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DOE/ER/13613--31

DE92 002428

CAPILLARY LIQUID CHROMATOGRAPHY USING LASER-BASED AND MASS SPECTROMETRIC DETECTION

TECHNICAL PROGRESS REPORT FOR THE PERIOD
FEBRUARY 1, 1991 - JANUARY 31, 1992

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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

MASTER

NOV 06 1991

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INTRODUCTION

Capillary electrokinetic separation techniques have been very prominent^e 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, some of which are included in this submission.

The DOE-supported research performed during the past year has mainly focused on investigating and minimizing the problems listed below 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. [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 due to a limited elution range (2). Because of the limited elution range, it is also beneficial to enhance separation selectivity through the use of non-traditional surfactants. [3] The very small solute band volumes require that "on-column" detection be performed (usually optical detection) and this seriously limits detectability. Laser fluorimetry is particularly amenable to on-column detection with these

capillary separation techniques. We have explored methods of on-column labeling and multi-wavelength detection to expand the utility of this mode of detection.

Because of a convenient overlap of equipment needs with the PI's other major area of interest, we have also included studies into the development of remote fiber-optic sensors to measure chemical carcinogens and other compounds in our DOE-supported research efforts.

I. Micellar Electrokinetic Capillary Chromatography: Technique Advancement

1. Refinement of Solvent Modification Techniques (gradient elution)

The efficient utilization of MECC, particularly in the separation of hydrophobic compounds, requires strict control of retention capacity factors, k' 's. In our previous work, gradient elution was shown to be useful in separations of complex samples (DOE/ER/13613-11). Recently, we devised and tested a model for predicting retention times based on k' values obtained from isocratic separations (DOE/ER/13613-23). The ultimate goal in this work is the time effective optimization of MECC separations. During the past year a more versatile gradient generating system has been developed. The new apparatus (see Figure 1) employs syringe pumps and a computer system purchased several years prior with DOE support to produce gradients of virtually any shape or duration. Because of the complex relationship between retention and mobile phase composition (DOE/ER/13613-23), the ability to generate unusual gradient shapes is particularly advantageous in MECC. This is illustrated in Figure 2. As seen in the figure, the gradient that "peaks-out" in $\%$ acetonitrile provides a more time effective separation of the test mixture.

FIGURE 1. MECC apparatus showing automated solvent gradient system

The ability to predict retention times provides a reasonable starting-point in the optimization of gradients in MECC. Refinement of the gradient is currently being accomplished using simplex methods (3) and a related computational algorithm (4). In our approach, the chromatographic response function (CRF), given in Equation 1,

$$CRF = \frac{N \times \sum_i \left(\frac{1}{t_{R_i} - t_{R_{i-1}}} + \frac{1}{t_{R_{i+1}} - t_{R_i}} \right)^{-1}}{t_m} \quad (1)$$

where N is the number of components in the sample, R_{t_i} is the retention time of the i^{th} component, and t_m is the micelle retention time, is maximized for separations of PAH test compounds (see Section III). Calculations are performed using Microsoft Quick Basic programs that are run on a Macintosh SE

30 PC. Preliminary results are being obtained by a visiting professor, Craig Powell, who recently left our research group (see accompanying proposal).

FIGURE 2. Illustration of the influence of solvent gradients in MECC

2. Exploring the Uses of Non-Traditional Organized Media to Separate Hydrophobic Compounds, Decrease Separation Times, and/or Provide Unique Selectivities

The majority of MECC separations are performed using SDS-formed micelles. This micelle system exhibits retention characteristics that resemble reversed phased HPLC and elution ranges that typically extend from roughly 6 minutes to 25 minutes. Unfortunately, moderately to highly

hydrophobic compounds tend to completely associate with the SDS micelles and coelute at the end of the elution range. Adding organic solvents to the mobile phase to reduce k' , as is done to control retention in reversed phase HPLC, only partially solves this problem, as at moderate concentrations organic solvents inhibit micelle formation. Other n-alkyl sulfate surfactants exhibit similar problems (DOE/ER/13613-10).

During the past couple of years we have explored the use of bile salts (naturally occurring physiological surfactants) as alternate pseudo-stationary phases in MECC. The unique "inverted" aggregates of the bile salts are more polar than SDS micelles and tolerate higher concentrations of organic solvent; both characteristics facilitate the separation of relatively polar compounds (DOE/ER/13613-30). Further evidence of the usefulness of bile salt-MECC for separations of hydrophobic compounds can be found in Section III of this report. Bile salts also provided opportunities for separations of certain chiral compounds (DOE/ER/13613-21). Most recently, we have conducted fundamental studies into the effects that experimental factors have on efficiency when extremely rapid (i.e., high field) bile salt-MECC separations are performed (DOE/ER/13613-35).

