CAPILLARY ELECTROPHORESIS OF LIPIDS DERIVED FROM SOME NATURAL PRODUCTS

By

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LIST OF SYMBOLS AND ABBREVIATIONS

ε	Dielectric constant of the medium
η	Viscosity of the medium
Ψ	Electric potential
ζ	Zeta potential
χ	Mole fraction
μ_{eo}	Electroosmotic mobility
μ_{ep}	Electrophoretic mobility
μ_{eff}	Effective electrophoretic mobility
t_0	Migration time of a neutral solute
ACN	Acetonitrile
AMP	Adenosine monophosphate
C16:0	Palmitic acid
C18:0	Stearic acid or octadecanoic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C19:0	Nonadecanoic acid
C24:0	Lignoceric or tetracosanoic acid

CD	Cyclodextrin
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
DMe	Dimethyl
DG	Diphosphatidylglycerol
EDL	Electric double layer
EOF	Electroosmotic flow
FA	Formamide
FFA	Free fatty acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
I.D.	Internal diameter
LIF	Laser induced fluorescence
LOD	Limit of detection
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
NACE	Nonaqueous capillary electrophoresis
NMF	*N*-Methylformamide
O.D.	Outer diameter
PA	Phosphatidic acid
PDA	Photo diode array
PG	Phosphatidyl glycerol
PI	Phosphatidylinositol

PS	Phosphatidylserine
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography
UV-Vis	Ultraviolet-visible

CHAPTER I

BACKGROUND AND SCOPE OF THE STUDY

Introduction

Over the recent years, capillary electrophoresis (CE) has gained wide acceptance among researchers and analysts and has become a common analytical separation technique in laboratories. CE is currently being applied in the analysis of a wide range of compounds [1-4]. The popularity of CE stems from its simplicity, relatively short analysis time, low sample consumption and high separation efficiency.

In CE, charged molecules are separated on the basis of difference in their migration velocity under the influence of a direct current electric field in a capillary filled with a conductive separation medium referred to as the running electrolyte or background electrolyte. The species being separated, depending on their charge and solvated ionic radius, migrate along the capillary axis at different rates thereby resulting in a separation based on their charge-to-mass ratio [5].

The CE technique was used in this study for the separation and quantitative analysis of some lipids. Lipids belong to a diverse group of naturally occurring organic compounds such as fats, oils, waxes, sterols,

vitamins, phospholipids, etc. Free fatty acids (FFA's) and phospholipids are the lipids that were investigated in this dissertation. In this regards, CE methods based on partially aqueous electrolyte systems were first developed using standard FFA´s and phospholipids and were then applied to the analysis of both FFA´s and phospholipids present in extracts derived from some natural food products, namely peanut seeds and watermelon.

In the first chapter, nonaqueous capillary electrophoresis (NACE) and indirect detection are discussed and background is provided due to their importance in the CE analysis of the lipids under investigation. The second chapter discusses the various electrolyte systems investigated for the CE separation of FFA´s and also covers the quantitative determination of FFA´s such as oleic acid and linoleic acid in oil derived from peanut seeds. The analysis was carried out in order to monitor the ratio of oleic and linoleic acids in various peanut-breeding lines. A high content of oleic acid increases peanut shelf life and is beneficial to human health. The CE method developed in chapter II could be used to quantify not only oleic and linoleic acids but also stearic acid, palmitic acid and linolenic acid. The third chapter deals with the development of a CE method for the separation and quantitative analysis of phospholipids. The CE method developed in chapter II, with some modifications, was applied to the analysis of phospholipids present in watermelon tissues. Five phospholipids were separated by the developed CE method and their presence in watermelon was quantitatively determined. These phospholipids include phosphatidylserine (PS),

phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA) and diphosphatidylglycerol.

Nonaqueous Capillary Electrophoresis

Historical and Overview of NACE

The majority of studies and applications of CE have been carried out using aqueous separation media. The reasons for the popularity of aqueous based CE could be the ready availability, low cost, relatively low viscosity and low volatility of water. Furthermore, the high solvating power, compatibility with different detectors and familiarity of acid-base chemistry in aqueous media have made water-based CE one of the commonly used separation techniques [6]. However, for nonpolar compounds, aqueous CE may not be the best choice. Also, conventional aqueous CE cannot separate compounds that are hydrophobic and/or have similar charge-to-mass ratios. In such cases, the selectivity can be enhanced by addition of a complexing agent in the electrolyte or by using pseudostationary phase e.g., in micellar electrokinetic capillary chromatography (MEKC) or a stationary phase, e.g., in capillary electrochromatography (CEC) [7]. Alternately, including an organic modifier (i.e., organic solvent) in the running electrolyte can also modify selectivity. Solvent characteristics (e.g. viscosity, dielectric constant, polarity, density, etc.) of organic solvents are different from those of water and therefore organic solvents will affect the analytes in a different

way than that of water, thus attributing to physiochemical differences between analytes and thereby increasing the separation selectivity [8].

Nonaqueous CE (NACE) has slowly matured into a separative approach having a great potential. The first work in nonaqueous separation media dates back to 1951, when Hayek investigated carbon black particles suspended in a medium of kerosene [9]. After Hayek's work, the nonaqueous media was applied to a variety of analytes [6] including weak acids and bases [10, 11], certain biological compounds such as fatty acids, steroids and cholesterol [12], lubricating oil additives [13, 14], metal ions [15, 16], anions [17, 18], and strong acids such as sulfuric and phosphoric acid [19]. Later on, in 1984, Wahlbroel and Jorgenson applied pure organic solvent in CE to separate five quinoline-type compounds using acetonitrile (ACN) containing tetraethylammonium perchlorate/hydrochloric as the running electrolyte [20]. After Wahlbroel and Jorgenson's work, organic solvents were generally used as organic modifiers in concentration of up to 40 % v/v [21-23]. Since then not much work was done in NACE up until early 1990's because of another technique that was competing against NACE. This technique was micellar electrokinetic capillary chromatography (MEKC), which was introduced in the same year, i.e., 1984, by Terabe *et al* [24]. The drawbacks of aqueous CE for neutral and hydrophobic analytes were compensated by MEKC.

Also, the delay in the development of NACE could be attributed in part to the lack of information about protolysis, different molecular interaction, common knowledge of electrochemistry and solvation performance in organic solvents

[25]. In addition, during early 1980's, the CE instruments were not intended for volatile solvents and most of the organic solvents are more volatile (*e.g.* ACN, alcohols, etc.) than water. The volatility of solvents could pose problems such as unstable/nonreproducible migration times and breakdown of electric current [7]. Later, in the beginning of the 1990's, NACE showed evidence of its advantages over aqueous CE for several compounds [26-29].

The work done by Benson *et al* in 1993 influenced the practice of NACE in the following years [26]. Benson *et al* separated pyrazoloacridine from metabolic degradation products and synthesis impurities in neat methanol (MeOH). The results obtained from NACE were more promising than any investigated water-MeOH electrolyte system. In 1994, Sahota and Khaledi separated peptides using pure formamide (FA) [27]. The separation of peptides was slow due to the high viscosity of formamide. NACE was compared to aqueous CE with respect to efficiency, selectivity and analysis time. The solubility of many supporting electrolytes in formamide was studied because of its high dielectric constant and low specific conductance. Also, good linearity between current and applied voltage was seen, enabling the use of wide bore capillaries and high concentration of electrolytes, consequently giving improved loadability and detectability. However, formamide suffered from reduced UV transparency and hydrolysis instability.

Ng *et al* and Tomlinson *et al* demonstrated the separation of hydrophobic drugs and metabolites in pure and mixed methanolic solvents [29-31]. In 1995, Bjørnsdottir and Hansen discussed the versatility of electrophoretic selectivities

in NACE [32] in the separation of amines having similar charge-to-mass ratio in many organic solvents (e.g., formamide, *N*-methylformamide, *N,N*-dimethylformamide, dimethyl sulfoxide, MeOH and ACN) using volatile salts such as ammonium acetate. Also, in 1995 for the first time a complete introduction of fundamental theory of CE with respect to organic media was published by Jansson and Roeraade [28]. In the following years, NACE began to develop rapidly and year-by-year applications of NACE increased.

Since few years, NACE has gained great popularity because of its successful applications in various disciplines such as pharmaceuticals, environmental analysis, food analysis, natural products, organic and inorganic chemistry, etc. In fact, NACE proved itself in the analysis of food micronutrients such as antioxidants [33], pharmaceuticals [34-38], phenols [39, 40], basic and acidic drugs [41], cholesterol [4], *N*-derivatized amino acids [42, 43], monocationic enantiomers [44], hydrophobic oligomers [45] and many more. Very recently, NACE was applied to the analysis of phosphatidylethanol, which is a new biomarker of ethanol intake [46] and *N,N'*-alkylmethylimidazolium cations [47].

As stated above, one of the major advantages of NACE resides in its superiority in the analysis of hydrophobic compounds such as lipids, which are the subject of this investigation. This is because lipids are characterized by their insolubility or low solubility in water (i.e., hydrophobicity). Although, NACE has been successfully applied for analyzing fatty acids and phospholipids [48-50], the exploitation of the full potentials of NACE in the analysis of various lipids in a

wide variety of natural matrices is yet to come. Thus, it is the aim of this dissertation to contribute to furthering the development of NACE and to enlarging its scope of applications in the analysis of natural products such as peanuts and watermelon.

