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Applications of Electrochemically-modulated Liquid
Chromatography (EMLC): Separations of Aromatic Amino Acids and
Polycyclic Aromatic Hydrocarbons

by

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CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

The research in this thesis explores the separation capabilities of a new technique termed electrochemically-modulated liquid chromatography (EMLC). The thesis begins with a general introduction section which provides a literature review of this technique as well as a brief background discussion of the two research projects in each of the next two chapters. The two papers which follow investigate the application of EMLC to the separation of a mixture of aromatic amino acids and of a mixture of polycyclic aromatic hydrocarbons (PAHs). The last section presents general conclusions and summarizes the thesis. References are compiled in the reference section of each chapter.

Literature Review

Since the late 1960's, high performance liquid chromatography (HPLC) has become one of the most widely used analytical techniques in pharmaceutical, medical, biochemical, and many other analytical laboratories [1]. This reliance reflects the capability of HPLC in separating a wide range of complex mixtures, given an appropriate choice of stationary phase material and mobile phase composition. Different kinds of columns are needed for different mechanistic forms of separations, such as adsorption [2], reversed-phase, normal-phase and ion-exchange [3-5], size-exclusion [6, 7], affinity [8, 9], and immunoaffinity [10-12]

processes. Normally, it is the mobile phase, and not the stationary phase, in HPLC that is manipulated to enhance the efficiency of a separation. Once the stationary phase is chosen, the mobile phase composition is adjusted by changing the percentage of the organic component or by adding a modifier to optimize separation efficiency. This approach, however, leads to a large amount of mixed waste generation and an ever increasing disposal cost.

To advance HPLC as a separation technique, several alternatives, such as the use of transformable stationary phases and dynamic coating techniques [13, 14] have been investigated. EMLC is one of the new alternatives, and combines electrochemistry and chromatography. This technique originated in early 1960's when Fujinaga et al. [15], as well as Strohl [16] and Roe [17], proposed a new concept which combined thin-layer electrochemical methods and liquid chromatographic concepts. With EMLC, the composition of the stationary phase is controlled electrochemically. Conductive stationary phases, such as glassy carbon [16] or amalgamated nickel [17], are used whereby their surface charge is manipulated through alterations of applied potential. In these early studies, the technique was mainly used in an anodic stripping mode in which metal ions were reductively deposited onto the stationary phase and then stripped in a stepwise manner to effect the separations of mixture composed of a few metal ions. Later, Fujinaga demonstrated that a voltage gradient could be applied to enhance the separations using an alternate column design [18, 19].

The technique was subsequently extended by Strohl in 1972 [20, 21] using “electrosorption” phenomena. The retention of electroactive organic species (e.g., mixtures of quinones) could be manipulated by changing their redox states on carbonaceous particles. In 1978, Strohl [22] further demonstrated that through alteration of applied potential, the composition (e.g., pH) of the mobile phase could be changed to manipulate the separation of inorganic cations. However, the performance of the technique was such that the widths of the elution profiles were not sufficient to prove tractable as a separation technique.

A significant advance in this area was brought by Antrim et al. [23, 24] in 1984. Components of this advancement include the proposal of “electrochromatography”, a term not to be confused with those presently in vogue in the capillary electrophoresis area, in which the capacity factors for analytes could be manipulated through the application of various fixed voltages to a carbonaceous column, and the development of a column with stainless steel used as the container for the stationary phase in order to withstand high operational pressures (~3000 psi).

As a means for tailoring the selectivity of such separations, some research groups have explored the modification of carbon surfaces with electroactive ionomers [25-28] and electroactive polymers [29-36] so that the stationary phase could be converted electrochemically between its oxidized and reduced forms with the concomitant uptake or expulsion of analyte ions. For example, Zumbrunnen and Anson reported in 1983 [27] that the equilibrium incorporation of a multiply-charged electroactive ion could be manipulated by changing both the pH of the electrolyte

and the oxidation state of pentacyanoferrate groups bound to poly(4-vinylpyridine). Ge and Wallace [30, 31] have indicated that, in addition to tuning ion-exchange capacity, alterations in the applied potential can modify the hydrophobicity, dipolar nature, and donor-acceptor strength of coatings like polypyrrole.