We have also investigated the utility of another class of organized media, cyclodextrins (CDs), to selectively modify retention in capillary electrokinetic separations. Cyclodextrins are natural macrocyclic polymers of glucose that are chiral in nature and, depending on the particular CD, possess structural cavities that can selectively interact with injected solutes when used in chromatographic systems (5). Cyclodextrins were employed in capillary electrokinetic separations as early as 1985 (6). We have recently explored the use of CDs for enantiomeric separations. Figure 3 demonstrates the

importance of cavity size for the CZE separation of charged binaphthyl enantiomers (note: the α -CD cavity is smaller than the β -CD cavity). This also illustrates a unique attribute of CZE/MECC, namely the fast and easy adjustment of mobile phase composition to influence separations. We have also discovered that certain substituted CDs (e.g., hydroxypropyl β -CD) provide unique selectivities (relative to their unsubstituted precursors) and can be used at higher concentrations without solubility problems. Further studies are being planned (see accompanying proposal). Most of the work involving non-traditional surfactants has been performed by Rod Cole, a fourth year graduate student who will be departing the group early in the proposed grant period.

FIGURE 3. CZE separation of 1,1'-binaphthyl-2,2'-diylhydrogenphosphate (eluting first) and 1,1'-binaphthyl-2,2'-carboxylic acid (eluting second) enantiomers using mobile phases containing (A) β -CD, (B) α -CD, and (C) a mixture of α - & β -CD

II. Investigating On-Column Chemistries to Facilitate Laser Fluorometric Detection and Manipulate Retention in Capillary Electrokinetic Separations

1. The Use of Hydrophobic Probes to Detect Separated Proteins and Surfactant Systems

The analysis of proteins by CZE is hampered by two problems: adsorption to the walls of the capillary and poor detector sensitivity. In collaboration with coworkers at Supelco, Inc., we have addressed the former of these problems. By modifying the walls of the capillaries with very stable hydrophobic and hydrophilic phases, protein adsorption problems were substantially reduced (DOE/ER/13613-32). Laser-based fluorometric detection schemes for proteins were evaluated and compared (DOE/ER/13613-33). Detection based on protein native fluorescence or pre-column labeling with fluorophores was limited by instrumental problems or a degradation of separation performance. However, a novel method of on-column labeling with hydrophobic probes (e.g., 2-p-toluidinonaphthlene-6-sulfonate, TNS) provided adequate detectability (nM concentration range for the proteins tested) and maintained the separation integrity of protein bands (see for example Figure 4 in the enclosed DOE/ER/13613-33). The on-column labeling procedure was very simple and involved doping the mobile phase with mM concentrations of TNS, a compound that experiences a large increase in fluorescence quantum yield when it intercalates into the hydrophobic regions of protein. A low cost, air cooled He-Cd laser is used for excitation. The effects of parameters such as pH and TNS concentration were investigated (DOE/ER/13613-33).

Surfactants, due to their ability to solubilize organic compounds in aqueous solutions, are very versatile chemical reagents. In addition to their

use as pseudostationary phases in MECC, they are used in other chromatographic methods, extraction procedures, and are found in various commercial products. Numerous analytical methods have been applied to the characterization of surfactant/micelle systems. Spectrophotometry has been employed, but its use is limited by the absence of suitable chromophores in most surfactants. The combination of CZE with the on-column labeling procedure described above is an attractive alternative to traditional methods that can be used to obtain analytical information as well as study certain physical and chemical properties of micelles. Detection is based on the solubilization of TNS (or related probes) into the hydrophobic core of a micelle. The analytical figures of merit of this technique are under investigation. Quantitative and qualitative analysis is complicated by the dynamics of the separation and detection processes, but calibration is possible. Examples of electrophorograms are shown in Figure 4. Preliminary experiments indicate that fundamental information about micellization (e.g., the stability of mixed micelle systems) can be gleaned from such experiments.

FIGURE 4. CZE separation of the micellar components of (A) sodium cholate, NaC, and sodium dodecyl sulfate, SDS, and (B) a commercial soap product. Elution times: water - 3.6 min., NaC - 6.3 min., SDS - 7.3 min., C₁₄ - C₁₆ olefin sulfonate - 10 min.

