Applications of partial filling micellar electrokinetic chromatography: from electrospray mass spectrometry to fluorescence polarization immunodetection

Wendy Marie Nelson

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Analytical Chemistry Commons, and the Biochemistry Commons

Recommended Citation


https://lib.dr.iastate.edu/rtd/11390
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600
Applications of partial filling micellar electrokinetic chromatography: from electrospray mass spectrometry to fluorescence polarization immunodetection

by

Wendy Marie Nelson

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Analytical Chemistry
Major Professors: Cheng S. Lee and Dennis C. Johnson

Iowa State University
Ames, Iowa
1996
Graduate College
Iowa State University

This is to certify that the Doctoral dissertation of

Wendy Marie Nelson

has meet the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College
DEDICATION

This thesis is dedicated to my husband, Bryan John Nelson. Only through his encouragement and trust does this dissertation exist.

Wendy Marie Nelson
TABLE OF CONTENTS

ABSTRACT........................................................................................................................vi

GENERAL INTRODUCTION ...............................................................................................1
Dissertation Organization .................................................................................................1
Triazine Herbicides ........................................................................................................1
Micellar Electrokinetic Chromatography (MEKC) ..........................................................2
Partial Filling Micellar Electrokinetic Chromatography (PF-MEKC) ...............................7
Detection Schemes Utilizing PF-MEKC ...........................................................................8
  On-Line PF-MEKC with Electrospray Mass Spectrometry (ESI-MS) .........................8
  On-Line PF-MEKC with Fluorescence Polarization Immunodetection ......................10
  Cross-Reactivity in Immunoassays .............................................................................11
Heterogeneous and Homogeneous Immunoassays ........................................................12
  On-Line Fluorescence Polarization Immunodetection ..............................................14

MECHANISTIC STUDIES OF PARTIAL FILLING
MICELLAR ELECTROKINETIC CHROMATOGRAPHY ........................................................17
Abstract ..........................................................................................................................17
Introduction .....................................................................................................................17
Experimental Section ......................................................................................................19
Results and Discussion ..................................................................................................20
  Theoretical Model of Partial Filling MEKC .................................................................22
  Band Broadening of Analyte Solutes in Partial Filling MEKC .....................................24
  Migration Times of Analyte Solutes in Partial Filling MEKC .....................................25
  Effect of Micelle Plug Length on Partial Filling MEKC Separation ..............................27
  Effect of SDS Concentration on Partial Filling MEKC Separation ............................28
Acknowledgment ...........................................................................................................29
References .......................................................................................................................29

ON-LINE PARTIAL FILLING MICELLAR ELECTROKINETIC
CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY ..............48
Abstract ..........................................................................................................................48
Introduction .....................................................................................................................49
Experimental Section ......................................................................................................51
  Micellar Electrokinetic Separations: UV Measurements .............................................51
  Mass Spectrometer and Electrospray Interface ............................................................51
  Chemicals .......................................................................................................................52
Results and Discussion ..................................................................................................53
Acknowledgments ...........................................................................................................56
References .......................................................................................................................56
ABSTRACT

The potential interference of surfactants used in micellar electrokinetic chromatography (MEKC) with on-line detection methods has limited the application of MEKC. We present a solution for eliminating the elution of surfactant used in MEKC into detectors. In comparison to conventional MEKC our separation method involves filling the capillary with a small plug of surfactant. We have coined this separation mode as partial filling micellar electrokinetic chromatography (PF-MEKC). The capillary in PF-MEKC is filled with electrophoresis buffer, followed by an introduction of micellar solution, and finally the sample injection. In PF-MEKC analytes first migrate into the micellar plug where the separation occurs, and then into the electrophoresis buffer which is free of surfactants, while the micellar plug remains in the capillary. Therefore, the elution of the analytes into a given detector occurs in the electrophoresis buffer. As a result, PF-MEKC provides a solution for coupling MEKC separation technique on-line to various detectors.

Before the application of PF-MEKC can be realized, it is important to investigate the overall mechanism and the effects PF-MEKC on analyte separation efficiency and resolution. The first paper in this work presents a theoretical model for predicting the separation behavior of triazine herbicides in PF-MEKC. The comparison between conventional and PF-MEKC in terms of separation efficiency and resolution of the triazines herbicides are presented and
discussed. In addition, optimization techniques, possible applications and advantages of PF-MEKC are similarly addressed.

The second paper describes the combination of PF-MEKC with electrospray ionization mass spectrometry (ESI-MS). Using PF-MEKC as the separation mode provides a mechanism for the separation and mass detection of neutral molecules without the interference of surfactant during detection. In this work, we discuss in detail the development and demonstration of PF-MEKC-ESI-MS using triazine herbicides as a model analyte system.

The third paper describes the combination of PF-MEKC with laser induced fluorescence polarization immunodetection. In analogy to a two dimensional detection system, PF-MEKC, as the first dimension, separates triazine analytes based on their difference in hydrophobicity. Fluorescence polarization immunodetection, as the second dimension, utilizes the antibody’s cross-reactivity for the recognition of triazine analytes with a common structural element (epitope).
GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction of theory and literature review related to this work. Relevant background concepts and the most recent progress in this area are also presented. The dissertation is arranged in a way such that the individual research publications of the author are presented as separate papers. Finally, a general summary presents comments and possible future directions for this work. A list of references cited in the general introduction conclude this dissertation.

Triazine Herbicides

We chose the class of triazine herbicides for this work as the model system for several reasons. Triazine herbicides are a heavily used class of agrochemicals worldwide. For example, atrazine, the main representative of the triazines, was the most commonly used herbicide in the United States in 1993\(^{(1)}\). Although of lower toxicological interest concerning human consumption, triazine herbicides are a valuable indicator of water pollution and have already been found in ground water in some areas\(^{(2)}\). Various detection methods for triazines have been developed in the past. Some of these detection methods are based on immunoassays\(^{(2-10)}\), mass spectrometry\(^{(11)}\), gas chromatography\(^{(12,13)}\), high performance liquid chromatography\(^{(14)}\), supercritical fluid extraction\(^{(15)}\), and immunoaffinity chromatography\(^{(16)}\). Since more than just one triazine herbicide may be used, especially in areas with extensive and diverse agriculture, there is a need to screen for multiple (triazine) herbicides. In light of this
requirement, our investigations have focused on five heavily used triazine herbicides including atrazine, simazine, propazine, ametryne, and prometryne.

The five triazine herbicides studied in this work are shown in Figure 1. Figure 1 depicts both the structures and pKₐ values of the triazines. The triazines exist as cationic species when the solution pH is near or below the pKₐ. Although the triazines are good candidates for separation by capillary zone electrophoresis (CZE), the low pKₐ values of the chloro-triazines do not permit separation in neutral pH buffers. Accordingly, micellar electrokinetic chromatography is applied in this work to successfully demonstrate the separation of the triazine herbicides.

**Micellar Electrokinetic Chromatography (MEKC)**

Micellar electrokinetic chromatography, MEKC, was developed by Terabe after a suggestion by Nakagawa, for the electrokinetic separation of neutral molecules. Others have preferred the terminology of micellar electrokinetic capillary chromatography (MECC). Following Otsuka and Terabe, I will continue to use MEKC to stress the electrokinetic nature of the separation mechanism. Due to the fact that MEKC has been described thoroughly in the literature, I will present only a brief overview of the technique. The purpose of this overview is two fold: one, to describe the main separation mechanism used throughout this work, and two, to serve as a foundation for the comparison between conventional and partial filling MEKC described in the first paper. Using the same instrumentation as CZE, MEKC requires only the addition of a surfactant to the background electrolyte. When the surfactant concentration is larger than the critical micelle concentration
Fig. 1 Triazine herbicides with corresponding $pK_a$ values.
CMC, micelles are formed. A micelle is comprised of aggregated surfactant monomers. The micelle is spherical in shape with the hydrophobic tail of the surfactant monomer orientated to the interior of the aggregate, allowing for the hydrophilic head to be exposed to the aqueous electrophoresis buffer. Solutes partition between the micelle and the bulk aqueous phase. More hydrophilic solutes reside primarily in the aqueous phase and elute closer to the electroosmotic flow. However, more hydrophobic solutes reside primarily in the micelle interior and, as a result, elute closer to the time of the micelle. Figure 2 shows the analysis of neutral molecules with an anionic surfactant and a neutral or alkaline buffer in fused silica capillaries. In this case, which is the most common situation, the electroosmotic flow, \( U_{eo} \), is directed toward the cathode. The micelles are negatively charged and, having their own negative electrophoretic velocity \( U_{ep,mc} \), will move with a net velocity \( U_{mc} = U_{eo} + U_{ep,mc} \). When the electroosmotic flow is fast enough, the micelles will move in the same direction as the electrophoresis buffer, but at a slower velocity.

Analyte molecules will be distributed between the aqueous and micellar phases. Very polar molecules that exist only in the aqueous phase will elute with a mobility \( U_{eo} \) and be detected at \( t_0 \). This is the shortest elution time possible in the system and can, by analogy, be compared to the void volume concept in column chromatography. Highly hydrophobic compounds, staying in the micelle interior all the time, will elute at the corresponding \( t_{mc} \). All other analytes must elute within the elution window of \( t_0 \) and \( t_{mc} \), depending upon their partitioning between the aqueous and the micellar phases.

In MEKC the capacity factor, \( k' \), is defined as

\[
k' = \frac{n_{mc}}{n_{eq}}
\]
Fig. 2 Separation mechanism in micellar electrokinetic chromatography.
where \( n_{mc} \) is the number of analyte molecules in the micelle interior and \( n_{aq} \) is the number of analyte molecules in the polar aqueous phase. The capacity factor of an analyte is directly related to the migration time of the analyte, \( t_r \), and the range of retention window defined by \( t_o \) and \( t_{mc} \). The fraction of molecules in the aqueous phase or the retention ratio, \( R_r \), is shown to be

\[
R_r = \frac{n_{aq}}{n_{aq} + n_{mc}} = \frac{1}{1 + k'}
\]

Thus the net velocity of an analyte, \( \nu_r \), can be expressed as

\[
\nu_r = R_r \nu_{eo} + (1 - R_r) \nu_{mc}
\]

where \( \nu_{eo} \) is the linear electroosmotic velocity, and \( \nu_{mc} \) is the net velocity of the micelle. After combination of equations 1-3, and using the inverse relationship between migration time and migration velocity, the expression of an analyte’s retention time, \( t_r \), in MEKC is given as

\[
t_r = t_o \{(1 + k') / [1 + (t_o / t_{mc}) k']\}
\]

Subsequently, the capacity factor, \( k' \), can be calculated from

\[
k' = (t_r - t_o) / \{t_o [1 - (t_r / t_{mc})]\}
\]

It should be stressed that a solute capacity factor of infinity in MEKC implies that the solute stays in the micellar phase at all times. Equations 4 and 5 are slightly different from their counterparts in liquid chromatography. This difference is caused by the fact that the micellar phase which functionally corresponds to the traditional stationary phase in liquid chromatography is actually moving in MEKC. Therefore, the micellar phase is called a “pseudo-stationary phase” in MEKC. If the electroosmotic flow is equal but in opposite direction to the electrophoretic velocity of the micelle, \( \nu_{eo} = - \nu_{ep,mc} \), \( t_{mc} \) becomes infinite and the micellar phase is truly stationary. Subsequently, equations 4 and 5 become
\[ t_r = (1 + k')t_o \]  \hspace{1cm} (6)

and

\[ k' = (t_r - t_o) / t_o \]  \hspace{1cm} (7)

respectively. Equations 6 and 7 represent the traditional equations in liquid chromatography.