More recently, Nagaoka investigated the influence of applied voltage on the retention of inorganic species using different kinds of stationary phases, including microporous glassy carbon [37] or glassy carbon spheres that were coated with crown ethers [32], polyaniline [35, 36], or polypyrrole [36]. Lam et al. [38] found the retention of β -lactoglobulin could be manipulated by changing the redox states of heme in heme-agarose bed with additives in the mobile phase. However, the performance of these column designs in all of the above reports still failed to yield separation with efficiencies that were competitive with conventional HPLC.

Our group started research on EMLC in the early 1990's, and the effect of column design on chromatographic performance continues to be a major concern. Deinhammer, Shimazu, and Porter reported some improvements in the preparation of a polypyrrole coating on glassy carbon, and applied such coatings to the separation of adenosine phosphates [39] and of dansyl amino acids [40]. These researchers demonstrated for the first time the ability to manipulate retention by changing electrochemically the composition of a stationary phase during elution. In these early investigations, a Nafion tube served both as a container for the stationary phase and as an ion exchange membrane to separate the working electrode from the counter and reference electrodes. Electrical contact to the

particulate phase was made by insertion of a gold wire spot-welded to a strip of gold mesh into the Nafion tube prior to column packing. Although this column proved effective in a proof-of-concept demonstration, the low physical strength of the Nafion tube resulted in the three serious limitations, all of which limited chromatographic performance. First, the column could not stand pressures higher than 10 psi. Second, the low pressure limit prevented the use of high pressure methods for column packing. Third, the column could not be used with mobile phases containing organic solvents because of a solvent-induced swelling of the Nafion tube.

In order to overcome these limitations, a new column design was developed [41, 42] in which a porous stainless steel column was used not only to support physically the Nafion tube but also make electrical contact to the particulate phase. The new column was able to withstand very high pressures (up to ~6000 psi), and, consequently, the use of conductive packing materials with diameters less than 10 μm became possible. Organic solvents could now also be used as mobile phase additives. For a stationary phase, small, uniformly-sized carbonaceous materials were used without subsequent surface modification. Results demonstrated that chromatographic efficiencies could be increased dramatically by using porous graphitic carbon (PGC) [43], with efficiencies competitive with conventional HPLC at last realized.

However, there were still some drawbacks with this column design, particularly the limited efficiency in electrochemical performance [44]. For example, only species with anodic formal reduction potentials were electrolyzed but those with

large cathodic formal reduction potential were not. After examining their column design, the research team found that the poor electrochemical performance originated from the connection of PGC and stainless steel tube together as a single working electrode and the resulting potential drop from the reduction of molecular oxygen at the stainless steel component of the working electrode. Upon redesign, whereby the stainless steel tube was insulated electrically from the PGC packing and now used as the counter electrode instead of as a part of the working electrode, this limitation was minimized. This new column design was used in the research described in this thesis.

Thesis Overview

Two novel applications of EMLC are presented in this thesis. Chapter 2 describes the separation of a mixtures of aromatic amino acids (i.e., phenylalanine, tyrosine, *m*-tyrosine, *o*-tyrosine, and dopa) using EMLC. This family of analytes are of great clinical importance. For example, phenylalanine and tyrosine are used in screening for phenylketonuria [45], and other diseases, and dopa is used for treatment of Parkinson's disease [46]. It is not surprising that the analysis and separation of these compounds have been an intensive research area in HPLC.

Aromatic amino acids have been separated on octydecyl silica (ODS) stationary phases [47-49]. However, the limit of the pH range with ODS precluded the study of the effect of pH on the retention of the naturally occurring amino acids [50]. Unlike ODS, PGC allows the use of a wide range of mobile phase pH values to

investigate the retentive behavior and to optimize the separation of ionizable analytes [51]. Wan et al. [52] reported results on the retention of ionizable aromatic compounds over mobile phase pH range between 2 and 9.4 at PGC stationary phase. Mama et al. [53] demonstrated the advantage of using strongly acidic conditions (pH 1) for the separation of aromatic amino acids at PGC.

