feel this system may fill an important void in the capabilities of CZE/MECC; namely the efficient separation of hydrophobic compounds. We are also interested in separating carbon cluster mixtures, including those reported to be chiral, using non-aqueous CZE. We have established that rapid electroosmotic flow can be generated in silica capillaries filled with solvents such as CH_3CN and CH_2Cl_2. The flow is modified by the addition of organic salts. Non-aqueous separations of certain PAHs have been performed, based on solvation within mobile phases containing organic salts. Mass spectral data suggests that our initial attempt to modify (impart a charge) buckyballs, by chemical reaction and sulfonation, have not been successful. Plans for further research in this area are presented in the proposal.

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*40. C. Copper, T. Staller, and M. J. Sepaniak, "Characterization of Polyaromatic Hydrocarbon Mixtures by MECC," DOE/ER/13613-40, *Poly. Arom. Compds.*, in press.

*41. S. Arrowood, A. M. Hoyt, and M. J. Sepaniak, "Monitoring Benzylpenicillin Decomposition in Gastric Contents by CZE," DOE/ER/13613-41, *J. Chromatogr. Biomed. Appl.*, submitted.

*42. R. D. Holland and M. J. Sepaniak, "Qualitative Analysis of Mycotoxins using MECC," DOE/ER/13613-42, *Anal. Chem.*, submitted.

*43. D. F. Swaile, M. J. Sepaniak, and D. E. Burton, "The Use of Fluorescent Probe Molecules for Detection in Capillary Electrokinetic Separations," DOE/ER/13613-43, *J. Microcol. Sep.*, preliminary copy for submission.

*44. A. C. Powell and M. J. Sepaniak, "Analytical Instrumentation and Computational Methods for Generating and Optimizing Solvent-Gradients in MECC,: DOE/ER/13613-44, *Anal. Instr.*, preliminary copy for submission.

*Recent Publications (copies enclosed)

PERSONNEL

Michael J. Capacci, MS, December 1986, Thesis: Biological Sample Injection for Open Tubular Liquid Chromatography.

Charles N. Kettler, Ph.D., March 1987, Dissertation: Photothermal Detection for Capillary Liquid Chromatography.

D. Edward Burton, Ph.D., March 1987, Dissertation: Development and Application of Micellar Electrokinetic Capillary Chromatography.

Anthony T. Balchunas, Ph.D., December 1987, Dissertation: Surfactant Mediated Development of High Resolution Open Capillary Liquid Chromatography.

Suzanne Landry-Baker, MS, August 1988, Thesis: Macromolecular/Particle Separations Using Capillary Electrokinetic Techniques.

*James Bowyer, Ph.D. December 1991, Dissertation: Construction, Evaluation, and Use of a Fluoroimmunochemical-Based Fiber Optic Microscale Regenerable Biosensor.

*David Swaile, Ph.D. December 1991, Dissertation: Alternate Methods of Laser-Based Fluorometric Detection in Capillary Electrokinetic Separations.

*Roderic Cole, Ph.D. December 1991, Dissertation: Applications of Non-Traditional Organized Media in Capillary Electrophoresis.

*Susan Finniss, M.S. May 1992, Thesis: Preliminary Studies of Immunoaffinity Capillary Electrophoresis.

*Ricky Holland, M.S. August 1992, Thesis: Evaluation and Improvement of the Qualitative Characteristics of MECC.

*A. Craig Powell, fifth year graduate student (on leave, Ph.D. expected 1992).

*Tracey Staller, fourth year graduate student.

*Beth Colburn, third year graduate student.

*Christine Copper, second year graduate student.

*Brian Clark, second year graduate student.

*Miland Nagale, first year graduate student.

Steve Cosgrove, undergraduate student.

*Boris McCubbin, undergraduate student.

*Andy Porter, undergraduate student.

Dr. Joe Gorse, visiting faculty research participant (Baldwin Wallace College).

*Dr. Art Hoyt, visiting faculty research participant (University of Central Arkansas).

*Dr. Chris McGowen, visiting faculty research participant (Tennessee Technical University).

*supported at least partially during current 41 month grant period.

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