Calculation of a solute's capacity factor requires knowledge of \( t_o \) and \( t_{mc} \). As in CZE, \( t_o \) is determined by measuring the migration time of small, neutral molecules, sufficiently polar to avoid partitioning with the micelles. The UV absorbance of formamide\(^{(31, 32)}\), acetone\(^{(33, 34)}\), and the baseline disturbance caused by the presence of methanol\(^{(23, 31, 32)}\) have been used for the measurement of \( t_o \). Following a practice in tracer electrophoresis\(^{(35, 36)}\), \( t_{mc} \) is measured by incorporating a hydrophobic dye to the sample. Being completely retained in the micelle, the migration behavior of the dye is considered to be representative for the micelle. Sudan III\(^{(23)}\) is the most common \( t_{mc} \) marker. Sudan IV\(^{(37, 38)}\) and quinine hydrochloride\(^{(39-41)}\) have been reported as suitable \( t_{mc} \) markers as well.

**Partial Filling Micellar Electrophoretic Chromatography (PF-MEKC)**

The potential interference of surfactants used in MEKC with on-line detection methods, such as mass spectrometry and fluorescence polarization immunodetection studied in this work, has limited the application of MEKC. We present a solution for eliminating the elution of MEKC surfactants into detectors while preserving the separation resolution provided by MEKC. In comparison to conventional MEKC, our separation method involves filling a small portion of the capillary with a micellar solution to achieve a separation. We have coined this separation mode as partial filling micellar electrokinetic chromatography (PF-MEKC). The
capillary in PF-MEKC is filled with electrophoresis buffer, followed by an introduction of micellar solution, and finally the sample injection. Analytes first migrate into the micellar plug where the separation occurs, and then into the electrophoresis buffer which is free of surfactant. Therefore, the elution of the analytes into various detectors occurs in the electrophoresis buffer while the surfactant plug remains in the capillary. As a result, the use of PF-MEKC provides a potential solution for coupling a MEKC separation on-line to electrospray mass spectrometry and fluorescence polarization immunodetection.

Before the application of PF-MEKC can be realized, it is important to investigate the overall mechanism of PF-MEKC and analyte separation efficiency and resolution. The first paper in this work presents a theoretical model for predicting the separation behavior of triazine herbicides in PF-MEKC. The comparisons between conventional and PF-MEKC in terms of separation efficiency and resolution of the triazine herbicides are presented and discussed. Optimization techniques, possible applications and advantages of PF-MEKC are similarly addressed.

Detection Schemes Utilizing PF-MEKC

On-Line PF-MEKC with Electrospray Ionization Mass Spectrometry (ESI-MS)

The potential interference of MEKC surfactants with ESI-MS has been investigated by several research groups \(^{(42-44)}\). In the presence of a relatively high concentration of nonvolatile surfactant, the electrospray efficiency and mass sensitivity of analytes decrease significantly relative to those observed in the absence of surfactant. The choice of surfactant added to the running buffer in MEKC is also highly significant because surfactants are nonvolatile and in
many cases cause background ions in the mass spectrum. Both positive and negative ESI mass spectra of sodium dodecyl sulfate (SDS), which is the most frequently used surfactant in MEKC, have been observed by Smith and coworkers\textsuperscript{(45)}.

The combination of MEKC with ESI-MS is very attractive for the possibility of selectivity enhancement, for identification of analytes, and for further structure determination of analyte or unknowns in a MS-MS mode. The need for direct coupling of MEKC with ESI-MS has led to several new approaches including the use of high-molecular weight surfactants\textsuperscript{(43)}, semi-permeable membrane interface\textsuperscript{(44)}, and a voltage switching and buffer renewal system\textsuperscript{(46)}. Ozaki and colleagues demonstrated the use of high molecular weight surfactants\textsuperscript{(43)} such as butyl acrylate-butyl methacrylate-methacrylic acid copolymer sodium salt. By using a high molecular weight surfactant, the authors avoided the production of high levels of low mass background ions in the mass spectrum. In other words, the mass range of high molecular weight surfactants in ESI-MS is far above those of small analytes, therefore, increasing the signal to noise ratio of the small analytes. Furthermore, high molecular weight surfactants can be used at very low concentrations because of the substantially low CMC.

The interface introduced by Foley et al.\textsuperscript{(44)} utilized a semi-permeable membrane which selectively permeated small analyte molecules to the mass spectrometer while retaining the relatively larger buffer additives such as surfactants. The voltage switching and buffer renewal system\textsuperscript{(46)} presented by Lamoree et al. allowed on-line heart-cutting of the analyte zones in the MEKC capillary with subsequent transfer via a second CZE capillary for introduction to the mass spectrometer.
In this work, we demonstrate the direct coupling of PF-MEKC with ESI-MS for the mass determination of triazine herbicides. The advantage of utilizing PF-MEKC as the separation mode for coupling with ESI-MS is two fold. First, it is possible to use a common and readily available surfactant such as SDS, therefore, avoiding timely synthesis or characterization of less common surfactants or polymers. Second, by utilizing PF-MEKC, it is also possible to avoid time consuming and expensive instrument modifications such as a voltage switching and buffer renewal system developed by Lamoree et al.\(^{(46)}\). Furthermore, the application of PF-MEKC as the on-line separation mode allows for long term stable operation of ESI-MS. The second paper of this dissertation discusses in detail the development and the demonstration of PF-MEKC-ESI-MS using triazine herbicides as a model analyte system.

**On-Line PF-MEKC with Fluorescence Polarization Immunodetection**

There has recently been a heightened interest in developing immunoassays for pesticides, herbicides and other environmental chemicals for residue analysis\(^{(47-53)}\) even though the use of antibodies as an analytical tool was first demonstrated many years ago\(^{(54)}\). The use of immunoassays for analysis of small molecules has been extensive in endocrinology, clinical chemistry, and other fields. However, applications of immunoassay technology by environmental chemists is well behind other fields, primarily because the compounds of interest can be analyzed by gas or liquid chromatography without extensive sample pretreatment. Nonetheless, the many advantages of immunoassays such as speed, specificity, sensitivity, and simplicity in sample treatment call for continued development in this field.
There are a number of steps in the development and implementation of an immunoassay. These steps include selection of hapten, synthesis and characterization of hapten, covalent binding of hapten to carrier molecules such as proteins, immunization, purification and characterization of the antibody, development and optimization of the immunoassay format, and final demonstration of application to field samples and validation of results. This general introduction will cover the primary disadvantage of current immunoassays, common detection schemes currently used in immunoassays, and finally focusing on the development of on-line fluorescence polarization immunodetection in PF-MEKC.

**cross-reactivity in immunoassays**

Immunoassays are based on an antibody-antigen interaction. However, it has been frequently observed that antibodies bind to a number of structurally similar compounds, rather than being monospecific for a single analyte. This phenomenon, which occurs with both mono- and poly- clonal antibodies is termed cross-reactivity. Cross-reactivity is the immunochemical analogue of interference in analytical measurements. A suitable example for non-immunochemical cross-reactivity is an ion-selective electrode. An ion-selective electrode typically responds to interfering ions with a radius of the same size as the measuring ion.

The problem of multiple cross-reacting analytes in a sample is a very general one, occurring in agricultural, clinical, environmental, and other branches of analytical chemistry. False qualitative and quantitative data will be obtained in the presence of cross-reacting compounds. On the other hand, cross-reactivity enables the use of antibodies as a screening
tool for multiple analytes or for a whole class of compounds. Usually this approach yields a
combined signal and indicates whether a certain class of analytes is present or absent. In
certain cases, this yes-no type answer is sufficient, but in other cases structurally similar
compounds need to be differentiated. The third paper in this work describes how the main
disadvantage of cross-reactivity in immunoassays can be utilized to develop multianalyte
immunodetection in PF-MEKC. However, it is first necessary to discuss the detection
schemes currently applied in immunoassays and the integration of fluorescence polarization
immunodetection with PF-MEKC.

**homogeneous and heterogeneous immunoassays**

Immunoassays are classified into the homogeneous and heterogeneous formats. Homogeneous assays are based on the fact that the measured property of the labeled molecule changes when bound to an antibody. The bound molecules can thus be distinguished from unbound molecules without a separation step. The advantage of the homogeneous assay is that it facilitates automation. Heterogeneous assays, in contrast, require the separation of free and bound species, which is a major disadvantage, especially with regard to automation.

Various heterogeneous immunoassays including the radioimmunoassay (RIA) and the enzyme linked immunosorbent assay (ELISA) have been developed. RIA involves the use of radioisotopes and requires special facilities including scintillation counting. Although RIA is among the most sensitive measuring techniques it has a number of drawbacks. Radiation can cause a health hazard which necessitates special handling techniques in the laboratory. Furthermore, long term assay standardization is prevented because the useful lifetime of the
reagents is governed by the half-life of the radioactive label.

The most common heterogeneous immunoassay which utilizes enzymes and enzymatic reactions in the detection scheme is ELISA. A typical ELISA is performed in a 96 well microtiter plate by coating antibody on the wall of each well. Sample and standard solutions are added to form antigen-antibody complexes on the wall. Another solution containing enzymes conjugated with antibodies is added to each well. The enzyme-antibody conjugates, which bind with the antigen-antibody complexes on the wall, are proportional to the antigen concentration. The enzymes catalyze a chromogenic reaction when a specific substrate is added. Subsequently, the antigen concentration is then determined by measuring the absorbance of enzymatic products. The primary disadvantages of ELISA are multiple washing steps and long incubation times. Numerous reviews and applications of ELISA have been published.

By comparison, fluorescence polarization immunoassay (FPIA), as first described by Dandliker et al., has enjoyed rising success among various types of homogeneous immunoassays. FPIA is based on the difference in fluorescence polarization of a small fluorochrome-labeled hapten (tracer) before and after it’s complexation with a much larger antibody. In 1984, Spencer reviewed the applications of this technique to clinical analysis. A more recent review of fluorescence polarization was offered by Gutierrez et al. where methods, applications and instrumentation were discussed. Furthermore, several research groups have developed immunoassays utilizing fluorescence polarization as the detection scheme.

On-line fluorescence polarization immunodetection utilizing PF-MEKC offers two
distinct advantages for coupling with immunoassays. One, PF-MEKC allows for an
electrokinetic separation of analytes without the use of immobilized matrix interaction as in
high performance liquid chromatography. Two, PF-MEKC allows for the electrokinetic
separation of analytes without the interference of surfactant into the detector region.
Consequently, it becomes natural to integrate the homogeneous separation technique of PF-
MEKC with the on-line homogeneous immunodetection based on the principle of FPIA.
Therefore, the final portion of the general introduction in this dissertation will focus on the
principles used to develop on-line fluorescence polarization immunodetection for the direct
coupling with PF-MEKC.

**on-line fluorescence polarization immunodetection**

The principles of fluorescence polarization have been known since the 1920’s\(^{(74)}\).
Somewhat later, Weber\(^{(75)}\) developed the instrumentation required for the application of this
technique. The theory of polarization is well documented in the literature\(^{(76,77)}\), so only a brief
description of the technique will be given here.

Fluorescence polarization is based on the observation that fluorescent molecules in
solution, excited with plane-polarized light, will emit light back into a fixed plane if the
molecules remain stationary between excitation and emission. Molecules in solution, however,
rotate and tumble, and thus the planes into which light is emitted can be very different from
the plane used for excitation. Subsequently, the polarization of a molecule is proportional to a
molecule’s rotational relaxation time. Rotational relaxation is related to viscosity (\(\eta\)), absolute
temperature (\(T\)), molecular volume (\(V\)), and the gas constant (\(R\)) therefore, polarization is
proportional to $3\eta V/RT$. Consequently, if viscosity and temperature are held constant, polarization is directly proportional to molecular volume. Fluorescent polarization can be measured by comparing the amount of light emitted into horizontal and vertical planes. Accordingly, if a fluorescent molecule excited with a plane of polarized light (for example, vertical plane), it is then possible to calculate the polarization value, $P$, according to the following equation:

$$ P = \frac{I_v - I_h}{I_v + I_h} $$

where $I_v$ and $I_h$ are the vertical and horizontal fluorescent intensities, respectively. The polarization value, $P$, is defined as the difference of the vertical and horizontal fluorescent intensities divided by the sum of these intensities. High polarization values mean that the molecule is large and rotates little during the excited state; low values mean that the molecule is small and rotates very quickly during the excited state.