Advantages and Limitations of NACE

As stated above NACE is the most common method alternative to traditional aqueous CE. Many advantages of NACE over aqueous CE have been reported in the literature: increased selectivity, higher efficiencies, higher separation voltage, shorter analysis time, enhanced dissolution and stability of analytes, etc. [51]. However, there are some advantages that lack fundamental basis and need justification for its existence. In this regards and in a recent review article, Porras and Kenndler [51] had scrutinized some of the advantages reported in earlier in the literature. In case of efficiency, it was concluded that water offers better efficiency than most organic solvents considering longitudinal diffusion as the only source for peak broadening. Also, adsorption, electromigration dispersion and joule heating, which are some of the main sources of peak broadening does not favor organic solvent. With the exception of ACN, electric current is lower in organic solvents because of lower electric conductivities of ions than in water. As a consequence, higher electric field or higher ionic strengths can be used in nonaqueous media, which ultimately leads to shorter analysis time. Following the argument that the mobilities of analytes

are lower in organic solvents (except in ACN), applying higher electric field strength does not result in faster analysis due to their lower velocity in the nonaqueous medium and application of higher field strength does not fully compensate for lower velocity (due to lower mobility). Moreover, there are instrumental limitations preventing the use of higher electric field strength. As mentioned above, lower conductivities make the application of higher current possible. Such decrease in conductivity in aqueous media can be achieved either by dilution or decreasing internal diameter of the capillary. In this case, the advantage of shorter analysis time in organic solvents is not completely justified.

With respect to high solubility of lipophilic compounds in organic solvents, NACE scores more than aqueous CE. Also, the compounds that are labile in aqueous media can be successfully evaluated in organic media. The chemical stability of certain compounds in organic solvents is one of the major advantages of NACE.

In NACE, the separation medium is either as one pure organic solvent or mixtures of different organic solvents. However, sometimes the background electrolyte can have a mixture of water (in low content) and organic solvents. MeOH and ACN are undeniably the most routinely used organic solvents. Application of these solvents can be found in early 1970s [17, 18] and 1980s [20, 52, 53]. There are several reasons behind their popularity such as easy to handle, sufficiently UV-transparent, relatively cheap, readily/commercially available at high purity and sizeable literature data can be found on these solvents. Some of the other organic solvents that are used in NACE are ethanol

[54], 1-propanol [55], formamide [56], *N*-methylformamide [57], *N,N*-dimethylformamide [58], etc.

Undoubtedly, a large number of different solvents are available but not all could be used as the solvent in NACE. To be used as a suitable solvent for NACE, the solvent should at least meet some of the following requirements [7, 25]: (i) suitable liquid range, (ii) low viscosity, (iii) sufficient relative permittivity, (iv) chemical stability, (v) good solvent properties, (vi) not too high volatility, (vii) easily available at reasonable cost and purity and (viii) should be compatible with instrumental requirements.

Considering all the advantages and disadvantages of NACE, it can be considered as a method of choice where analyte solubility and chemical stability are important. With large choice of solvents and solvent mixtures, the desired goal can be readily accomplished by NACE.

Indirect Detection

Capillary electrophoresis has been used to analyze solutes varying in sizes including small molecules such as inorganic ions, amino acids, etc. and large molecules such as proteins, DNA, etc. in a variety of complex sample matrices such as food products, river water, blood, urine, etc. When selecting a detection method, interferences from these matrices should be considered. Also, the characteristics of the solutes to be analyzed, the composition of the sample

matrix and the required detection limits are the most important factors that should be taken into consideration for selecting an appropriate detection scheme [59].

Some of the common detection schemes successfully implemented in CE are UV-Vis absorbance, fluorescence, mass spectrometry and electrochemical detection. The overall analytical performance of CE determination depends greatly on the detector properties. Therefore, choosing an appropriate detection method for a given analysis is of prime importance. Optical detection is the most commonly used detection method in CE separations. Today under optical mode, UV-Vis absorbance is the most popular technique employed whereas fluorescence being the second most common in CE separations.

In the following sections, an overview of indirect UV and fluorescence detection is provided. Since the analytical problem under investigation involved relatively concentrated samples, detection on trace level was not an issue, and therefore, indirect UV detection was preferred over the more sensitive indirect fluorescence detection due to the simplicity of the former mode of detection and availability of instrumentation for this project.

Overview of Indirect UV and Fluorescence Detection

For an analyte to be detected in CE, it should possess a suitable detection property, e.g., chromophore, fluorophore or electrochemical activity. For UV detection, analytes that lack this property or does not possess sufficient UV absorbance can still be detected either by derivatization of the analytes or by

indirect detection [60]. Indirect UV detection would be the preferred choice because of its simplicity and moreover by this method, precolumn or postcolumn derivatization procedures can be eliminated, which are often time-consuming. Hjerten *et al* were the first to propose the possibility of indirect detection in CE methods [61] followed by some investigations in the late 1980's [62, 63].

Fluorescence is the second most common detection technique employed in CE. The reason for its popularity is due to the fact that relatively high sensitivity can be gained by fluorescence. In fluorescence [59], the molecule absorbs a fraction of incident photon of a particular wavelength and goes to an excited state. Fraction of the excited molecules emits photons at a different wavelength upon returning to the ground state thereby giving a fluorescence signal, which is detected. Absorbtivity of the molecule, fluorescence quantum yield and photostability are the important properties that determine the use of a fluorophore. Low detection limits can be attained by fluorescence detection due to the fact that excitation wavelength is different from that of fluorescence emission wavelength, which results in low background signal and a single analyte can repeatedly fluoresce several times (~ 10^5 cycles in aqueous medium).

Laser induced fluorescence (LIF) is the most sensitive detection mode for CE. LIF was first utilized by Gassmann et al in 1985 [64]. It has the potential to detect few molecules or a single molecule. By using special designs, stray light from the optical components and Raman scattering from the solvents can be minimized, thus resulting in lower limit of detection (LOD) [65]. Generally, lasers

are used as the source for excitation of the molecules, and possess many advantages namely lasers can be focused near the diffraction limit of light, offer simple spectral backgrounds and depending on the analyte under study, excitation power can be varied over a wide range for optimization [59]. In LIF, labeling dyes that absorb in the 350-650 nm range are mostly used because of the available lasers and detection efficiency of the majority of photomultipliers in the visible region [65].

Not all molecules can fluoresce, and therefore to analyze molecules that lack fluorophore they are often derivatized, a process in which fluorescent probe molecules are attached to the analyte of interest making them detectable. Derivatization can be done by employing either pre-column [66-68] or on-column [69] methods using reactive functional groups such as amines or thiols. Analytes such as carboxylic acids or inorganic ions are not easily derivatized and in such cases, indirect detection seems to be the best alternative choice [70]. In 1988, Kuhr and Yeung first reported the use of indirect LIF detection [71]. Native amino acids were studied using CE coupled with indirect fluorescence [63]. Indirect LIF detection has been applied for analyzing analytes such as fatty acids [72, 73], amines [74], coumarin compounds [75], arsenic species [76], diuretics [77], carbohydrates [78], etc. employing CE.

In this dissertation, since the fatty acids and phospholipids under investigation lacked sufficient UV absorbance, indirect UV detection was employed in their CE analysis. Indirect UV detection has been successfully applied to a variety of analytes because of its simplicity, ease of use and

relatively low cost. These analytes include lipids [57, 79-81], carbohydrates [82, 83], detergents [84], metal ions [85, 86], anions [87, 88], enantiomers [89], etc.

Brief Description of the Principle of Indirect UV and Fluorescence Detection

In indirect UV detection [60, 90], the key principle is to maintain a large and constant background absorbance signal, which can be achieved by adding a chromogenic ion to the running electrolyte having strong response at the detector. The concentration of the chromophore should be constant in the electric double layer conferring a steady background signal in the form of a stable baseline (see Fig.1A). The chromophore ions should have the same charge as that of the analyte ions and also have similar electrophoretic mobilities, thus preventing asymmetry in peak shapes [91]. As the analytes (e.g., free fatty acids and phospholipids) that are deficient in chromophore passes through the detector, the original level of detector signal drops resulting in a negative peak (see Fig.1B). When the analyte has completely passed through the window, the original level of background signal is regained (see Fig.1C). The peak detected in the indirect detection is not from the analyte but results from the displacement of the background chromophore ions.

The principle of indirect fluorescence detection is the same as indirect UV detection, where the electrophoretic buffer has the fluorophore ion as the main component and the nonfluorescing ions are detected through charge displacement of the fluorophore ions, see Fig. 1.