2. Metal Ion Separations Using On-Column Chelation

Although CZE exhibits exceptional separation power for ions, it has largely been ignored in metal ion analysis, presumably due to the scarcity of suitable detection schemes. We have reported the use of on-column chelation using 8-hydroxyquinoline-5-sulfonic acid (HQS) to facilitate detection and manipulate retention in the CZE separation of selected metal ions (DOE/ER/13613-22). The free HQS that is added to the mobile phase does not fluoresce appreciably, while many of the HQS-metal complexes that are formed on-column fluoresce efficiently when excited with the 488 nm output of an argon ion laser. In addition to the potential for sensitive laser fluorometric detection, the HQS chelation reduces interactions between positive metal ions and the capillary wall and permits control of mobility (hence separation selectivity) via adjustments of HQS concentration and pH.

Since our earlier work was aimed at demonstrating the analytical feasibility of on-column chelation in CZE, the methodology was applied to an "uninteresting" metal ion system for which the metal-HQS solution chemistry was well-established. A minor effort has been made during the current grant period to investigate the use of on-column chelation in CZE for separations of more interesting metals such as the rare earths. Para-azoresorcinol (PAR) are being used in conjunction with spectrophotometric detection (see Figure 5) and 2-naphthoyltrifluoroacetate and 2,2'-dihydroxy azobenzene are being investigated in conjunction laser fluorometric detection. Preliminary results indicate that the conditions for the complex formation and detection of a particular metal ion are so highly specific that it is difficult to detect different separated metals with one set of conditions. Thus, the efficiency and selectivity of the CZE separation process are generally not fully

exploited. It may be necessary to use multiple chelates, indirect detection methods, or post capillary reactors to produce a more universal method for metal ion detection. We plan to pursue this avenue of research more rigorously in the future (see accompanying proposal). Most of the on-column labeling research was performed by a Ph.D. candidate, David Swaile, who is currently with Proctor & Gamble Co.

SEPARATION CONDITIONS:

Voltage - 15 kV

Capillary - 50 μ m x 40 cm (to detection)

Mobile phase - Phosphate borate buffer
containing SDS (0.02 M) and para-azo-
resorcinol, PAR (1 x 10⁻⁴ M)

DETECTION:

Spectrophotometric at 500 nm using a
Linear Model 204 HPLC detector

SAMPLE:

Standard containing U (5 x 10⁻⁴ M),
Th (5 x 10⁻⁴ M), and PAR (2.5 x 10⁻³ M)
(about 2 nL injected)

FIGURE 5. CZE separation of U and Th using on-column chelation with PAR. Elution order: U - 6.5 min. and Th 7.5 min. (see insert for details)

III. Micellar Electrokinetic Capillary Chromatography: Utilizing Technique Advances for the Characterization of Polyaromatic Hydrocarbons in Environmental/Energy Samples

The characterization of the polyaromatic hydrocarbons (PAHs) in geochemical (e.g., fossil fuels) and environmental (e.g., air particulates) samples is a formidable analytical challenge. The complexity, diversity (in size, polarity, and functionality, etc.), and significance of isomers among the aromatic components of such samples dictates that numerous analytical

techniques and methodologies be utilized. While chromatographic methods have been used extensively, the MECC technique, because of its novelty and the problems cited in the introduction of this report, has not been used as an analytical tool in the characterization of these samples. Nevertheless, the efficiency, speed, and ease with which selectivity can be altered in MECC are potential advantages of the technique.

During the current grant period we have begun exploiting the technique advancements discussed above and in our previous publications and reports in attempts to characterize environmental/energy samples using MECC and laser fluorometric detection. Mixed aqueous-organic mobile phases have provided

SEPARATION CONDITIONS:

Voltage - 24 kV

Capillary - 25 μ m x 60 cm (to detection)

Mobile phase - sodium phosphate buffer with 0.1 M sodium cholate. (A) contains 25 % (v/v) acetonitrile and (B) contains 25% & 10% (v/v) acetonitrile & 2 propanol, respectively.

DETECTION:

Laser fluorimetry using 2 mW He-Cd laser at 325 nm for excitation.

Emission isolated around 420 nm with broad bandpass filter.

SAMPLE:

Shale Oil (NBS standard # 1580) extracted with acetonitrile and diluted 2:1 with mobile phase.