The third paper in this work demonstrates the separation and immunodetection of triazine herbicides by PF-MEKC equipped with post-column laser induced fluorescence polarization immunodetection. In analogy to a two-dimensional detection system, PF-MEKC, as the first dimension, separates triazine analytes based on their difference in hydrophobicity. The second dimension, fluorescence polarization immunodetection, utilizes the antibody’s cross-reactivity for the recognition of triazine analytes with a common structural element (epitope). As mentioned before, the overall advantage of utilizing PF-MEKC eliminates the introduction of surfactant into the immunoreactor.

More specifically, fluorescence polarization immunodetection in PF-MEKC is designed so that a liquid junction between the separation and detection capillaries allows for
the introduction of antibody as well as the transfer of triazine analyte-antibody complex into the detection capillary. In this scheme, the separation buffer contains a hapten-fluorochrome conjugate (tracer) which competes with the analyte for limited antibody in the liquid junction. In the situation when only tracer is present, the binding of tracer to antibody induces the emission of polarized fluorescence. Competition occurs when a triazine analyte is also present in the system. When the trizaines are separated by PF-MEKC and eluted into the liquid junction the antibody preferentially binds the triazine analyte. Consequently, the analyte’s binding to the antibody decreases the degree of tracer-antibody complexation, and therefore, results in a change in the emitted fluorescence polarization of tracer. Thus, the change in the emitted fluorescence polarization due to analyte competition identifies the presence of triazine herbicides as they sequentially elute from the PF-MEKC capillary. In conclusion, the antibody's cross reactivity to each herbicide analyte in combination with laser induced fluorescence polarization detection actualizes multiresidue immunodetection of environmental contaminants separated by PF-MEKC.
MECHANISTIC STUDIES OF PARTIAL FILLING
MICELLAR ELECTROKINETIC CHROMATOGRAPHY

A paper accepted by Analytical Chemistry

Wendy M. Nelson and Cheng S. Lee

ABSTRACT
The need for coupling micellar electrokinetic chromatography (MEKC) with electrospray mass spectrometry initiates the development of partial filling MEKC. In comparison with conventional MEKC, only a small portion of the capillary is filled with a micellar solution for performing the separation in partial filling MEKC. Analytes first migrate into the micellar plug where the separation occurs, and then into the leading electrophoresis buffer which is free of surfactants. A theoretical model is proposed for predicting the separation behavior of triazine herbicides in partial filling MEKC. The comparisons between conventional and partial filling MEKC in terms of separation efficiency and resolution of triazine herbicides are presented and discussed. The optimization techniques, possible applications and advantages of partial filling MEKC are similarly addressed.

INTRODUCTION
Micellar electrokinetic chromatography (MEKC) was introduced by Terabe\(^1\,^2\) for the separation of neutral compounds. MEKC involves the solubilization of surfactant monomers
in solution at a concentration above the critical micelle concentration. At these concentrations, surfactant monomers form roughly spherical aggregates, or micelles. Neutral solutes differentially partition between the aqueous phase and the hydrophobic interior of the micelles. The partitioning difference among neutral solutes contributes to the separation in MEKC.

The potential interference of surfactants used in MEKC with on-line electrospray mass spectrometry detection has been presented by several research groups\(^3\)\(^-\)\(^5\). In the presence of a relatively high concentration of nonvolatile surfactant, the electrospray efficiency and the mass sensitivity of analytes decrease significantly relative to those observed in the absence of surfactant. The need for coupling MEKC with electrospray mass spectrometry has led to several new approaches including the use of high-molecular weight surfactants\(^6\), semi-permeable membrane interface\(^5\), and partial filling MEKC\(^4\). The advantage of high-molecular weight surfactants is their low critical micelle concentration. Additionally, the use of high-molecular weight surfactants 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. The new interface introduced by Foley et al. \(^5\) utilizes a semi-permeable membrane that selectively permeates small analyte molecules to the mass spectrometer while retaining the relatively larger surfactants.

In comparison with conventional MEKC, partial filling MEKC involves filling a small portion of the capillary with a micellar solution to achieve a separation. The capillary in partial filling MEKC mode is filled with electrophoresis buffer, followed by an introduction of micellar solution, and finally sample injection. Analytes will first migrate into the micellar plug where the separation occurs, and then into the electrophoresis buffer which is free of
surfactants. This paper describes the mechanistic studies of partial filling MEKC by a
comparison with conventional MEKC using triazine herbicides as a model system. The
effects of micellar concentration, plug length, and overall capillary length on the separation
resolution and efficiency of partial filling MEKC are investigated both experimentally and
theoretically. Optimization of partial filling MEKC and advantages of partial filling MEKC in
the manipulation of retention window are also presented and discussed.

EXPERIMENTAL SECTION

Electrokinetic separations were performed using fused silica tubing from Polymicro
Technologies (Phoenix, Arizona). Capillary dimension consisted of 50 mm i.d. x 360 mm o.d.
with a total length of 45 cm, 30 cm to the detector. Detection was carried out by a Linear
UVIS 200 detector from Linear Instruments (Reno, Nevada) modified for on-column
detection. Detection wavelength was set at 226 nm. A Spellman CZE 1000R High Voltage
Power Supply (Plainview, New York) delivered -11.25 kV to the detector end of the capillary
for electrokinetic injection and for electrophoretic separation. All experiments were
performed at room temperature, 24 °C. Data collection was performed by HP 35900D analog
to digital interface board with the HP G1250C General Purpose Chemstation Software
(Hewlett Packard, Fullerton, California).

Sodium dodecyl sulfate (SDS) of protein research grade was purchased from
Boehringer Mannheim GmbH (Mannheim, Germany), and used as received. Phosphate buffer
was prepared by titrating 10 mM solutions of monobasic with dibasic sodium phosphate
(Fisher Scientific, Pittsburgh, Pennsylvania) to a pH of 7. HPLC grade methanol (Fisher
Scientific, Pittsburgh, Pennsylvania) and quinine hydrochloride (Aldrich, Milwaukee, Wisconsin) were used as the electroosmotic flow marker and the micellar marker, respectively.

Ametryne, Atrazine, prometryne, propazine, and simazine were purchased from ChemService (West Chester, Pennsylvania). Triazine herbicides with an individual concentration of $10^{-5}$ M were prepared in phosphate buffer containing 5% (v/v) methanol. The standard herbicide solution did not contain any SDS surfactant. All solutions were prepared using water purified by a NANOpure II system (Dubuque, Iowa) and further filtered with a 0.2 mm Supor-200 membrane filter from Gelman Sciences (Ann Arbor, Michigan).

RESULTS AND DISCUSSION

For conventional MEKC separation of triazine herbicides, a fused silica capillary was filled with electrophoresis buffer containing 20 mM SDS and 10 mM phosphate buffer at pH 7. The standard herbicide solution with the addition of $10^{-5}$ M quinine hydrochloride was electrokinetically injected for 3 sec at the running voltage. The running voltage of -11.25 kV was applied at the cathodic end (the UV detector end) for obtaining the separation of triazine herbicides shown in Fig. 1. Triazine herbicides with their corresponding structures and $pK_a$ values were listed in Table I. The elution order and the capacity factor of triazine herbicides were summarized in Table II with the more hydrophobic herbicides eluting latter in the micelle phase. Propazine coeluted with ametryne.

To compare with conventional MEKC, a fused silica capillary was initially filled with 10 mM phosphate buffer at pH 7 containing no SDS. A 20 mM SDS solution (in 10 mM
phosphate buffer at pH 7) and a standard herbicide solution were sequentially injected at the anodic end of capillary by using a -11.25 kV for 20 sec and 3 sec, respectively. The separation of triazine herbicides in partial filling MEKC was shown in Fig. 2. To identify the elution of micellar plug, quinine hydrochloride was added into a 20 mM SDS solution with a final concentration of $3.2 \times 10^{-4}$ M. To mimic the injection conditions in Fig. 2, a SDS solution containing quinine hydrochloride and a phosphate buffer free of SDS and triazine herbicides were injected sequentially by using a -11.25 kV for 20 sec and 3 sec, respectively. As shown in Fig. 3, a SDS plug with a peak width of approximate 20 sec was eluted at 7.38 min. In comparison with Fig. 2, all triazine herbicides other than prometryne were eluted ahead of the SDS micelle plug. Prometryne was eluted within the SDS micelle plug.

Several dramatic differences between conventional and partial filling MEKC were observed by comparing the electropherograms shown in Figs. 1-3. The decrease in the migration times of triazine herbicides was observed in partial filling MEKC. The separation efficiencies of triazine herbicides in conventional and partial filling MEKC were summarized and compared in Table III. The separation efficiencies of triazine herbicides other than prometryne were lower in partial filling than in conventional MEKC. However, prometryne coeluted within the SDS micelle plug and exhibited a greater separation efficiency in partial filling than in conventional MEKC. Propazine and ametryne were baseline separated and resolved in partial filling MEKC, but coeluted in conventional MEKC. In contrast, the separation resolutions of the other triazine herbicides were reduced in partial filling MEKC. Furthermore, the migration time of SDS micelle, $t_{MC}$, was reduced from 15.95 min in conventional MEKC to 7.38 min in partial filling MEKC.
Theoretical Model of Partial Filling MEKC

A theoretical model for partial filling MEKC separation of triazine herbicides was proposed and illustrated in Fig. 4. Due to the presence of 20 mM SDS, the SDS micelle plug exhibited a higher solution conductivity and a correspondingly lower electric field strength than those of surrounding phosphate buffer. In partial filling MEKC, a constant electric current, $I$, was applied across the entire capillary as

$$I = \frac{V_s}{L_s R_s} = \frac{V_B}{L_B R_B} \quad (1)$$

where $V$, $L$, and $R$ were the applied electric potential, capillary length, and specific resistance in each region with the subscripts of S and B for the surfactant plug and the phosphate buffer. The total capillary length, $L$, was 45 cm and the total applied electric potential, $V$, was 11.25 kV. The total capillary length and the total applied electric potential were given as

$$L = L_s + L_B \quad (2)$$

and

$$V = V_s + V_B \quad (3)$$

The micellar electrophoretic mobility, $\mu_{MC}$, which was measured from the quinine hydrochloride peak in conventional MEKC (Fig. 1) was $-4.75 \times 10^{-4}$ cm$^2$/V-sec. In
comparison with phosphoric acid, the ionic mobilities of phosphoric acid were reported as -3.4, -5.8, and \(-7.2 \times 10^{-4}\) cm\(^2\)/V-sec with the corresponding pK\(_a\) values of 2.1, 7.2, and 12.3\(^{(10)}\). The effective ion mobility of our background phosphate buffer at pH 7 was therefore estimated around \(-4.6 \times 10^{-4}\) cm\(^2\)/V-sec and was close to that of SDS micelles. Thus, the SDS micellar plug of partial filling MEKC separation could be treated, in first order approximation, as a steady migrating zone. A SDS plug which was injected by a \(-11.25\) kV for 20 sec was shown in Fig. 3 with a peak width of approximately 20 sec. No significant band broadening of SDS micellar plug was observed. The SDS plug length, \(L_s\), in partial filling MEKC was estimated as

\[
L_s = \frac{L_{o_{inj}}}{t_{MC}}
\]

where \(L_o\) and \(t_{inj}\) were the distance between the injection point and the UV detector and the electrokinetic injection time, respectively. The calculated value for \(L_s\) was 1.32 cm.