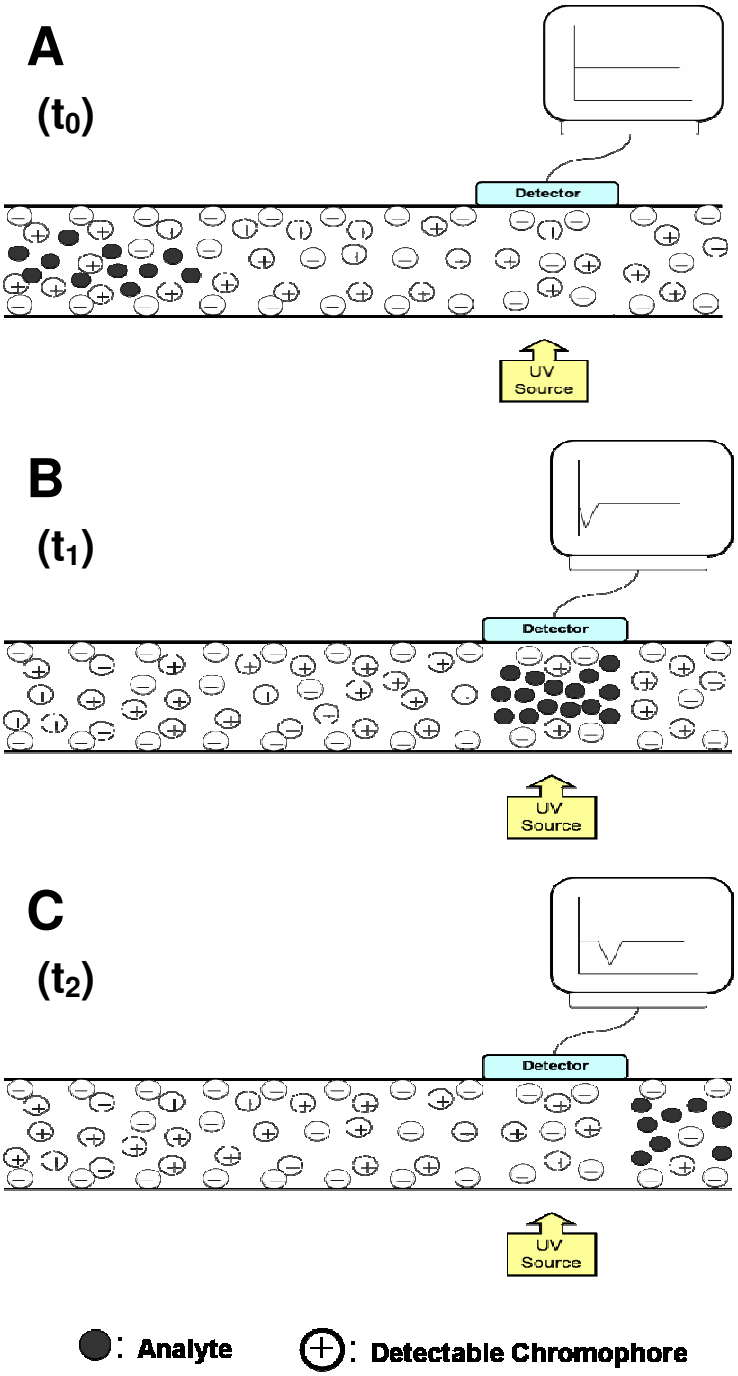

Figure 1. *Schematic representation of displacement mechanisim of detectable chromophore in indirect detection at different residence time in the capillary.*

Conclusions

This chapter provided the background and rationale of the study including an overview of NACE and indirect UV detection. Historical background and advances in NACE has been discussed. CE is a growing field with ample potentials in various applications. Using nonaqueous media in CE further broadens the scope of the technique. Wide range of solutes can be analyzed by carefully selecting an appropriate organic solvent. Indirect UV and fluorescence are popular detection schemes employed in CE. For analytes that do not possess chromophore or fluorophore, the application of indirect detection seems to be the best choice.

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CHAPTER II

CAPILLARY ELECTROPHORESIS OF SOME FREE FATTY ACIDS USING PARTIALLY AQUEOUS ELECTROLYTE SYSTEMS AND INDIRECT UV DETECTION. APPLICATION TO THE ANALYSIS OF OLEIC AND LINOLEIC ACIDS IN PEANUT BREEDING LINES

Introduction

Fatty acids are an important class of organic compounds known as "lipids". Fatty acids encountered in plants and animals are the carboxylic acids often having a long, unbranched aliphatic carbon chain, which can be saturated or unsaturated [1]. In nature, fatty acids are bound to other molecules forming triglycerides and the breakdown of these triglycerides yields free fatty acids (FFA's) and glycerol.

Due to their importance in the food industry, the analysis of fatty acids in food matrices plays a key role in controlling various parameters like product stability, quality and chemical properties of edible oils and fats. In this study, peanut (*Arachis hypogaea* L.) oil was evaluated for its fatty acids composition, e.g., oleic acid and linoleic acid contents. Peanut oil is very common in daily

household cooking because of its high smoke point relative to other cooking oils. Also, peanut seeds hold fourth rank in world production [2]. Peanuts are composed principally of oils (44-56%) and proteins (22-30%) [3, 4]. The oil consists mainly of unsaturated fatty acids making it more susceptible to oxidation. The stability and nutritional quality of the oil depends on the relative proportion of saturated and unsaturated fatty acids. Palmitic, oleic and linoleic acids comprise approximately 90% of the total fatty acid composition along with some other fatty acids [5, 6]. In this work, the quantitative determination of oleic acid and linoleic acid content of peanut oils were of major interest. Peanuts with normal oleic content go rancid on the shelf in about a year. High oleic acid content along with low linoleic acid content is beneficial in increasing product shelf life [7], product flavor, decreasing rancidity and also offers consumers health advantage by reducing the blood level of LDL cholesterol [8, 9]. Peanut seeds are classified as high oleic if the oleic acid content is 70% or greater while they fall under normal oleic acid category if the oleic acid content is less than 70 % [10].

To produce peanut oils with high oleic acid content, peanut breeding is carried out where genetic manipulation is employed to develop peanut lines with elevated oleic acid content. The genetics of the trait conferring a high ratio of oleic acid to linoleic acid is known to be controlled by at least two recessive genes [11]. Incorporation of high oleic acid genes into new peanut breeding lines results in high oleic acid peanut cultivars. In early generations of peanut lines, the range of oleic acid can vary to some extent. Many early generations of

peanut lines will produce mid-oleic acid seeds. These seeds will then produce plants that eventually result in high-oleic seeds after self-pollination over generations. Screening for those individual peanut seeds, which are high in oleic acid content is an important part of breeding programs.

To screen for the fatty acids content of early generation seeds, a small portion (0.2 to 0.3 g) of the peanut seed is usually cut for FFA´s analysis, keeping the rest of the seed intact for germination and subsequent plant generation. Therefore, there is a need for an analytical separation technique that is ideal for analyzing small amount of peanut oil derived from a small portion of a single peanut seed. Capillary electrophoresis (CE) with its small sample requirement offers the ideal analytical platform for small amounts of peanut oils. In addition, CE is a versatile separation technique that is increasingly employed in the analysis of complex natural matrices, e.g., food (for a recent review see Ref. [12]), and systems biology [13] due to the fact that CE offers high separation efficiency and unique selectivity.

Despite the fact that CE has been employed in the analysis of a wide variety of compounds, it has been rarely applied to peanut oil fatty acids investigations. The most traditionally used methods for the determination of fatty acids in natural samples including peanut oils have been gas chromatography (GC) [14, 15] and high performance liquid chromatography (HPLC) [16, 17], with HPLC methods offering slightly better precision than the GC-flame ionization detector methods [18]. However, both techniques require time-consuming steps

of derivatization of fatty acids to improve volatility in GC and increase detectability in HPLC.

One of the major concerns in analyzing FFA´s by CE has been their limited solubility in aqueous electrolyte systems. This particular problem has been overcome by introducing nonaqueous CE (NACE) more than a decade ago [19], and the technique has been reviewed in Ref. [20]. NACE as well as partially aqueous CE (i.e., organic rich CE) have found use in the separation of saturated and unsaturated FFA´s. *N*-Methylfomamide (NMF) – dioxane based electrolyte systems [21, 22], and 100% methanol with 12.5 mM tetraethylammonium chloride [23] were effective in the separation of some long-chained fatty acids (≥ C16). Also, effective in the separation of fatty aids have been some partially aqueous electrolyte systems containing 60% acetonitrile [24], 60% methanol [25], 50% methanol [26], mixture of 10 mM sodium dodecyl benzenesulfonate (SDBS), 50% acetonitrile and Brij [27], sodium dodecyl sulfate (SDS) micelles with 20% methanol [28], mixture of 40% acetonitrile and 30% ethanol [29], or mixture of 4 mM SDBS, 10 mM Brij 35, 2% 1-octanol and 45% acetonitrile [30].

An additional difficulty in CE of fatty acids is the absence of strong chromophores in their structures that prohibits their sensitive detection in direct photometric detection. A solution to this problem was provided by the introduction of indirect UV and indirect fluorescence detection in CE well over a decade ago for the sensitive detection of compounds that lack chromophores and fluorophores (for a review see Ref. [31]). In indirect detection, a detectable co-ion which possesses higher molar absorptivity or high fluorescence quantum

yield is added to the running electrolyte, thus providing a continuous detector response. Since the charge neutrality must be maintained, an analyte of the same charge as the detectable co-ion will therefore displace this co-ion causing a decrease in the detector response. Thus the resulting peak is derived from the detectable background co-ion rather than the analyte itself forming the basis of indirect detection. Among the various UV detectable co-ions (i.e., background UV absorber) that proved useful in the indirect UV detection of FFA´s are anthraquinone-2-caroxylic acid [21], adenosine-5'-monophosphate (AMP) [22], 3,5-dinitrobenzoic acid or trinitrobenzenesulfonic acid [24], *p*-anisate [25, 26], and SDBS surfactant [27, 30]. The choice of these background UV absorbers was dictated by the nature of the nonaqueous electrolyte systems or the partially aqueous electrolyte systems. Indirect laser induced fluorescence (LIF) detection of FFA´s was carried out with the fluorescing dye co-ion merocyanine 540 [29]. On the other hand, direct UV detection of parinaric acid and conjugated linoleic acid at 305 nm and 234 nm [28], respectively, while saturated linear fatty acids derivatized with near-infrared absorbing fluorescent dye were detected by direct LIF [23].