FIGURE 6. MECC separation of shale oil extract illustrating the effects of (A) mixed aqueous-acetonitrile and (B) mixed aqueous-acetonitrile/2-propanol mobile phases (see insert for details)

longer elution ranges (particularly with alcohol organic modifiers) and more optimum k' values for the relatively hydrophobic PAHs. The MECC chromatograms shown in Figure 6 of an extract of shale oil reveal the complexity of the sample and the utility of mixed aqueous-organic mobile phases in separating the components in the extract. These chromatograms were obtained using bile salt-formed micelles (DOE/ER/13613-30). The use of bile salts and CDs for isomeric discrimination will be investigated in the future (see proposal). We have also begun utilizing the simplex optimization procedures discussed above to choose solvent gradients for separations of standard mixtures of PAHs.

The advantages of laser fluorometric detection in capillary electrokinetic separations, that have been demonstrated throughout our work, are being realized in this application as well. Figure 7 illustrates the detectability afforded by laser fluorimetry. Separation conditions for the figure were as described for Figure 6. Fluorescence detection was accomplished by using the output of a frequency doubled argon ion laser (about 5 mW at 257 nm) for excitation and isolating the PAH emissions with a broad bandpass filter assembly and a PMT. Detectability was limited by the fluorescence background produced by the fused silica capillary. Field modulation may improve detectability in future work (7). The shale oil chromatograms shown in Figure 6 were obtained similarly using the 325 nm output of a He-Cd laser for excitation. Using the He-Cd excitation source and data collection software modified for use with the LDC spectromonitor photodiode array purchased previously (see DOE/ER/13613-19), we have also obtained distinctive temporal emission profiles during the shale oil separation. Signal/noise was rather poor but should improve with better extraction procedures and baseline noise smoothing routines. By invoking the

high efficiency and selectivity of these techniques, we plan to advance the methodologies available to analytical chemists to characterize the PAHs and biomarkers in environmental/energy samples (see accompanying proposal).

FIGURE 7. MECC separation of a standard mixture of PAH compounds: (a) acridine (2.0 fmol), (b) naphthylene (20 fmol), (c) carbazole (2.0 fmol), (d) anthracene (0.3 fmol), (e) aminopyrene (0.3 fmol), (f) fluorene (2.0 fmol), (g) phenanthrene (1.0 fmol), (h) aminochrysene (1.0 fmol), (i) pyrene (0.6 fmol), (j) chrysene (2.0 fmol), (k) benzo(a)pyrene (0.03 fmol). See Figure 6 and report for details.

IV. Refinement of a Fiberoptic Regenerable Sensor

The previous technical progress report (DOE/ER/13613-19) and several publications (DOE/ER/13613-26-29) describe a fiber optic sensor that combines a fiber optic/capillary column delivery system with fluoroimmunoassay principles to remotely monitor chemicals. This sensor has been applied in the monitoring of toxins and their metabolites. In addition to providing high sensitivity and selectivity, the sensor can be regenerated in situ, an important attribute for true sensing. Research during the current grant

period has focused on improving the ease and reproducibility of fabricating the sensors and improving the flow characteristics of the sensor to increase sensitivity. This was accomplished by developing the casting forms and procedures to mold acrylic sensing tips (8). Improvements in sensitivity were realized and the performance of the new design evaluated by monitoring fluorescein isothiocyanate, FITC (a dye that can be used to monitor the movement of materials in and around waste sites). The limit of detection for FITC was in the low nM range. The sensor work was performed largely by James Bowyer, who will be receiving his Ph.D. this fall.

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VI. PUBLICATIONS AND REPORTS

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*Recent Publications (copies enclosed)

VII. 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, fourth year graduate student (Ph.D. expected Spring 1992).

*A. Craig Powell, fifth year graduate student (Ph.D. expected Fall 1991).

*Susan Finniss, third year graduate student (M.S. expected Fall 1991).

*Tracey Staller, third year graduate student.

*Beth Colburn, second year graduate student.

*Rick Holland, second year graduate student.

*Christine Copper, first year graduate student.

*Brian Clark, first year graduate student.

Steve Cosgrove, undergraduate student.

Boris McCubbin, undergraduate student.

Dr. Joe Gorse, visiting faculty research participant.

*Dr. Art Hoyt, visiting faculty research participant.

*supported at least partially during current or proposed grant period

note: Four Ph.D. candidates (Powell, Bowyer, Swaile, and Cole) will be graduating or finishing their research during the second half of 1991. Several relatively new students (Staller, Colburn, Holland, Copper, and Clark) will be working on DOE projects during the proposed continuation grant period.

END

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12 / 16 / 91