According to the current measurements, the solution resistances of phosphate buffer and 20 mM SDS solution in a 50 mm i.d. x 360 mm o.d. capillary, \(R_B\) and \(R_s\), were calculated as \(3.55 \times 10^7\) \(\Omega/cm\) and \(1.85 \times 10^7\) \(\Omega/cm\), respectively. By using eqs. (1)-(4), the electric potentials and the electric field strengths in the micelle plug and in the phosphate buffer were calculated and summarized in Table IV. In comparison with a field strength of 254 V/cm at phosphate buffer region, a much lower electric field strength of 132 V/cm was estimated in the micelle plug.
Band Broadening of Analyte Solutes in Partial Filling MEKC

Since the electric field strength in the micelle plug was significantly different from the electric field strength in the phosphate buffer, additional band broadening occurred in the analyte band. More specifically, the analyte band in the micelle plug migrated slower due to its partitioning with the micelle and the presence of a lower electric field. Thus, all analyte molecules that diffuse past the micelle front on the left (see Fig. 4) encountered a higher electric field, and therefore migration speed driven by electroosmosis increased. In comparison with conventional MEKC, the presence of additional band broadening phenomenon at the interface between the micelle plug and the leading phosphate buffer contributed to a generally lower separation efficiencies of the triazine herbicides in partial filling MEKC (see Table III). As shown in Figs. 2 and 3, prometryne, however, coeluted within the SDS micelle plug at 7.33 min. By reducing its migration time from 12.50 min in conventional MEKC to 7.33 min in partial filling MEKC, the increase in the separation efficiency of prometryne was thus observed in partial filling MEKC.

In partial filling MEKC, the electroosmotic velocities were different between the micelle plug and the phosphate buffer region due to their difference in the electric field strength. However, the bulk velocity of fluid at each cross section in the capillary had to be the same for a noncompressible fluid. The difference between the electroosmotic velocities and the bulk velocity generated hydrostatic pressures and additional laminar flows in both the micelle plug and the phosphate buffer region\(^\text{11}\). In general, laminar flows display a characteristic parabolic flow pattern which give rise to additional band broadening of analytes in partial filling MEKC.
Migration Times of Analyte Solutes in Partial Filling MEKC

According to Chien’s theory\(^{(11)}\), the bulk velocity, \(v_b\), in partial filling MEKC is shown to be

\[
v_b = \frac{(L_S v_{eo,S} + L_B v_{eo,B})}{L}
\]

(5)

where \(v_{eo,S}\) and \(v_{eo,B}\) were the electroosmotic velocities in the surfactant plug and the phosphate buffer, respectively. By assuming the same electroosmotic mobility across the entire capillary, the bulk velocity in partial filling MEKC would therefore be the same as the electroosmotic velocity in conventional MEKC. This was confirmed by comparing the migration times of methanol peaks shown in Figs. 2 and 3.

The bulk velocity, \(v_b\), in partial filling MEKC was measured as \(30 \text{ cm}/3.33 \text{ min} = 9.01 \text{ cm/min}\). The micellar electrophoretic mobility, \(\mu_{MC}\), was \(-4.75 \times 10^{-4} \text{ cm}^2/V\text{-sec}\). The migration times of triazine herbicides in partial filling MEKC, \(t_R\), was given as

\[
t_R = t_S + t_B
\]

(6)

where \(t_S\) and \(t_B\) were the migration times of analyte band moving through the SDS micelle plug and the phosphate buffer, respectively. The migration velocity of any analyte molecule in the SDS micelle plug, \(v_n\), was estimated as
\[ v_s = v_b + \left[ \mu_{MC} E_s k'/(1 + k') \right] \]  

(7)

where \( k' \) was the capacity factor measured in conventional MEKC and summarized in Table II. The difference in the migration velocity between the analyte band and the SDS micelle plug, \( v' \), was obtained as

\[ v' = v_s - (v_b + \mu_{MC} E_s) = -\mu_{MC} E_s/(1 + k') \]  

(8)

Thus, \( t_s \) as the migration time of analyte band moving through the SDS micelle plug was calculated as

\[ t_s = L_s/v' \]  

(9)

The remaining migration distance and the migration time in the phosphate buffer before the analyte band reached the UV detector window were given as

\[ L' = L_0 - t_s v_s \]  

(10)

and

\[ t_b = L'/v_b \]  

(11)
By using eqs. (6)-(11), the migration times of triazine herbicides in partial filling MEKC were predicted and compared with the measured migration times in Table V. Clearly, the predicted migration times of triazine herbicides other than prometryne were in good agreement with the observed migration times in partial filling MEKC. The difference between the predicted and the measured migration times of prometryne was mainly contributed by the estimation of SDS micelle plug length, $L_S$.

Furthermore, the capacity factor in partial filling MEKC, $k'_{PF\text{-MEKC}}$, was given as

$$k'_{PF\text{-MEKC}} = \frac{k' (L_O - L')}{L_O}$$

(12)

where $k'$ was the capacity factor in conventional MEKC. On the basis of eq. (10), $L'$ was the remaining migration distance in the phosphate buffer before the analyte reached the UV detector window. Before the analyte migrated into the phosphate buffer, the analyte remained in the SDS micelle plug and traveled within the micelle plug for the distance of $L_O - L'$. The capacity factors in partial filling MEKC were calculated and listed in Table V. In comparison with conventional MEKC, the reduction of the capacity factors for propazine and ametryne contributed to the baseline separation resolution obtained in partial filling MEKC.

**Effect of Micelle Plug Length on Partial Filling MEKC Separation**

To demonstrate the effect of micelle plug length on the separation of triazine herbicides in partial filling MEKC, the electrokinetic injection of a 20 mM SDS solution was reduced
from 20 sec to 10 sec with the application of a -11.25 kV. As shown in Fig. 5, prometryne with a migration time of 4.27 min was ahead of the SDS micelle plug with a migration time of 6.88 min (not shown). A significantly lower separation efficiency of prometryne was thus observed in Fig. 5 due to the experience of additional band broadening phenomenon at the interface between the micelle plug and the leading electrophoresis buffer. In the presence of a shorter SDS micelle plug, all triazine herbicides eluted earlier and separated within 5 min of the analysis time. However, a 10 sec SDS micelle plug was too short to achieve any measurable separation resolution between propazine and ametryne.

Effect of SDS Concentration on Partial Filling MEKC Separation

A 30 mM SDS solution (in 10 mM phosphate buffer at pH 7) and a standard herbicide solution were sequentially injected at the anodic end of the capillary by using -11.25 kV for 15 sec and 3 sec, respectively. By comparing the electropherograms shown in Figs. 5 and 6, the separation resolutions of simazine/atrazine and propazine/ametryne pairs were enhanced by raising the SDS concentration from 20 mM to 30 mM in the micelle plug. The selection of a 15 sec micelle injection in partial filling MEKC also ensured the elution of prometryne ahead of the SDS micelle plug which was eluted with a migration time of 7.87 min (not shown).

According to the theoretical model of partial filling MEKC proposed in this study, the effects of micellar concentration, plug length, and overall capillary length on the separation resolution and the migration time of analytes can be predicted. All these predictions only require the solution conductivity and the capacity factor obtained from conventional MEKC experiments. The use of partial filling MEKC not only provides a potential solution for
interfacing MEKC separation with on-line electrospray mass spectrometry detection, but also contributes to an additional manipulation of separation resolution in MEKC.

ACKNOWLEDGMENT

Support for this work by an EPA grant (R 823292-01) and the Microanalytical Instrumentation Center of the Institute for Physical Research and Technology at Iowa State University is gratefully acknowledged. W.M.N. is a recipient of the Graduate Assistantship in Areas of National Need (GAANN) Fellowship from the US Department of Education. C.S.L. is a National Science Foundation Young Investigator (BCS-9258652).

REFERENCES


TABLE I
Triazine Herbicides with Corresponding Structures and pK\textsubscript{a} Values\textsuperscript{a}

![Triazine Structure](image)

<table>
<thead>
<tr>
<th>Triazines</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>pK\textsubscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simazine</td>
<td>Cl</td>
<td>Ethyl</td>
<td>Ethyl</td>
<td>1.65-1.80</td>
</tr>
<tr>
<td>Atrazine</td>
<td>Cl</td>
<td>Isopropyl</td>
<td>Ethyl</td>
<td>1.68-1.85</td>
</tr>
<tr>
<td>Propazine</td>
<td>Cl</td>
<td>Isopropyl</td>
<td>Isopropyl</td>
<td>1.50-1.85</td>
</tr>
<tr>
<td>Ametryne</td>
<td>S-methyl</td>
<td>Ethyl</td>
<td>Isopropyl</td>
<td>4.00-4.10</td>
</tr>
<tr>
<td>Prometryne</td>
<td>S-methyl</td>
<td>Isopropyl</td>
<td>Isopropyl</td>
<td>4.05-4.10</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Taken from references (6-9).
### TABLE II

Elution Order and Capacity Factor for Triazine Herbicides

<table>
<thead>
<tr>
<th>Analyte Peak</th>
<th>Elution Order</th>
<th>Capacity Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Simazine</td>
<td>2</td>
<td>1.66</td>
</tr>
<tr>
<td>Atrazine</td>
<td>3</td>
<td>3.27</td>
</tr>
<tr>
<td>Propazine</td>
<td>4</td>
<td>6.62</td>
</tr>
<tr>
<td>Ametryne</td>
<td>4</td>
<td>6.62</td>
</tr>
<tr>
<td>Prometryne</td>
<td>5</td>
<td>12.79</td>
</tr>
<tr>
<td>Quinine hydrochloride</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE III
The Separation Efficiency of Triazine Herbicides in Conventional and Partial Filling MEKC

<table>
<thead>
<tr>
<th>Triazine Herbicides</th>
<th>The Number of Theoretical Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional MEKC</td>
</tr>
<tr>
<td>Simazine</td>
<td>64,000</td>
</tr>
<tr>
<td>Atrazine</td>
<td>85,000</td>
</tr>
<tr>
<td>Propazine</td>
<td>114,000</td>
</tr>
<tr>
<td>Ametryne</td>
<td>114,000</td>
</tr>
<tr>
<td>Prometryne</td>
<td>240,000</td>
</tr>
</tbody>
</table>
### TABLE IV

The Distribution of Applied Electric Potentials and Electric Field Strengths in Partial Filling and Conventional MEKC

<table>
<thead>
<tr>
<th>Capillary Region</th>
<th>Length in Each Region</th>
<th>Electric Potential</th>
<th>Electric Field Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial Filling MEKC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micelle Plug</td>
<td>1.32 cm</td>
<td>174 V</td>
<td>132 V/cm</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>43.68 cm</td>
<td>11,076 V</td>
<td>254 V/cm</td>
</tr>
<tr>
<td>Conventional MEKC</td>
<td>45 cm</td>
<td>11,250 V</td>
<td>250 V/cm</td>
</tr>
</tbody>
</table>
### TABLE V

The Migration Time and The Apparent Capacity Factor of Triazine Herbicides in Partial Filling MEKC