In this chapter, the electromigration behaviors of five standard FFA's, which are common in most oils, namely palmitic, stearic, oleic, linoleic and linolenic acids were first investigated in CE under various nonaqueous and partially aqueous electrolyte systems in order to achieve a baseline separation for these FFA's. The optimal electrolyte system was applied to the determination of oleic acid and linoleic acid in peanut oils in order to provide a

CE method for the screening of high oleic peanut seeds that are essentials in breeding programs.

Experimental

Instrumentation

The CE analysis of all standards and peanut samples was performed on a P/ACE MDQ (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a photodiode array detector and a 0-30 kV high-voltage power supply. The data were collected on an IBM personal computer configured with P/ACE MDQ gold software version 1.5. The capillary columns used for separation were untreated fused-silica capillaries with 50 μm I.D. and 363-359 μm O.D from Polymicro Technologies (Phoenix, AZ, USA). The total and effective lengths were 60.2 cm and 50 cm, respectively. The experiments were performed at a constant voltage of 28 KV and the temperature was maintained at 20 ˚C. All standards and samples were injected hydrodynamically for 3 sec by application of a pressure of 0.5 psi. The indirect UV detection was carried out at a wavelength of 254 nm using anthraquinone-2-carboxylic acid, *p*-anisate or adenosine 5'-monophosphate (AMP) as the background UV absorbers.

Refluxing of oil extracts was done in a Thermolyne Dri-bath (Dubuque, IA, USA). The samples were centrifuged in a 1550-RPM Centricone (Precision Scientific, Chicago, IL, USA) and sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonic Corp., Danbury, CT, USA). The other equipments used were Speed Vac (model-SC110) equipped with a Refrigerated Vapor Trap

(Model-RVT4104) along with vacuum pump components Model-VLP120 (Savant Instruments, Inc., Holbrook, NY, USA) and a pH meter from Jenco electronics, Ltd. (San Diego, CA, USA).

Materials and Reagents

Saturated free fatty acid (FFA's) standards such as stearic acid (C18:0), palmitic acid (C16:0) and nonadecanoic acid (C19:0) and unsaturated FFA's such as oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3), adenosine 5'-monophosphate (AMP) monohydrate from yeast, tris (hydroxymethyl) aminomethane (Tris), α-cyclodextrin (α-CD) and β-CD were obtained from Sigma (St. Louis, MO, USA). Dioxane, HCl, diethyl ether and anhydrous sodium sulphate were from Fischer Scientific (Fairlawn, NJ, USA) and *N*-methyformamide (NMF), anthraquione-2-carboxylic acid and 4-methoxybenzoic acid were from Aldrich (St. Louis, MO, USA). Dimethyl β-cyclodextrin (DMe β-CD) was obtained from Fluka (Fluka Chemie, Buchs, Switzerland), hexane from EM Science (Cherry Hill, NJ, USA) and KOH was from Mallinckrodt, Inc. (Paris, Kentucky, USA). Ethanol and methanol were purchased from AAPER Alcohol and Chemical Co. (Shelbyville, Kentucky, USA).

Preparation of Solutions

Three different electrolyte systems were evaluated in this study. The first non-aqueous electrolyte system (i.e., running electrolyte) that was tried consisted of 40 mM Tris and 2.5 mM anthraquinone-2-carboxylic acid in NMF-dioxane (3:1,

v/v) [21]. The various FFA's standard stock solutions were prepared using neat NMF as the solvent to give a concentration of 5 mM. All standard solutions used in the CE experiments were prepared by diluting an aliquot of the stock solutions in the running electrolyte.

The second electrolyte system was hydro-organic system of water-methanol (1:1, v/v) containing of 10 mM Tris-5 mM *p*-anisate and 1 mM dimethyl-β-CD [26] Tris was partially neutralized with *p*-anisic (4-methoxybenzoic) acid to pH 8.1. The stock solutions of the various standard FFA's were prepared in neat methanol.

The third running electrolyte system in its final and optimal composition consisted of 40 mM Tris, 2.5 mM AMP and 7 mM of α-CD in a mixture of NMF-dioxane-water (5:3:2, v/v). The stock solutions of all standard FFA's were prepared by dissolving appropriate amount of acids in NMF-dioxane (4:1 v/v) to give concentration of 5 mM. All standard solutions were prepared by diluting an aliquot of the stock solutions in the running electrolyte.

The concentrations of standard solutions for the two FFA's oleic acid (C18:1) and linoleic acid (C18:2) used for calibration were 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mM while the FFA C19:0 used as the internal standard had a concentration of 0.5 mM. The internal standard was first dissolved in dioxane, and then NMF was added. All stock and standard solutions were stored in the refrigerator.

Electrophoretic Conditions

A new capillary column was flushed successively with a manual syringe using the following solutions and duration: 1M NaOH for 10 min, followed by water for 3 min, 0.1 M HCl for 10 min, water again for 3 min, and finally with the running electrolyte for 5 min. This successive washing of the capillary was done daily at the start of the experiments using the P\ACE MDQ instrument setting and applying a pressure of 65 psi to the vial for each washing step. Before injections, equilibration of the capillary was carried out at the running voltage (i.e., 28 kV) for 20-30 min with freshly prepared running electrolyte solution.

Hydrodynamic injection of all standards and samples was done for 3 sec at 0.5 psi (1psi = 6895 Pa) at the anodic end. The capillary was pressure rinsed with the running electrolyte for 2 min at 65 psi between injections. Each run was performed at 28 kV with the capillary and sample temperature maintained at 20 ˚C, and the detection was set at 254 nm. The running electrolyte in the inlet reservoir was changed several times a day while the outlet reservoir electrolyte was changed daily. During weekends and at nights, the capillaries were stored in water.

Peanut Breeding

The analysis of fatty acid was carried out primarily on two peanut lines (Okrun and ARSOK-R1) and to a lesser extent on some other lines. Okrun is a peanut cultivar that was released in 1980's [32] while ARSOK-R1 is an advanced breeding line under development for variety release [33]. Okrun, classified as the runner variety results from the cross of Florunner and Spanhoma [32]. Both

lines, Okrun and ARSOK-R1 were planted in the test plots of the Oklahoma State University Agricultural Experiment Station in Ft. Cobb, Oklahoma around May 15, 2006 and harvested around October 15, 2006. To harvest, the plants were first dug (uprooted and turned over) and then they were left in the windrows for 3 days, which is sufficient, for the peanut hay to dry. After 3 days, the hay is dry enough to be separated from the pods (thrashed). The pods are placed in a peanut dryer which forces warm air through a trailer and dries the pod out. Finally, the pods are shelled and the seeds are stored at 4 ˚C until analysis.

Oil and Fatty Acids Extraction From Single Peanut Seeds

The seeds used for the determination of FFA's were sound and mature. Prior to analysis, the seeds were brought to room temperature. After removing the seed coat, a small portion of the seed was cut from the distal end (away from the embryo), weighed and ground to paste in a mortar-pestle in a sufficient volume of hexane. The slurry was transferred into a vial. The remaining paste was scraped and washed into the vial. The mortar was washed 2-3 times with hexane as required for complete transfer of the contents. The vial was vortexed for ca. one min and centrifuged for about 20-25 min at 10,000 rpm. The supernatant layer was collected and the hexane was evaporated using speed vacuum. The peanut oil left behind was further used for extraction of FFA's.