In Chapter 2, we present the results on the effect of the ionization of aromatic amino acids on their retention at PGC. Comparisons to predictions from acid-base equilibria [50] indicate that the retention behavior at different mobile phase pH values can be correlated with the degree of ionization.

As a stationary phase, PGC is largely selective to differences in double bond structures, but less so to differences in functional groups [54]. However, based on one of our previous studies [55], the selectivity of PGC stationary phases to differences in functional groups can be greatly enhanced by the manipulation of applied potential (E_{appl}). The EMLC-based separation of a mixture of five aromatic amino acids is also presented in Chapter 2. This chapter also shows that chromatographic performance can be enhanced by the modification of E_{appl} during elution, an approach somewhat analogous to gradient elution in conventional HPLC.

Chapter 3 presents the investigation of PGC as a stationary phase in the separation of a mixture of PAHs. The environmental and physiological importance of PAHs has made this class of molecules the subject of a vast separation literature [56]. One of the challenges in the application of PGC in the separation of PAHs is the unusually strong retention of such species by the column material [54, 57, 58].

However, this complication can be overcome by EMLC. Coupled with the use of a strongly eluting mobile phase (i.e., methylene chloride), negative values of E_{appl} were found effective to elute and in some cases, separate a simple mixture of PAHs that contain four aromatic rings. This result is the first successful example of the use of PGC in the separation of PAHs of this extensive of ring size.

Together, the results in this thesis demonstrate the prospects of EMLC to the separation of two new type of analytes, and continue to expand the breadth of this exciting, new separation technique.

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CHAPTER 4. GENERAL CONCLUSIONS

This thesis has described two new explorations of the range and scope of porous graphitic carbon (PGC) as a stationary phase coupled to electrochemically modulated liquid chromatography (EMLC).

In Chapter 2, the mobile phase with pH between 1.11 and 10.83 has been used to study the retention behavior of a mixture of aromatic amino acids at PGC to develop a basis for enhancement of their separation. Although none of the mobile phase conditions resulted in baseline separation for such a mixture, the results indicate that the retention of the amino acids can be correlated with the degree of ionization, with those more ionized being less strongly retained. In addition, this correlation served as a starting point for assessing the potential utility of EMLC in separating mixtures of such ampholytes.

Chapter 2 also demonstrated the improvement of the separation of such mixture via EMLC. The chromatographic performance was enhanced by the modification of E_{appl} both prior to and during elution. The effect of the alteration of E_{appl} on retention of these aromatic amino acids at different mobile phase pHs was also investigated. Generally, the retention of anionic forms increases as E_{appl} increases and the retention of cationic forms decreases as E_{appl} increases. However, the retention strength of some of the analytes at a low mobile phase pH reflected the superposition of ion-pair formation between cationic analytes and the anions of the supporting electrolyte in the mobile phase. Due to the dipolar

properties of amino acids, the success of the application of EMLC in the aromatic amino acids can likely be extended to all naturally occurring amino acids. However, a detector more sensitive than those presently available in our laboratory is needed to test this possibility.

Chapter 3 has demonstrated the successful application of EMLC in separating strongly retaining analytes like PAHs at a PGC stationary phase. Preliminary results show that coupled with the use of methylene chloride as a strongly eluting mobile phase, simple mixtures containing four-ring PAHs were eluted and, in some case, effectively resolved. As carbonaceous stationary phases have shown a strong selectivity for PAHs, the elution and separation of PAHs larger than those tested in this chapter may be realized by EMLC with GC as the stationary phase. Efforts focused on removal of oxygen-containing functional groups on the GC surface are, however, likely needed for success, coupled with the use of a suitable mobile phase modifier.

In summary, the above results hint that EMLC has the potential to be used as a separation tool for a large variety of analytes, and ongoing efforts (e.g. the separation of the many positional isomers of benzene) in our laboratory are directed along these lines.

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