<table>
<thead>
<tr>
<th>Analyte Peak</th>
<th>Migration Time</th>
<th>Capacity Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted</td>
<td>Measured</td>
</tr>
<tr>
<td>Simazine</td>
<td>3.57 min</td>
<td>3.66 min</td>
</tr>
<tr>
<td>Atrazine</td>
<td>3.80 min</td>
<td>3.94 min</td>
</tr>
<tr>
<td>Propazine</td>
<td>4.29 min</td>
<td>4.42 min</td>
</tr>
<tr>
<td>Ametryne</td>
<td>4.29 min</td>
<td>4.69 min</td>
</tr>
<tr>
<td>Prometryne</td>
<td>5.20 min</td>
<td>7.20 min</td>
</tr>
</tbody>
</table>
Fig. 1 Conventional MEKC separation of $10^{-5}$ M each of (2) simazine, (3) atrazine, (4) propazine and ametryne, (5) prometryne. (1) methanol and (6) quinine hydrochloride were used as the electroosmotic flow marker and the micellar marker, respectively. Buffer, 10 mM phosphate/20 mM SDS, pH 7; capillary, 45-cm total length, 50-mm i.d. and 360-mm o.d., length to detector 30 cm; voltage, -11.25 kV and 3 s for injection, -11.25 kV for electrophoresis; UV detection at 226 nm.
Absorbance (226 nm)
Fig. 2 Partial filling MEKC separation of $10^{-5}$ M each of (2) simazine, (3) atrazine, (4) propazine, (5) ametryne, and (6) prometryne. (1) methanol was used as the electroosmotic flow marker. Electrophoresis buffer: 10 mM phosphate and pH 7; capillary, 45-cm total length, 50-mm i.d. and 360-mm o.d., length to detector 30 cm; voltage, -11.25 kV and 20 s for SDS injection (20 mM SDS in 10 mM phosphate buffer at pH 7), -11.25 kV and 3 s for triazine injection, -11.25 kV for electrophoresis; UV detection at 226 nm.
Absorbance (226 nm)
Fig. 3  Partial filling MEKC separation of $3.2 \times 10^{-4}$ M quinine hydrochloride in 20 mM SDS, 10 mM phosphate buffer, pH 7. Electrophoresis buffer: 10 mM phosphate and pH 7; voltage, -11.25 kV and 20 s for quinine hydrochloride injection, -11.25 kV and 3 s for electrophoresis buffer injection, -11.25 kV for electrophoresis. Other conditions are the same as in Fig. 2.
Fig. 3

Absorbance (226 nm)

Time (min)

10.00
Fig. 4 Schematic diagram of partial filling MEKC. B: the background phosphate buffer; S: the SDS micelle plug; A: the sample analytes; L_s: the SDS micelle plug length; L_o: the distance between the injection point and the UV detector; L: the total capillary length.
Time $t_0$

- $B$

UV Detector

Time $t$

- $L_b$

$L_0$

$L$

Fig. 4
Fig. 5 Partial filling MEKC separation of $10^{-3}$ M each of (2) simazine, (3) atrazine, (4) propazine and ametryne, and (5) prometryne. (1) methanol was used as the electroosmotic flow marker. Voltage, -11.25 kV and 10 s for SDS injection (20 mM SDS in 10 mM phosphate buffer at pH 7), -11.25 kV and 3 s for triazine injection, -11.25 kV for electrophoresis. Other conditions were the same as in Fig. 2.
Partial filling MEKC separation of $10^{-3}$ M each of (2) simazine, (3) atrazine, (4) propazine, (5) ametryne, and (6) prometryne. (1) methanol was used as the electroosmotic flow marker. Voltage, -11.25 kV and 15 s for SDS injection (30 mM SDS in 10 mM phosphate buffer at pH 7), -11.25 kV and 3 s for triazine injection, -11.25 kV for electrophoresis. Other conditions were the same as in Fig. 2.
ON-LINE PARTIAL FILLING MICELLAR ELECTROKINETIC
CHROMATOGRAPHY- ELECTROSPRAY IONIZATION MASS SPECTROMETRY

A paper accepted by Journal of Chromatography A

Wendy M. Nelson, Qing Tang, A. Kamel Harrata, and Cheng S. Lee

ABSTRACT

On-line combination of partial filling micellar electrokinetic chromatography (PF-MEKC) and electrospray ionization mass spectrometry (ESI-MS) is demonstrated for the analysis of triazine herbicides including atrazine, propazine, ametryne, and prometryne. In comparison with conventional micellar electrokinetic chromatography (MEKC), PF-MEKC involves filling a small portion of the capillary with a sodium dodecyl sulfate (SDS) micellar solution for achieving the separation. In PF-MEKC the triazine analytes first migrate into the micellar plug where the separation occurs, and then into the electrophoresis buffer which is free of surfactant. Consequently, the electroosmotic transfer of neutral triazine herbicides to ESI-MS at the end of PF-MEKC capillary is comparable to conventional capillary zone electrophoresis ESI-MS. Therefore, PF-MEKC-ESI-MS provides a mechanism for the separation and mass detection of neutral molecules without the interference of surfactant.
INTRODUCTION

The potential interference of surfactants used MEKC with on-line electrospray ionization (ESI) mass spectrometry (MS) has been investigated by several research groups. Direct coupling of MEKC to MS is hazardous due to the elution of nonvolatile surfactants from the MEKC capillary into the MS. The presence of a relatively high concentration of nonvolatile surfactants results in a significant loss of electrospray efficiency and sensitivity of analytes relative to those observed in the absence of surfactant. The choice of surfactant added to the running buffer in MEKC is also highly significant because surfactants are nonvolatile and in many cases may cause background ions in the mass spectrum. Both positive and negative ESI mass spectra of sodium dodecyl sulfate (SDS), which is the most frequently used surfactant in MEKC, have been observed by Smith and coworkers. To date, the use of a low molecular weight surfactant, such as SDS, has not yet been successfully demonstrated for the direct coupling of MEKC with ESI-MS.

The combination of MEKC with ESI-MS is very attractive for the possibility of selectivity enhancement, for the direct identification of analytes, and for the structure analysis of analyte molecules in a MS-MS mode. The need for coupling MEKC with ESI-MS has led to several new approaches including the use of high-molecular weight surfactants, a semi-permeable membrane interface, and a voltage switching and buffer renewal system. Ozaki and colleagues demonstrated the use of high molecular weight surfactants such as butyl acrylate-butyl methacrylate-methacrylic acid copolymer sodium salt and as a result avoided the production of high levels of low mass background ions in the mass spectrum. The interface introduced by Foley and Masucci utilized a semi-permeable membrane that
selectively permeates small analyte molecules to the mass spectrometer while retaining large
buffer additives such as surfactants. The voltage switching and buffer renewal system\(^4\)
introduced by Lamoree et al. allowed on-line heart-cutting of the analyte zones in the MEKC
capillary with subsequent transfer via a second CZE capillary for introduction to the mass
spectrometer.

Successful demonstration of on-line coupling of MEKC to ESI-MS involves the
elimination of surfactant introduction into the mass spectrometer. PF-MEKC was first
introduced by Terabe et al.\(^6\) as a potential solution for interfacing MEKC separations with
ESI-MS. In comparison with conventional MEKC (see Fig. 1), PF-MEKC involves filling a
small portion of the capillary with a micellar solution to achieve a separation. The capillary in
PF-MEKC is filled with electrophoresis buffer, followed by an introduction of micellar
solution, and finally a sample injection. In PF-MEKC the analytes first migrate into the
micellar plug where the separation occurs, and then into the electrophoresis buffer, which is
free of surfactant. The analytes in the electrophoresis buffer sequentially elute out of the
MEKC capillary and are subsequently introduced into ESI-MS while the surfactant plug
remains behind in the capillary. Once the analytes are detected by the mass spectrometer,
electrophoresis is terminated to avoid the surfactant plug eluting into the detector.

Mechanistic studies of PF-MEKC and it's comparison with conventional MEKC were
discussed by Nelson and Lee\(^7\). The effects of micellar concentration, plug length, and overall
length on separation efficiency and resolution of PF-MEKC were investigated both
experimentally and theoretically. A thorough understanding of the separation mechanism in
PF-MEKC offered the opportunity for on-line coupling of partial filling MEKC to ESI-MS. In
this paper, PF-MEKC-ESI-MS is presented using triazine herbicides as a model system.

EXPERIMENTAL SECTION

Micellar electrokinetic separations: UV measurements. Electrokinetic separations were performed using fused silica tubing from Polymicro Technologies (Phoenix, Arizona USA). Capillary dimension consisted of 50 μm i.d. and 360 μm o.d. with a total length of 45 cm, 30 cm to the detector. Detection was carried out by a Linear UVIS 200 detector from Linear Instruments (Reno, Nevada USA) modified for on-column detection. Detection wavelength was set at 226 nm. A Spellman CZE 1000R High Voltage Power Supply (Plainview, New York USA) delivered -9.0 kV to the detector end of the capillary for electrokinetic injection and for electrophoretic separation. All experiments were performed at room temperature, 24 °C. Data collection was performed by a Hewlett Packard HP 35900D analog to digital interface board with the HP G1250C General Purpose Chemstation Software (Hewlett Packard, Fullerton, California USA).

Mass spectrometer and electrospray interface. The mass spectrometer used in this study was a Finnigan MAT TSQ700 (San Jose, California USA) triple quadrupole equipped with an electrospray ionization source. The Finnigan MAT electrospray adapter kit, containing both gas and liquid sheath tubes, was used to directly couple PF-MEKC with ESI-MS without modifications. The electrospray needle was maintained at 5 kV for all ESI-MS measurements. The first quadrupole was used in selected ion monitoring mode at m/z ratios of 216 (atrazine), 228 (ametryne), 230 (propazine), and 240 (prometryne) at a scan rate of 0.125 sec/scan. The electron multiplier was set at 1.4 kV, with 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 methionine-arginine-phenylalanine-alanine (MRFA).

Interfacing PF-MEKC to ESI-MS was accomplished by using a 45 cm long, 50 μm i.d. and 192 μm o.d. fused silica capillary mounted within the electrospray probe. The capillary tip was fixed at 0.5 mm outside the electrospray needle. The inlet reservoir contained a 20 mM ammonium acetate buffer, pH 6.8, and was maintained at the same height as the electrospray probe. The mass spectrometer operated in the positive ion mode, therefore requiring an acidic sheath liquid to assist positive ion formation. The sheath liquid was a water/methanol/acetic acid solution (50:49:1, v/v/v) delivered by a Harvard Apparatus 22 Syringe Pump (South Natick, Massachusetts USA) at 5 μl/min. No sheath gas was employed during the PF-MEKC-ESI-MS measurements. Two Spellman CZE 1000R High Voltage Power Supplies (Plainview, New York USA) delivered electric potentials of 14 kV and 5 kV at the inlet electrode and electrospray needle, respectively. Therefore, a constant electric field of 200 v/cm was maintained during the PF-MEKC separation. A resistor wired in parallel with the high voltage electrode connecting the electrospray needle was incorporated as a current sink to protect the high voltage power supply.

**Chemicals.** SDS of protein research grade was purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and used as received. Background electrophoresis buffer was prepared by titrating a 20 mM ammonium acetate solution with a 50% (v/v) sodium hydroxide solution to pH 6.8. Ammonium acetate is needed to better maintain the efficiency and stability of the electrospray process, even though the buffer is within 0.2 pH units of its relative
minimum buffering capacity at pH 7.0. HPLC grade methanol and quinine hydrochloride (Aldrich, Milwaukee, Wisconsin USA) were used as the electroosmotic flow marker and the micellar marker, respectively. Ammonium acetate, sodium hydroxide, and methanol were purchased from Fisher Scientific (Pittsburgh, Pennsylvania USA).

Atrazine, propazine, ametryne and prometryne were purchased from ChemService (West Chester, Pennsylvania USA). The chemical structures of four triazine herbicides were included in Fig. 2. A standard triazine herbicide solution with an individual concentration of $10^{-4}$ M was prepared in 20 mM ammonium acetate buffer containing 10% (v/v) methanol. The standard herbicide solution did not contain any SDS surfactant. All solutions were prepared using water purified by a NANOpure II system (Dubuque, Iowa USA).

RESULTS AND DISCUSSION

In order to obtain a conventional MEKC separation of the triazine herbicides, a fused silica capillary was filled with electrophoresis buffer containing 25 mM SDS and 20 mM ammonium acetate buffer at pH 6.8. The standard herbicide solution with the addition of $2.8 \times 10^5$ M quinine hydrochloride was electrokinetically injected at the anodic end of the capillary by applying -9.0 kV for 1 sec to the cathodic end (the UV detector end). The same -9.0 kV was applied for the separation of triazine herbicides shown in Fig. 3. The elution order and capacity factor of triazine herbicides were summarized in Table I with the more hydrophobic herbicides eluting latter in the micelle phase.