The extraction of peanut lines was accomplished using the method of Dermaux *et al*. with some modifications [34]. To 20-25 mg of oil, 0.55 mL of 1 M potassium hydroxide in 95 % ethanol solution was added and subjected to

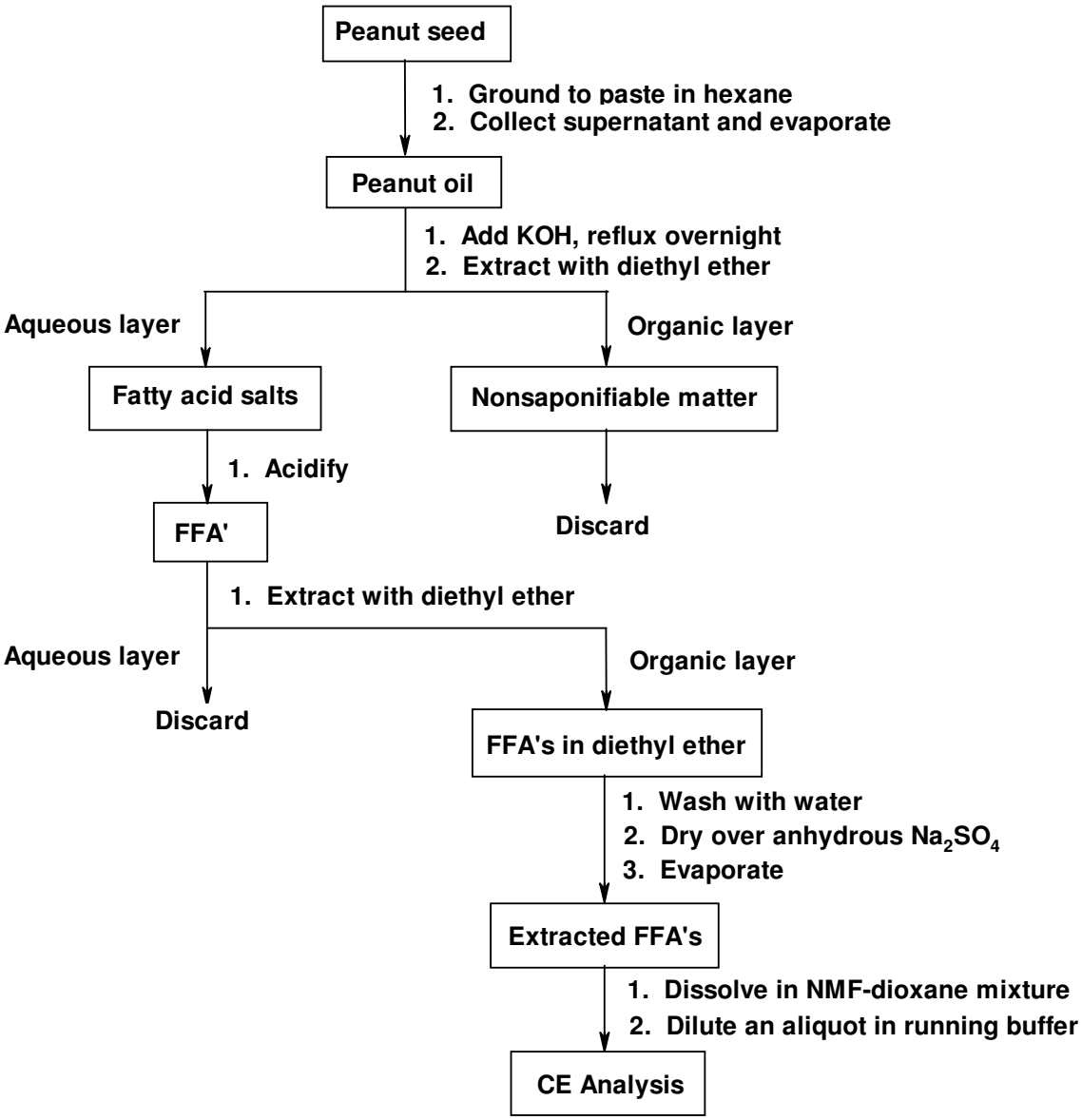

Figure 1. *Chart showing the various steps for oil and free fatty acids extraction from a portion of a single peanut seed*

overnight reflux in a dri-bath. Thereafter, the mixture was cooled to room temperature, and then transferred to a separatory funnel by rinsing the reflux vial with 1.5 mL water. The non-saponifiable matter was extracted using 1.5 mL of diethyl ether. The aqueous layer containing fatty acid salts was separated from the organic layer and then acidified to pH 2.0 with 1 M HCl. The FFA's formed were extracted with 6 $\times$ 1 mL diethyl ether. All organic layers were collected, washed with 1 mL of water and dried over anhydrous sodium sulphate. The solvent was evaporated at room temperature in the fume hood. The extraction procedure is summarized in Fig. 1.

Sampling of Extracted Fatty Acids From Peanut Oil for CE Analysis

For direct CE analysis, the extracted FFA's were dissolved in 0.5 mL of NMF-dioxane (1:1, v/v) mixture. This was done by first dissolving the FFA's in dioxane followed by addition of NMF and then vortexing for 10-15 s. An aliquot (1 or 2 μL) from this solution was taken and diluted (50-fold or 100-fold) in the final running buffer and vortexed for 4-5 sec. The sample was then pressure injected in the CE instrument.

Results and Discussion

CE of Fatty Acids

Because of their weak carboxylic acid groups, free fatty acids ionize readily in basic solutions forming FFA anions, which favor their differential migration in CE. One important factor that should be considered during FFA's

separation in CE especially in aqueous media is their solubility, which tends to decrease as the hydrophobic alkyl chain of the fatty acid increases. This fact has led to developing and investigating non-aqueous CE systems. Furthermore, FFA's lack chromophoric centers that would allow their sensitive detection in the UV, thus the decision to perform indirect UV detection.

The first electrolyte system that was examined in the present CE study consisted of a non-aqueous medium which was described by Drange and Lundanes [21]. It was composed of 2.5 mM anthraquinone-2-carboxylic acid and 40 mM Tris in NMF-dioxane (3:1, v/v). Anthraquinone-2-carboxylic acid was the background UV absorber in the running electrolyte having UV-maximum at 263 nm. The FFA´s were detected by indirect UV detection at the wavelength of 254 nm. A typical electropherogram is shown in Fig. 2, and the CE run was achieved in a slightly over 10 min. This was facilitated by a relatively fast EOF with a migration time t_0 of ~ 6.6 min. This may be attributed to the high dielectric constant to viscosity ratio of the NMF [35]. However, and as shown in Fig. 2, this non-aqueous electrolyte system yielded poor resolution between the FFA's investigated. The palmitic acid (C16:0) and the linolenic acid (C18:3) formed the critical pair, which was not resolved.

The second electrolyte system investigated in this report consisted of a partially aqueous medium containing 10 mM Tris, 5 mM *p*-anisate and 1 mM dimethyl-**β**-CD in a binary solvent mixture of water-methanol (1:1, v/v). This electrolyte system, which was first described by Collet and Gareil [26], had the *p*-anisic acid as the background UV absorber while dimethyl-β-CD was introduced

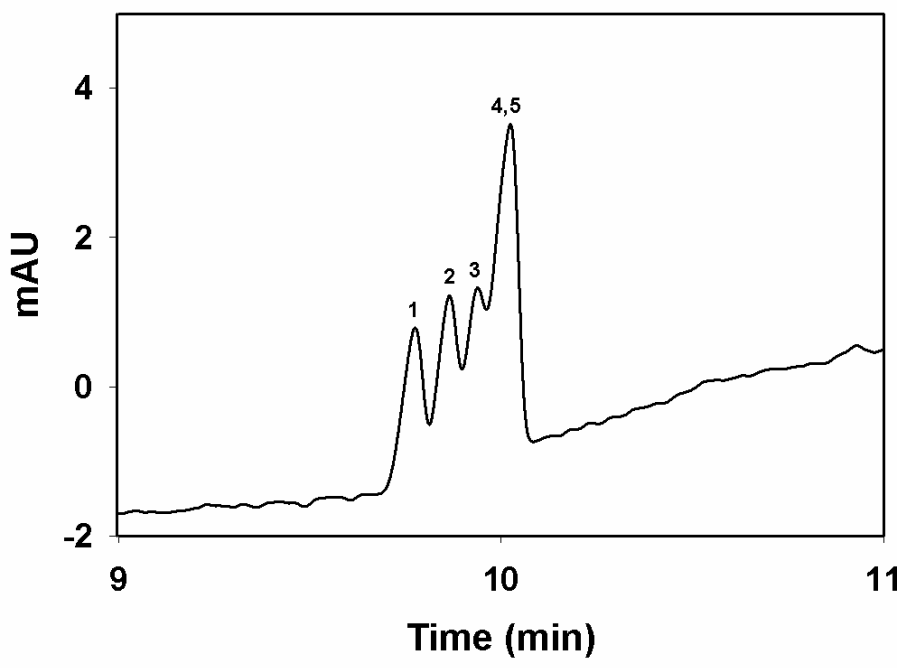

Figure 2. *Electropherogram of five standard FFA's using a nonaqueous electrolyte system consisting of 2.5 mM anthraquinone-2-carboxylic acid (UV absorber) and 40 mM Tris in NMF-dioxane (3:1, v/v), pH 10-11. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: 1, C18:0; 2, C18:1; 3, C18:2; 4, C18:3; 5, C16:0*

to adjust the selectivity, which is related to the difference in effective mobilities. The standard FFA´s analyzed in this hydro-organic solvent mixture are shown in Fig. 3 where palmitic acid (C16:0) was not well resolved from oleic acid (C18:1). In addition, baseline instability and peak fronting were observed and problems regarding reproducibility were also encountered reason for which the system was not further pursued.