For comparison with conventional MEKC, PF-MEKC was accomplished by employing a fused silica capillary filled with 20 mM ammonium acetate buffer at pH 6.8 containing no
SDS. A 25 mM SDS solution (in 20 mM ammonium acetate buffer, pH 6.8) and a standard herbicide solution were sequentially injected at the anodic end of the capillary by applying a -9.0 kV for 25 sec and 1 sec, respectively. The separation of triazine herbicides in PF-MEKC was shown in Fig. 4. To identify the elution of the micellar plug, quinine hydrochloride was added to a 25 mM SDS solution with a final concentration of $4.0 \times 10^{-4}$ M. By assuming each SDS micelle contained approximately 62 monomers, the addition of $4.0 \times 10^{-4}$ M quinine hydrochloride gave a ratio of one quinine hydrochloride molecule to one SDS micelle. To mimic the injection conditions in Fig. 4, a SDS solution containing quinine hydrochloride and a ammonium acetate buffer free of SDS and triazine herbicides were injected sequentially by applying a -9.0 kV for 25 sec and 1 sec, respectively. As shown in Fig. 5, a SDS plug with a peak width of approximately 26 sec eluted at 8.5 min.

Several dramatic differences between conventional and PF-MEKC were observed when comparing the electropherograms shown in Figs. 3, 4, and 5. A decrease in the migration times and separation efficiencies of triazine herbicides was observed in PF-MEKC when compared to conventional MEKC. In addition, the migration time of SDS micelle, $t_{mc}$, was significantly reduced from 11.9 min in conventional MEKC to 8.5 min in PF-MEKC. Mechanistic studies of PF-MEKC and a theoretical model for the prediction of separation behavior in partial filling MEKC were presented by Nelson and Lee. Optimization techniques, possible applications and advantages of partial filling MEKC were similarly addressed.

Interfacing PF-MEKC with ESI-MS was similar to CZE-ESI-MS with respect to the electroosmotic transfer of the neutral triazine herbicides to ESI-MS at the end of the MEKC
capillary. The analyte zones in PF-MEKC were first separated in the SDS micellar plug and subsequently migrated into the electrophoresis buffer which was free of SDS surfactant. In the electrophoresis buffer, the neutral triazine herbicides were driven towards the electrospray tip as the result of electroosmotic flow. In the PF-MEKC-ESI-MS experiments, the separation conditions including capillary dimensions and electrokinetic voltages were identical to those employed in PF-MEKC-UV studies.

The choice of the electrophoresis buffer in PF-MEKC has proven to be important with regard to the maintenance and efficiency of the electrospray process. On the basis of volatility, the ammonium acetate buffer was chosen over phosphate buffer for PF-MEKC-ESI-MS experiments. As shown in Fig. 6, the triazine herbicides including atrazine, propazine, ametryne, and prometryne were sequentially eluted and monitored by ESI-MS at m/z values of 216, 230, 228, and 242, respectively. In comparison with PF-MEKC-UV experiments (see Fig. 4), the longer migration distance towards the end of the capillary in PF-MEKC-ESI-MS accounted for the increase in the migration time. There was no further enhancement in the separation resolution of triazine herbicides after their migration into the electrophoresis buffer. Prometryne appeared as a shoulder and overlapped with ametryne in the reconstructed ion electropherogram. The mixing between the capillary eluent and the sheath liquid at the capillary tip contributed to the decrease in the separation resolution of triazine herbicides in PF-MEKC-ESI-MS. However, the selected ion monitoring of ESI-MS provided the direct identification of triazine herbicides.

As demonstrated in this study, the application of PF-MEKC provides an alternative approach for the direct coupling of MEKC separation with ESI-MS. Based on the
mechanistic studies of PF-MEKC\(^{(7)}\), it is possible to directly transfer the conventional MEKC separations to PF-MEKC-ESI-MS measurements without any instrument modifications. Due to the absence of surfactant in the electrospray process, long-term stable operation of PF-MEKC-ESI-MS is ensured. In conclusion, PF-MEKC-ESI-MS enables the separation and mass detection of neutral triazine herbicides that are difficult to analyze by CZE-ESI-MS. Further applications of PF-MEKC-ESI-MS involve obtaining structure information of analyte molecules in a MS-MS operation mode. The utilization of MS-MS would be particularly important for the analysis of unknown analytes.

ACKNOWLEDGMENTS

This work was supported by an EPA Grant (R 823292-01) and the Microanalytical Instrumentation Center of the Institute for Physical Research and Technology at Iowa State University is gratefully acknowledged. W.M.N. is a recipient of the Graduate Assistantship in Areas of National Need (GAANN) fellowship from the US Department of Education. C.S.L. is a National Science Foundation Young Investigator (BCS-9258652).

REFERENCES


<table>
<thead>
<tr>
<th>Analyte Peak</th>
<th>Elution Order</th>
<th>Conventional MEKC Retention Time</th>
<th>Capacity Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>1</td>
<td>3.92 min</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>2</td>
<td>8.69 min</td>
<td>4.48</td>
</tr>
<tr>
<td>Propazine</td>
<td>3</td>
<td>9.88 min</td>
<td>8.84</td>
</tr>
<tr>
<td>Ametryne</td>
<td>4</td>
<td>10.1 min</td>
<td>10.1</td>
</tr>
<tr>
<td>Prometryne</td>
<td>5</td>
<td>10.9 min</td>
<td>19.8</td>
</tr>
<tr>
<td>Quinine Hydrochloride</td>
<td>6</td>
<td>11.9 min</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Schematic diagrams comparing conventional and PF-MEKC. A: sample analytes; B: background electrophoresis buffer; S: surfactant in background electrophoresis buffer.
Figure 2: Structures of four triazine herbicides examined in this study.
Fig. 3  Conventional MEKC separation of $10^{-4}$ M each of (2) atrazine, (3) propazine, (4) ametryne, (5) prometryne. (1) methanol and (6) quinine hydrochloride were used as the electroosmotic flow marker and the micellar marker, respectively. Electrophoresis buffer: 20 mM ammonium acetate and 25 mM SDS, pH 6.8. Capillary: 50 μm i.d. and 360 μm o.d., 45 cm total length with 30 cm to detector. Applied voltage: -9.0 kV and 1 sec for injection, -9.0 kV for electrophoresis. UV detection at 226 nm.
Fig. 4 PF-MEKC separation of $10^{-4}$ M each of (1) atrazine, (2) propazine, (3) ametryne, (4) prometryne. Electrophoresis buffer: 20 mM ammonium acetate, pH 6.8. Applied voltage: -9.0 kV for 25 sec SDS injection (25 mM SDS in 20 mM ammonium acetate buffer, pH 6.8), -9.0 kV for 1 sec triazine injection, -9.0 kV for electrophoresis. Other conditions are the same as in Fig. 3.
Figure 4

Absorbance (226 nm)

1 (3.96)
2 (5.04)
3 (5.77)
4 (6.09)
Fig. 5  PF-MEKC separation of $4.0 \times 10^{-4}$ M quinine hydrochloride in 25 mM SDS, 20 mM ammonium acetate buffer, pH 6.8. Electrophoresis buffer: 20 mM ammonium acetate buffer, pH 6.8. Applied voltage: -9.0 kV for 25 sec quinine hydrochloride/SDS injection, -9.0 kV for 1 sec electrophoresis buffer injection, and -9.0 kV for electrophoresis. Other conditions are the same as in Fig. 3.
Fig. 6  Positive electrospray ionization mass electropherogram of triazine herbicides in 
PF-MEKC-ESI-MS. Applied voltage: 5 kV for electrospray, 14 kV for 
electrophoresis. Sheath liquid: methanol/water/acetic acid (50:49:1 v/v/v) at pH 
2.6, 5μl/min flow rate. Separation conditions of partial filling MEKC are the 
same as in Fig. 4.
Figure 6
POST-CAPILLARY FLUORESCENCE POLARIZATION
IMMUNODETECTION IN PARTIAL FILLING MICELLAR ELECTROKINETIC CHROMATOGRAPHY

A paper submitted to Journal of Chromatography A

Wendy M. Nelson and Cheng S. Lee

ABSTRACT

The separation and post-capillary immunodetection of triazine herbicides is demonstrated by partial filling micellar electrokinetic chromatography (MEKC) equipped with laser-induced fluorescence polarization detection. Partial filling MEKC is employed for the separation of neutral triazine herbicides without the introduction of MEKC surfactant into the post-capillary immunoreactor. A liquid junction between the separation and reaction capillaries in the post-capillary immunoreactor is created for the introduction of antibody as well as the transfer of antigen (triazine)-antibody complexes into the reaction capillary. In analogy to fluorescence polarization immunoassay, the separation buffer contains a hapten-fluorescein conjugate (tracer) which competes with triazine analyte for antibody binding in the liquid junction. The competition results in a change in the degree of tracer-antibody complexation, and therefore reduces the emitted fluorescence polarization of tracer. Consequently, the decrease in the emitted fluorescence polarization monitored by an on-column laser-induced fluorescence detection identifies the presence of triazine herbicides as
they sequentially elute from the separation capillary. The utilization of an antibody's cross-reactivity in combination with the resolving power of electrokinetic separation actualize multiresidue analysis of environmental contaminants.

INTRODUCTION

The use of immunoassays in trace analysis has proven to be a sensitive and selective analytical tool for various agricultural, biological, chemical, clinical, and environmental applications. The high degree of selectivity of antigen-antibody binding is the basis of immunochemical techniques and allows for quantification of trace amount of antigens in the presence of a large excess of other compounds. Despite the simplicity and sensitivity of immunoassays, a common drawback for all antibody based immunoassays is that antibodies cross react with structurally related compounds. This is because antibodies target epitopes, not the whole antigen. This is particularly the case in environmental analysis, where a number of structurally related compounds such as the parent compound, with its metabolites and degradation products, can be present in the sample matrix.

A broad spectrum of cross-reactivity is very useful for screening purposes within a class of compounds such as triazine herbicides. Because of the triazines' differences in cross-reactivity, the use of immunoassay for quantifying individual or total triazine composition in a complex mixture, however, has been limited. A series of monoclonal antibodies has been screened by Muldoon and colleagues for within-class specificity and sensitivity using various enzyme-linked immunosorbent assay (ELISA) formats. Three antibodies with different
within-class specificities were chosen and used with the modified ELISA format to quantify individual and total triazines by solving simultaneous equations derived from each antibody.

Similarly, immunoassays of triazine herbicides against panels of antibodies have been used to define reproducible patterns of cross-reaction which represent distinctive "spectra" of various triazines\(^2\). In this approach a sample containing triazines was analyzed by applying pattern matching algorithms to the spectral signatures. Karu et al.\(^3\) have investigated four alternative methods of multivariate analysis including discriminate analysis, maximum likelihood analysis, classification and regression trees, and computational neuronal networks. The use of immunoassays for multianalyte analysis of triazines, phenyl urea herbicides, and avermectins has been demonstrated.

Alternatively, the antibody based immunoassay has been used as a detection method following HPLC separation of a complex mixture\(^4\). HPLC/immunoassay offered up to a 1,000 fold increase in sensitivity over HPLC with ultraviolet absorption detection. The major limitation of HPLC/immunoassay was the absence of an on-line immunodetector allowing for real time immunochemical sensing of the column effluent. Specifically, fractions were collected and the immunoassays were performed on the individual fractions. In spite of the labor intensive and cumbersome nature of this combination, the analytical power of retention time and immunochemical selectivity of this hybrid analytical procedure was impressive for the identification of unknowns in complex sample matrices.