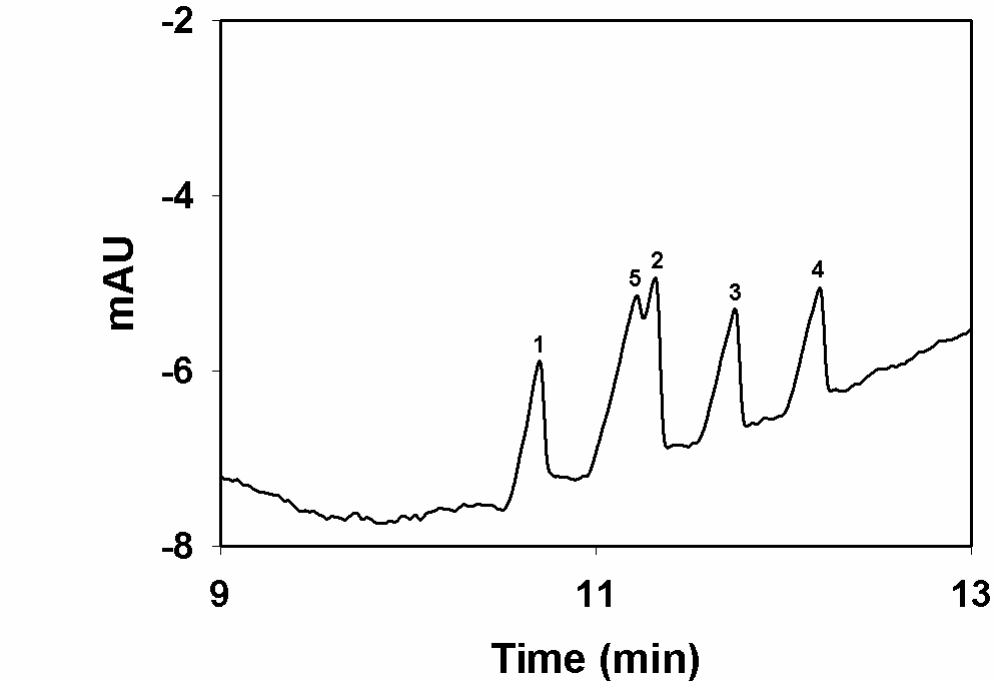

Figure 3. *Electropherogram of five standard FFA's using a partially aqueous electrolyte system consisting of 10mM Tris-5 mM p-anisate and 1 mM dimethyl-β-CD in a water-methanol (1:1, v/v) mixture whose pH was adjusted to 8.1. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: 1, C18:0; 2, C18:1; 3, C18:2; 4, C18:3; 5, C16:0*

The next electrolyte system under study was described by Haddadian *et al.* [22], and consisted of 40 mM Tris and 2.5 mM adenosine-5'-monophospahte (AMP) in NMF-dioxane-water (5:4:1, v/v) mixture. The standards FFA's were detected by indirect UV detection at 254 nm. The elution order of the FFA's was the same as in the nonaqueous NMF-dioxane system (see above), and the

palmitic acid (C16:0) co-eluted with linolenic acid (C18:3). The resolution in this partially aqueous system is superior to that in the neat NMF-dioxane system, compare Fig. 4A to Fig. 2. This observation revealed the importance of adding water to the electrolyte system in terms of improved FFA's resolution. The effect of the water content of the running electrolyte on resolution was studied by varying its percentage in the final running electrolyte. At 15 % water (Fig. 4B), there was a slight improvement, which was further enhanced by adding 20% of water (Fig. 4C) to the non-aqueous mixture. At 30 % water (Fig. 4D), no significant effect was observed and thus, 20% was considered as the best water content in NMF-dioxane-water mixture. Increasing the water content in the running electrolyte was done at the expense of decreasing the percentage of dioxane while keeping the NMF content constant. Decreasing the percent of NMF instead of decreasing the percent dioxane would have been detrimental to EOF [22], which in turn would have increased the analysis time.

However, adjusting the percentage of water did not lead to separating palmitic acid from linolenic acid. To improve the selectivity between these two co-eluting acids, a selectivity modulator that affects the effective mobilities of the two acids was needed. In this regards, 0.2 % (w/v) Brij 35 was added to the running electrolyte consisting of 40 mM Tris and 2.5 mM AMP in NMF-dioxane-water (5:3.5:1.5, v/v) mixture. Although the non-ionic surfactant Brij 35 would enhance the solubility of FFA's in the running electrolyte, its presence as a hydrophobic selector that would bind to the various FFA's and modulate their migration did not resolve the co-eluting pair, palmitic acid and linolenic acid.

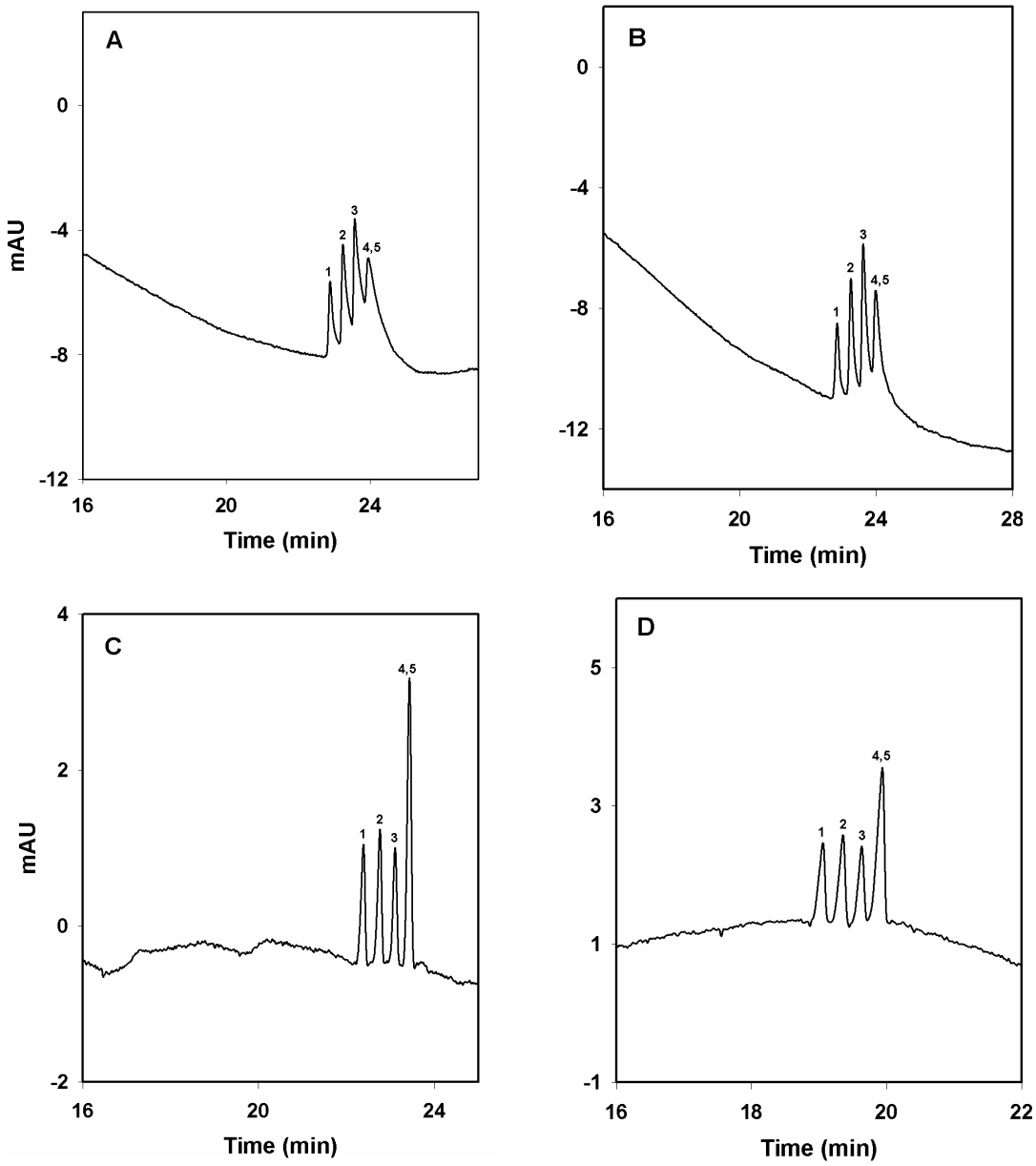

Figure 4. *Effect of water on FFA´s resolution. Electropherograms of five standard FFA's using partially aqueous electrolyte consisting of 40 mM Tris and 2.5 mM AMP in (A) NMF-dioxane-water (5:4:1, v/v), (B) NMF-dioxane-water (5:3.5:1.5, v/v), (C) NMF-dioxane-water (5:3:2, v/v) and (D) NMF-dioxane-water (5:2:3, v/v) mixture, pH in the range of 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: 1, C18:0; 2, C18:1; 3, C18:2; 4, C18:3; 5, C16:0.*

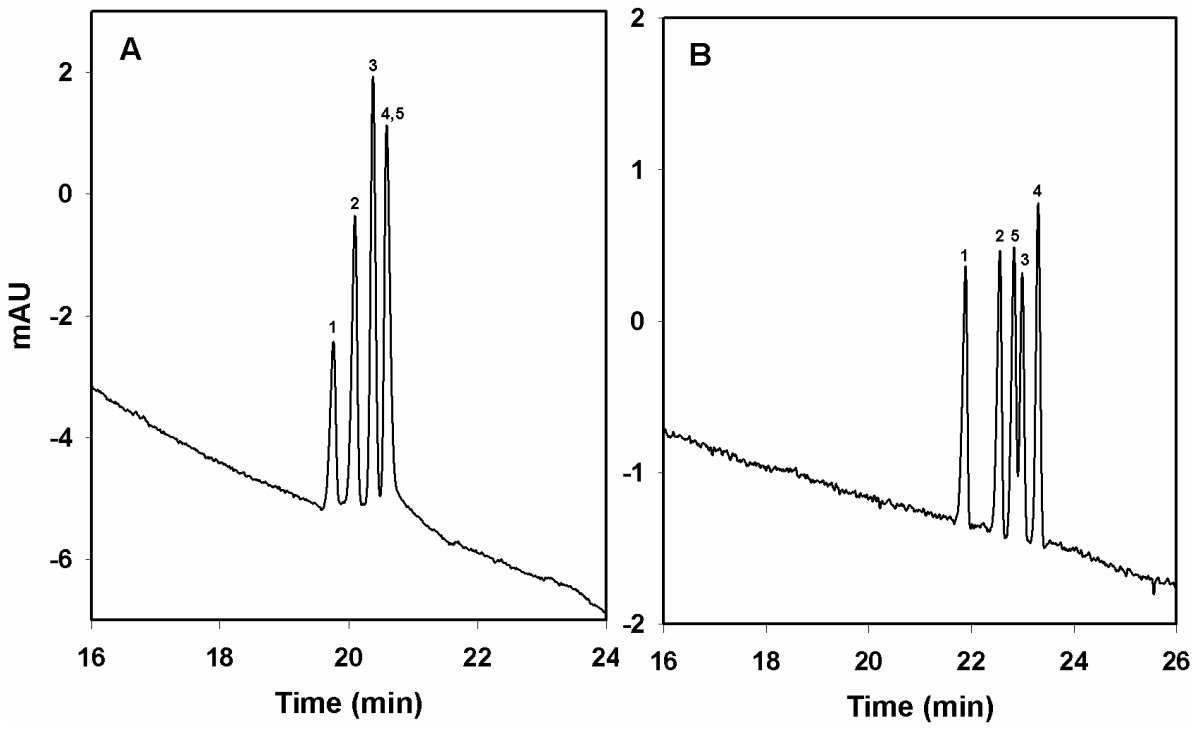

Figure 5. *Electropherograms of five standard FFA's in presence of (A) 5 mM β-CD and (B) 5 mM α–CD in the partially aqueous electrolyte consisting of 40 mM Tris and 2.5 mM AMP in NMF-dioxane-water (5:3:2, v/v) mixture, pH between 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: 1, C18:0; 2, C18:1; 3, C18:2; 4, C18:3; 5, C16:0.*

Next, cyclodextrins were incorporated into the running electrolyte to modify FFA´s electromigration and in turn selectivity. In fact, CD's have been known to resolve difficult to separate solute pairs [36, 37]. In the case of FFA´s presented here, α-cyclodextrin (α-CD) was more effective than β-CD in modifying the

selectivity under otherwise identical running conditions; compare Fig. 5B to 5A. On this basis, the electrolyte system incorporating α-CD was further investigated and different concentrations of α-CD were examined and the results are presented in Fig. 6. As mentioned above, in the absence of α–CD, the palmitic acid (C16:0) co-migrated with linolenic acid (C18:3) whereas between 2 mM to 4 mM (see Fig. 6A), it co-eluted with linoleic acid (C18:2) indicating that the palmitic acid formed a strong host-guest interaction with α–CD. Palmitic acid was partially co-eluting with linoleic acid (C18:3) at 5 mM concentration of α-CD and complete resolution was observed in the range of 6 mM to 7 mM (Fig. 6B). Above 8 mM concentration of α–CD, the mobility of palmitic acid showed extreme reduction and was almost equal to the mobility of the oleic acid (C18:1), Fig. 6C. The 7 mM concentration of α–CD (see Fig. 6B) was used for the analysis of FFA's in the peanut oil, see next section.

The effects of the concentrations of α–CD on the electroosmotic mobility, apparent mobility and effective electrophoretic mobility are shown in Fig. 7. As can be seen in Fig. 7A, the effective electrophoretic mobility of the FFA's decreased with increasing α–CD due to the decrease in the charge-to-mass ratio of the FFA^- ion upon its binding to α–CD to produce the complex $CDFFA^-$. Thus, the electrophoretic mobility of $CDFFA^-$ is less than that of FFA^-. That is $\mu_{ep,CDFFA^-} < \mu_{ep,FFA^-}$. The effective electrophoretic mobility (μ_{eff}) of FFA^- is given by Eq. 1:

$$\mu_{eff} = \mu_{ep,FFA^-}\chi_{FFA^-} + \mu_{ep,CDFFA^-}\chi_{CDFFA^-} \qquad (1)$$

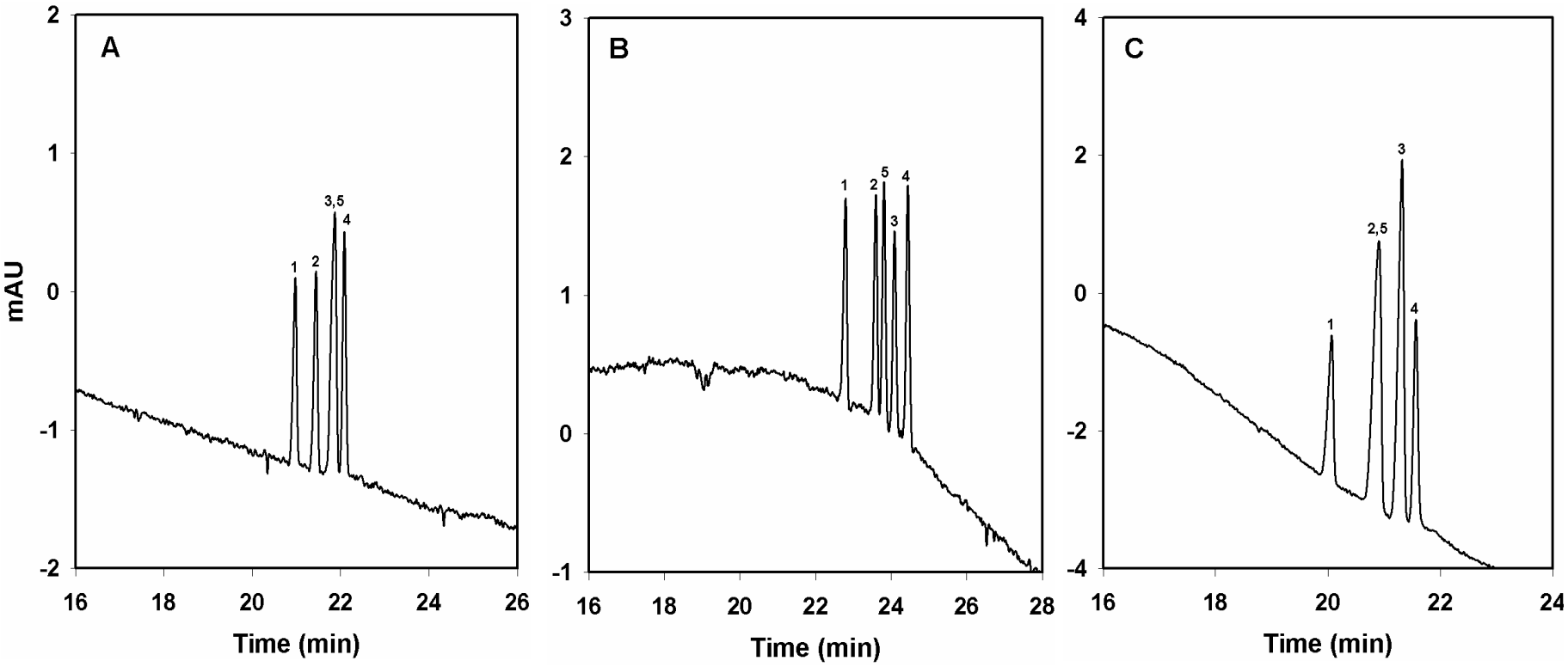

Figure 6. *Electropherograms of five standard FFA's in presence of (A) 2 mM α–CD, (B) 7 mM α–CD and (C) 10 mM α–CD in the partially aqueous electrolyte consisting of 40 mM Tris and 2.5 mM AMP in NMF-dioxane-water (5:3:2, v/v) mixture, pH between 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: 1, C18:0; 2, C18:1; 3, C18:2; 4, C18:3; 5, C16:0.*