To perform rapid yet sensitive analyte screening advanced bioanalytical techniques for multiresidue analysis of cross-reacting compounds are essential. In this study post-capillary immunodetection of four triazine herbicides including simazine, atrazine, propazine, and
ametryne (Fig. 1), is presented and demonstrated as a potential solution for measuring cross-reacting analytes in a single assay. Post-capillary immunophoresis, shown in Fig. 2, combines the separation power of micellar electrokinetic chromatography (MEKC) with the specificity of antigen-antibody interactions. In analogy to a two-dimensional separation system, MEKC, as the first dimension, separates triazine herbicides based on their differences in hydrophobicity. Post-capillary immunoreaction as the second dimension, utilizes the antibody's cross-reactivity for the recognition of triazines with a common structural element (epitope).

A liquid junction between the separation and reaction capillaries in the post-capillary immunoreactor allows for the introduction of antibody as well as the transfer of antigen (triazine)-antibody complexes into the reaction capillary. For the direct sensing of the antigen-antibody reaction, a hapten-fluorescein conjugate (tracer) is added in the separation buffer. In the absence of triazine analytes, complexation of the tracer with a high molecular weight antibody slows down the rotational diffusion of the tracer, and the fluorescence emission of the tracer remains polarized. Once the analytes reach the liquid junction, the competition occurs between the tracer and triazine analytes for limited antibody. This competition decreases the degree of tracer-antibody complexation, and therefore reduces the emitted fluorescence polarization of the tracer. The change in emitted fluorescence polarization, monitored by an on-column laser-induced fluorescence polarization detection (see Figs. 2 and 3), allows for the identification of triazine analytes as they sequentially elute from the separation capillary.
EXPERIMENTAL SECTION

Partial Filling Micellar Electrokinetic Chromatography

To avoid the potential interference of sodium dodecyl sulfate (SDS) surfactant used in MEKC with the antigen-antibody reaction in the post-capillary immunoreactor, partial filling MEKC was employed for the separation of triazine analytes. In comparison with conventional MEKC, partial filling MEKC involved filling a small portion of the capillary with a SDS micellar solution to achieve a separation. The capillary in partial filling MEKC was filled with the electrophoresis buffer, followed by an introduction of micellar solution, and finally a sample injection. In partial filling MEKC, the analytes first migrated into the micellar plug where the separation occurred, and then into the electrophoresis buffer, which was free of surfactant$^5$.

Partial filling MEKC separations were performed using fused silica tubing from Polymicro Technologies (Phoenix, Arizona). Capillary dimension consisted of 50 μm i.d. and 360 μm o.d. with a total length of 45 cm, 30 cm to the detector. Detection was carried out by a linear UVIS 200 detector from Linear Instruments (Reno, Nevada) modified for on-column detection. Detection wavelength was set at 226 nm. A Spellman CZE 1000R High Voltage Power Supply (Plainview, New York) delivered -11.25 kV to the detector end of the capillary for electrokinetic injection and for electrophoretic separation. All experiments were performed at room temperature, 24 °C. Data collection was performed by HP 35900D analog to digital interface board with the HP G1250C General Purpose Chemstation Software (Hewlett Packard, Fullerton, California).
SDS of protein research grade was purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and used as received. The electrophoresis buffer was prepared by titrating a solution containing 10 mM monobasic sodium phosphate with dibasic sodium phosphate to a pH of 8, with the addition of 18 mM sodium chloride. HPLC grade methanol and quinine hydrochloride (Aldrich, Milwaukee, Wisconsin) were used as the electroosmotic flow marker and the micellar marker, respectively. Monobasic and dibasic sodium phosphate, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, Pennsylvania).

Simazine, atrazine, propazine, and ametryne were purchased from ChemService (West Chester, Pennsylvania). Triazine herbicides with an individual concentration of 10^{-4} M were prepared in phosphate buffer containing 10% (v/v) methanol. The standard herbicide solution did not contain any SDS surfactant. All solutions were prepared using water purified by a NANOpure II system (Dubuque, Iowa) and further filtered with a 0.2 μm Supor-200 membrane filter from Gelman Sciences (Ann Arbor, Michigan).

Fluorescence Polarization Immunoassay

The serum containing polyclonal antibody (rabbit number 841) raised against simazine was generously donated by Dr. Bruce D. Hammock at University of California, Davis. The cross-reactivity of antiserum for various triazines in competitive inhibition ELISA has been studied and reported by Harrison and co-workers(6). 5-(4,6-dichlorotriazynyl)aminofluorescein and 5-(4-ethylamino, 6-isopropylamino-triazinyl) aminofluorescein (Fig. 4), were purchased from Molecular Probes (Eugene, Oregon) and were used as tracers 1 and 2, respectively.

Fluorescence polarization measurements were performed with a Perkin Elmer LS50B spectrophotometer equipped with steady state polarizers (Norwalk, Connecticut).
Fluorescence was excited at 488 nm with a slit width of 9 nm. Fluorescence polarization was collected at 515 nm with a slit width of 7 nm. The antiserum was diluted in the assay buffer (10 mM phosphate buffer at pH 7) with various dilution ratios from 20 to 800. The serum dilution curves were constructed by measuring fluorescence polarization of reaction mixtures containing tracer and diluted antiserum in a 200 µL quartz cell with a 3 mm path length (Starna Spectrophotometer Cells, Atascadero, California). Fluorescence polarization immunoassays for each triazine analyte were carried out by mixing the solution containing tracer and triazine with diluted antiserum in a quartz cell. In a competitive format, the fluorescence polarization of the reaction solution was measured immediately after mixing the reagents.

Post-Capillary Immunoreactor

The post-capillary immunoreactor shown in Fig. 2 consisted of the separation and reaction capillaries separated by a 20 µm gap. The separation and reaction capillaries with the dimension of 50 µm i.d and 360 µm o.d. were fused silica tubing from Polymicro Technologies (Phoenix, Arizona). The capillaries were aligned at the same height and lateral position for the generation of a 20 µm gap between the ends of both capillaries. The end to end distance between the capillaries was adjusted manually and visualized by a custom microscope equipped with a 10x objective and a 10x eyepiece (Edmund Scientific, Barrington, New Jersey). The use of a micrometer disc reticle allowed measurement of the 20 µm gap between the capillaries.

The capillaries were mounted in a microcentrifuge vial (Midwest Scientific, St. Louis, Missouri) and were secured by using epoxy resin. For the post-capillary immunoreaction, the
phosphate buffer containing diluted antiserum was placed in the post-capillary immunoreactor and vacuum was applied to the outlet reservoir (see Fig. 2) via a piper-aid (Drummond Scientific, Broomall, Pennsylvania).

**Laser-Induced Fluorescence Polarization Detection**

The 488 nm line of an argon ion laser (Ion Laser Technology, Salt Lake City, Utah) was selected by using two 488 nm filters for laser-induced fluorescence polarization detection. The optical layout shown in Fig. 3 was a typical dual T-format detection system equipped with excitation and emission polarizers. Specifically, a mirror directed the laser beam through a focusing lens, a polarizer positioned vertically, and finally onto the reaction capillary. A 5 mm section of polyimide coating on the reaction capillary was removed by hot sulfuric acid. Two separate fluorescence polarization emission measurements were made on the reaction capillary, 3.5 cm from the capillary junction. Each emission path contained a 10x objective mounted on a moveable stage, an emission polarizer positioned either vertically or horizontally, a 515 nm filter, and a photomultiplier tube. The objectives shown in Fig. 3 were positioned at 90° with respect to the incident (excitation) beam. The polarizers and the photomultiplier tubes were purchased from Melles Griot (Irvine, California) and Hamamatsu (Bridgewater, New Jersey), respectively. All other optic components were obtained from Edmund Scientific (Barrington, New Jersey).

**RESULTS AND DISCUSSION**

In this study, the success of post-capillary immunophoresis for multiresidue analysis of cross-reacting compounds requires the accomplishment of two discrete events in a two-
dimensional separation system. In the first dimension, partial filling MEKC involves the separation of neutral triazine herbicides as a model system while avoiding the introduction of surfactant into the post-capillary immunoreactor. The second dimension, laser-induced fluorescence polarization detection, utilizes the antibody’s cross-reactivity for the identification of triazine analytes eluted from partial filling MEKC capillary.

**Partial Filling Micellar Electrokinetic Chromatography**

Triazine herbicides exist as cationic species when the solution pH is near or below their pKₐ’s. Although triazine herbicides can be good candidates for the separation in capillary zone electrophoresis (CZE), the low pKₐ values of chloro-triazines (Fig. 1) do not allow for separation in neutral pH buffers. To achieve the separation of triazine herbicides based on their differential partitioning with SDS micelles, a fused silica capillary was initially filled with the electrophoresis buffer containing 18 mM sodium chloride and 10 mM sodium phosphate at pH 8. A 30 mM SDS solution (in 10 mM phosphate buffer, pH 8) and a standard herbicide solution were sequentially injected at the anodic end of the capillary by using a -11.25 kV for 30 sec and 1 sec, respectively. The separation of triazine herbicides in partial filling MEKC was shown in Fig. 5 with the elution order of simazine, atrazine, propazine, and ametryne.

To identify the elution of micellar plug, quinine hydrochloride was added into a 30 mM SDS solution with a final concentration of $4.8 \times 10^{-4}$ M. By assuming each SDS micelle contained approximately 62 monomers\(^7\), the addition of $4.8 \times 10^{-4}$ M quinine hydrochloride gave a ratio of one quinine hydrochloride molecule to one SDS micelle. To mimic the injection conditions in Fig. 5, a SDS solution containing quinine hydrochloride and a phosphate buffer free of SDS and triazine herbicides were injected sequentially by using
-11.25 kV for 30 sec and 1 sec, respectively. As shown in Fig. 6, a SDS plug with a peak width of approximately 36 sec eluted at 5.18 min. In comparison with Fig. 5, all triazine herbicides eluted ahead of the SDS micelle plug.

Mechanistic studies of partial filling MEKC and its comparison with conventional MEKC were discussed by Nelson and Lee. The effects of micellar concentration, plug length, and overall capillary length on separation efficiency and resolution in partial filling MEKC were investigated both experimentally and theoretically. Furthermore, on-line combination of partial filling MEKC and electrospray ionization mass spectrometry (ESI-MS) has been demonstrated for the analysis of triazine herbicides. Partial filling MEKC-ESI-MS avoided the introduction of SDS micelles into the electrospray process while enabling the separation and mass detection of neutral triazine herbicides.

**Fluorescence Polarization Immunoassay**

To integrate post-capillary immunoreaction with partial filling MEKC, it is essential to characterize the fluorescence polarization immunoassay of triazine herbicides in a competitive format. Fluorescence polarization immunoassay, developed by Dandliker et al., is based on the difference in molecular volume of a small tracer when it is free or bound to a large antibody. The polarized fluorescence emitted by the free tracer is low because of the small molecular volume and fast rotational motion, while that emitted by the tracer-antibody complex is high as a result of the increase in molecular volume and the decrease in rotational motion. Fluorescent polarization analyzer measures the fluorescence intensities emitted into horizontal and vertical planes by tracer excited with plane polarized light (for example, vertical
plane), and subsequently calculates the degree of polarization according to the following
equation:

\[ P = \frac{(I_v - I_h)}{(I_v + I_h)} \]  

(1)

where \( I_v \) and \( I_h \) are the vertical and horizontal fluorescent intensities, respectively.

Due to their similar chemical structures to triazine herbicides, 5-(4,6-
dichlorotriazynyl)aminofluorescein and 5-(4-ethylamino, 6-isopropylamino-triazinyl)
aminofluorescein shown in Fig. 4 were employed as tracers 1 and 2 in this study. The
antiserum dilution curves (Figs. 7 and 8) for tracers 1 and 2 were constructed by measuring
the degree of polarization in the reaction mixtures containing tracer and antiserum at various
dilution ratios. In comparison with tracer 1, tracer 2 clearly exhibited the larger degree of
polarization upon the binding with antiserum, possibly contributed by a greater binding
constant of tracer-antiserum complex.

By comparing the serum dilution curves at two different tracer concentrations of \( 10^{-6} \)
and \( 10^{-8} \) M, the decrease in tracer concentration resulted in the increase of fluorescence
polarization of both tracers at various serum dilution ratios. A reduction of free tracer and the
increase in the ratio of bound to free tracer at lower tracer concentration accounted for the
enhancement in fluorescence polarization. Furthermore, the polarization value at serum
dilution ratio of 400 was close to 50% of maximum fluorescence polarization at lowest serum
dilution ratio. Thus, the dilution ratio of 400, possibly with the greatest response to analyte
competition in the fluorescence polarization immunoassay\(^{9,10}\), was selected together with the use of \(10^{-8}\) M tracer concentration for the analysis of triazine herbicides.

As shown in Table I, appreciable reduction in the degree of polarization due to triazine competition was only observed with each individual triazine concentration as high as \(10^{-5}\) M. The competition results indicated that both tracer 1 and tracer 2 had significantly higher binding constants towards antiserum than those of triazine herbicides. The competition order among triazine herbicides was atrazine with the greatest reduction in the degree of polarization, followed by propazine, simazine, and ametryne. This competition order was in good agreement with the reported cross-reactivity of triazine herbicides measured by competitive inhibition ELISA\(^{(6)}\).

**Post-Capillary Immunodetection in Partial Filling Micellar Electrokinetic Chromatography**

The exhibition of larger binding constant of tracer 2 in comparison with tracer 1 was further supported by the competition results shown in Table I. Tracer 1 was selected and added into the electrophoresis buffer of partial filling MEKC for the post-capillary immunodetection of triazine herbicides due to the apparent smaller binding constant. By applying vacuum to the outlet reservoir (Fig. 2), the dilution ratio of tracer caused by the transfer across the post-capillary immunoreactor was studied. A dilution ratio around 2.75 was estimated by comparing the fluorescence intensities of tracer front in the separation and reaction capillaries. On the basis of dilution ratio in the post-capillary immunoreactor, the tracer with a concentration of \(2.75 \times 10^{-8}\) M and the antiserum with a dilution ratio of 100 were placed in the electrophoresis buffer and the liquid junction, respectively.
A 30 mM SDS solution (in 10 mM phosphate buffer at pH 8) and a standard herbicide solution were sequentially injected at the anodic end of capillary by using a 11.25 kV for 30 sec and 2 sec, respectively. As the triazine herbicides sequentially eluted from the separation capillary, the reaction mixture containing the tracer, the triazine, and the antibody was siphoned into the reaction capillary by applying vacuum to the outlet reservoir. As shown in Fig. 9, three negative peaks representing the post-capillary immunodetection of simazine, atrazine, and propazine were measured by an on-column laser-induced fluorescence polarization detector. In the presence of triazine herbicides, the competition between triazine and tracer for the limited antibody decreased the emitted fluorescence polarization of the tracer and resulted in the negative triazine peaks. The peak height ratios between simazine, atrazine and propazine were in good agreement with the competition order measured in the fluorescence polarization immunoassay (Table I). The percent change in fluorescence polarization due to the presence of ametryne was too low to be observed in the electropherogram.

The present fluorescent polarization immunodetection of triazine herbicides is limited by the unusually tight binding between the antiserum and the tracers used in this study. By incorporating a spacer arm between hapten and fluorescein in the tracer design, Onnerfjord et al. have demonstrated the fluorescent polarization immunoassay of atrazine and simazine in the low ppb concentrations\(^{(11)}\). Our future research plans are thus directed towards the better selection of triazine tracer and the improvements in the detection limits and dynamic ranges of multiresidue analysis by post-capillary immunophoresis.
ACKNOWLEDGMENTS

The authors would like to thank Dr. Bruce D. Hammock (University of California, Davis) for generously providing the antiserum used in this study. Assistance and discussion by Dr. Edward S. Yeung in the design and development of laser-induced fluorescence polarization detection is appreciated.

This work was supported by an EPA grant (R 823292-01) and the Microanalytical Instrumentation Center of the Institute for Physical Research and Technology at Iowa State University. W.M.N. is a recipient of the Graduate Assistantship in Areas of National Need (GAANN) Fellowship from the US Department of Education. C.S.L. is a National Science Foundation Young Investigator (BCS-9258652).

REFERENCES


TABLE I
Fluorescence Polarization Immunoassay of Triazines in Competitive Format

<table>
<thead>
<tr>
<th>Triazine</th>
<th>P</th>
<th>% of Reduction$^a$</th>
<th>Triazine</th>
<th>P</th>
<th>% of Reduction$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer Only</td>
<td>0.1428</td>
<td></td>
<td>Tracer Only</td>
<td>0.2710</td>
<td></td>
</tr>
<tr>
<td>Simazine</td>
<td>0.1123</td>
<td>21%</td>
<td>Simazine</td>
<td>0.2400</td>
<td>11%</td>
</tr>
<tr>
<td>Atrazine</td>
<td>0.0788</td>
<td>45%</td>
<td>Atrazine</td>
<td>0.2110</td>
<td>22%</td>
</tr>
<tr>
<td>Propazine</td>
<td>0.0987</td>
<td>31%</td>
<td>Propazine</td>
<td>0.2300</td>
<td>15%</td>
</tr>
<tr>
<td>Ametryne</td>
<td>0.1375</td>
<td>4%</td>
<td>Ametryne</td>
<td>0.2711</td>
<td>0%</td>
</tr>
</tbody>
</table>

$^a$ % of Reduction = $[P \text{ (tracer only)} - P \text{ (tracer and triazine)}]/P \text{ (tracer only)}$
### Fig. 1  Structures and pKₐ values of four triazine herbicides used in this study.

<table>
<thead>
<tr>
<th>Triazines</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>pKₐ&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simazine</td>
<td>Cl</td>
<td>Ethyl</td>
<td>Ethyl</td>
<td>1.65 - 1.80</td>
</tr>
<tr>
<td>Atrazine</td>
<td>Cl</td>
<td>Isopropyl</td>
<td>Ethyl</td>
<td>1.68 - 1.85</td>
</tr>
<tr>
<td>Propazine</td>
<td>Cl</td>
<td>Isopropyl</td>
<td>Isopropyl</td>
<td>1.50 - 1.85</td>
</tr>
<tr>
<td>Ametryne</td>
<td>S-methyl</td>
<td>Ethyl</td>
<td>Isopropyl</td>
<td>4.00 - 4.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Taken from references 12-15.
Fig. 2  Schematic diagram of post-capillary immunophoresis with expanded view of post-capillary immunoreactor. A 20 μm gap between the separation and reaction capillaries was filled with antibody solution.
Fig. 3  (A) Optical layout of laser-induced fluorescence polarization detection;  
(B) spatial configuration of laser-induced fluorescence polarization detection.
1. 488nm Filters
2. Mirror
3. Focusing Lens
4. Excitation Polarizer
5. Reaction Capillary
6 & 9. Objectives
7 & 10. Emission Polarizers
8 & 11. 515nm Filters

Polarization = \frac{I_v - I_h}{I_v + I_h}

Fig. 3
Fig. 4  Molecular structures of 5-(4,6-dichlorotriazinyl)aminofluorescein and 5-(4-ethylamino, 6-isopropylamino-triazinyl)aminofluorescein as tracers 1 and 2.
Fig. 5 Partial filling MEKC separation of $10^{-4}$ M each of (2) simazine, (3) atrazine, (4) propazine, and (5) ametryne. (1) methanol was used as the electroosmotic flow marker. Electrophoresis buffer: 10 mM sodium phosphate/18 mM sodium chloride, pH 8; capillary, 45 cm total length and 30 cm to detector, 50 μm i.d. and 360 μm o.d.; applied voltage, -11.25 kV and 30 sec for SDS injection (30 mM SDS in 10 mM phosphate buffer, pH 8), -11.25 kV and 1 sec for triazine injection, -11.25 kV for electrophoresis; UV detection at 226 nm.
Fig. 5

Absorbance

Time (min)
Fig. 6 Partial filling MEKC separation of $4.8 \times 10^4$ M quinine hydrochloride in 30 SDS, 10 mM phosphate buffer, pH 8. Electrophoresis buffer; 10 mM sodium phosphate/18 mM sodium chloride, pH 8; applied voltage, -11.25 kV and 30 sec for quinine hydrochloride injection, -11.25 kV and 1 sec for electrophoresis buffer injection, -11.25 kV for electrophoresis. Other conditions are the same as Fig. 5.
Fig. 6
Fig. 7 Serum dilution curves for tracer 1 with concentrations of $10^{-6}$ and $10^{-8}$ M.
Serum Dilution Curve

Tracer 2

Fig. 8 Serum dilution curves for tracer 2 with concentrations of $10^{-6}$ and $10^{-8}$ M.
Fig. 9 Post-capillary fluorescence polarization detection of triazine herbicides with the elution order of (1) simazine, (2) atrazine, and (3) propazine. Separation and reaction capillaries were fused silica each with a total length of 45 cm (50 μm i.d. and 360 μm o.d.) and were separated by a 20 μm gap. Electrophoresis buffer; 10 mM sodium phosphate/18 mM NaCl and 2.75 x 10^{-8} M tracer 1 at pH 8. 11.25 kV was applied sequentially for 30 sec injection of 30 mM SDS (10 mM sodium phosphate at pH 8) and for 1 sec injection of 10^{-4} M trazine standard. A constant electric field of 250 V/cm was maintained in the separation capillary. Laser-induced fluorescence polarization detection (excitation: 488 nm, emission: 515 nm) was monitored in the reaction capillary, 3.5 cm from the capillary junction. Vacuum was applied to the outlet reservoir and an antiserum dilution ratio of 100 was placed in the immunoreactor.
Fig. 9
GENERAL SUMMARY

Micellar electrokinetic chromatography (MEKC) has experienced rapid advances in the last few years in both theoretical developments and applications. The progress recently achieved in MEKC affords it the flexibility for further application development. The success of MEKC in fields such as clinical chemistry, environmental chemistry, and analytical chemistry demonstrate the versatility of sample components easily separated and detected in MEKC. However, the requirements for more sensitive, yet versatile detection schemes and adaptable separation methods create many opportunities for future development in MEKC. To assist the progress of development for new detection schemes this work introduced partial filling micellar electrokinetic chromatography (PF-MEKC) as an additional separation mode in MEKC. With this novel approach to a micellar separation more flexible detection schemes and applications of PF-MEKC can be developed in the future.

This work has presented a investigation of the mechanism of PF-MEKC and the effects of PF-MEKC on analyte separation efficiency and resolution. We have also presented the development and demonstration of electrospray ionization mass spectrometry (ESI-MS) on-line detection utilizing PF-MEKC as the separation mode. The capability of PF-MEKC couple to ESI-MS allows for added sample identification and characterization in MEKC. In addition, utilizing PF-MEKC as an on-line separation mechanism for multi-residue immunodetection of truly cross reacting analytes provides an entirely new direction in the development of immunoassays. Based on the protocols developed in this work, future studies could be extended to further development of on-line detection modes with using PF-MEKC as the separation mechanism.
ACKNOWLEDGMENTS

The following people have contributed either their magic, faith, time, energy, vision, passion, support or friendship in an important and appreciated way.


For support through my graduate career I gratefully acknowledge Edward Yeung and Dennis Johnson. And to those who scientific vision enabled me to complete this dissertation, Sigeru Terabe and Bruce Hammock.

This work was performed at Ames Laboratory under Contract No. W-7405-Eng-82 with the U.S. Department of Energy. The United States government has assigned the DOE report number IS-T1795.
REFERENCES