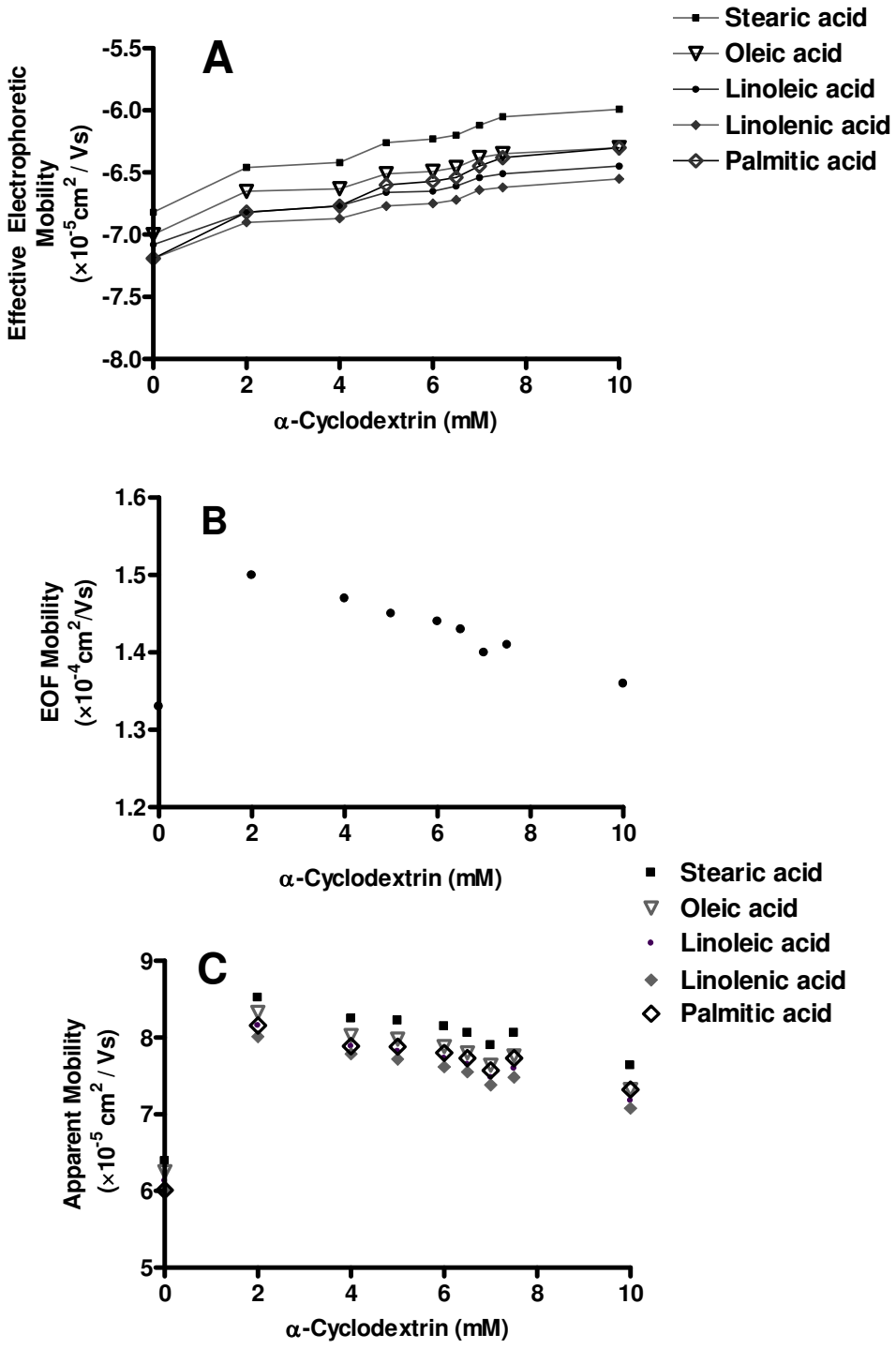

Figure 7. *Effect of the concentration of α–CD on (A) effective electrophoretic mobilities, (B) electroosmotic mobility and (C) apparent mobilities of five standard FFA's in a NMF-dioxane-water (5:3:2,v/v) mixture.* .

where μ_{ep,FFA^-} is the electrophoretic mobility of the free acid, $\mu_{ep,CDFFA^-}$ is the electrophoretic mobility of the complex $CDFFA^-$, X_{FFA^-} is the mole fraction of the free acid and X_{CDFFA^-} is the mole fraction of the complex $CDFFA^-$ given by $\chi_{CDFFA^-} = 1 - \chi_{FFA^-}$. On this basis, it is clear that as the amount of added α–CD is increased the mole fraction X_{CDFFA^-} is increased while that of the FFA- is decreased. The net result is a decrease in the effective electrophoretic mobility of the solute. This behavior is seen in Fig 7A by the monotonous decrease in the effective electrophoretic mobility in the concentration range studied.

Since the decrease in the effective electrophoretic mobility is continuous in the concentration range studied of α-CD, the apparent mobility of the FFA´s almost paralleled that of the EOF upon varying α-CD concentration, compare Fig. 7B and 7C. In general, the EOF increased first when going from 0 to 2 mM α-CD, and then decreased slightly thereafter in the range between 2 mM and 10 mM α-CD, Fig. 7B. This behavior of EOF may be explained by the fact that the addition of α-CD to the running electrolyte would increase the dielectric constant of the medium, which would lead to an abrupt increase in the EOF. However, as the α-CD concentration is further increased the viscosity of the medium is increased and also a thicker layer of α-CD is then bound to the capillary wall, which would lead to an increase in the local viscosity at the wall where the EOF usually develops [38]. The following equation (Eq. 2) that relates the zeta potential ζ in the electric double layer (EDL) and EOF supports the above explanation for the change in EOF upon varying α-CD concentration in the running electrolyte [38, 39]:

$$\mu_{eo} = \frac{\varepsilon}{\eta}\zeta \approx \varepsilon \int_0^{\zeta} \frac{1}{\eta} d\Psi \qquad (2)$$

where μ_{eo} is the electroosmotic mobility, ε is the medium dielectric constant, η is the viscosity of the medium and ψ is the electric potential. Hjerten through the use of Eq. 2 [38] illustrated that the EOF mobility will go to zero as the viscosity of the buffer inside the EDL, due adsorption of polymer, approaches infinity even though the viscosity of the bulk solution remains unchanged.

Analysis of Oleic Acid and Linoleic Acid in Peanut Oil by CE

The various steps involved in the preparation of a given peanut oil sample derived from a small portion of a single peanut seed, and the subsequent extraction of FFA´s from peanut oil are described in the Experimental section, and summarized in Fig. 1. Okrun (moderate oleic acid with respect to linoleic acid) and ARSOK-R1 (high oleic with respect to linoleic acid) were the primary plant lines under study. In these peanut oil samples, oleic and linoleic acids were readily detected by CE using the partially aqueous electrolyte system described in the above section. Also, a tiny peak of stearic acid (C18:0) could be seen in these samples as peanut oils are known to have about 2-4 % of stearic acid [5], see Fig. 8.

For the quantitative determination of oleic and linoleic acids in peanut oil samples, an internal standard that shares similar properties with these solutes is the best choice. In this regards, the nonadecanoic acid (C19:0), which is usually

absent or present in trace amounts in peanut oils [5] was selected as the internal standard for establishing the standard calibration curve. The C19:0 being one

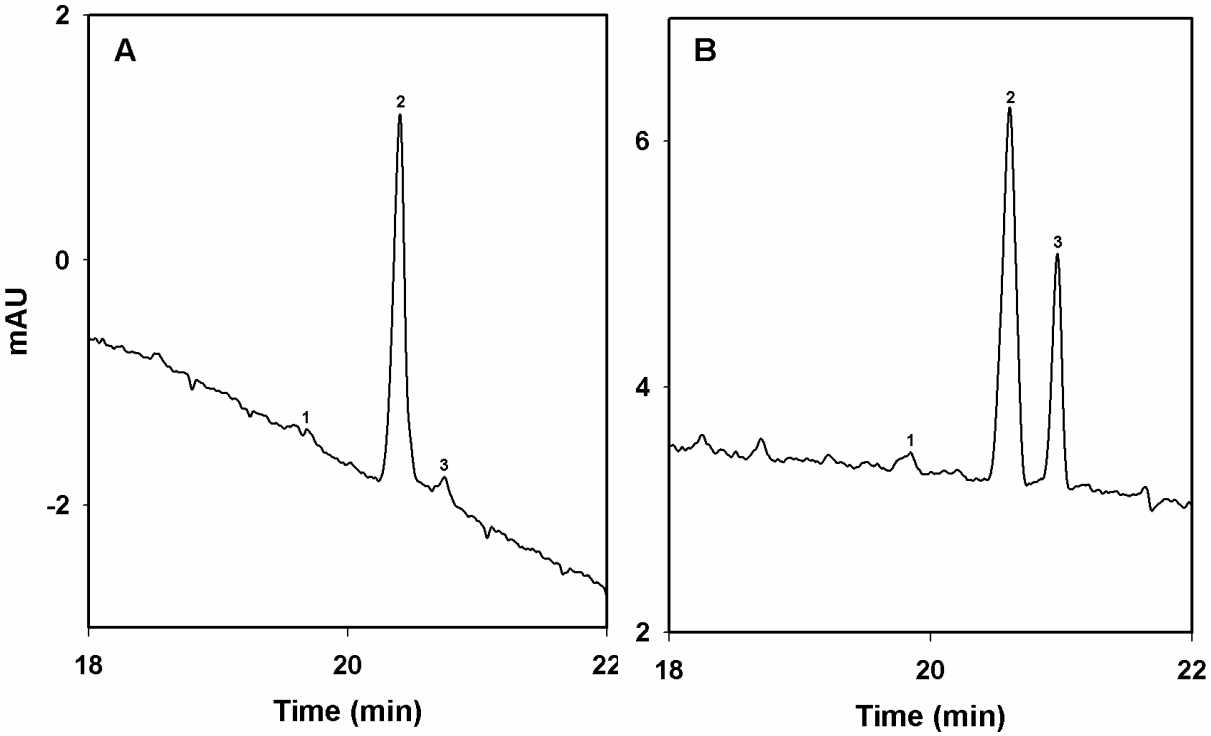

Figure 8. *Electropherograms of peanut oil samples from small portions of single peanut seeds of two peanut lines, (A) ARSOK-R1 and (B) Okrun using a partially aqueous electrolyte consisting of 40 mM Tris, 2.5 mM AMP and 7 mM α–CD in NMF-dioxane-water (5:3:2, v/v) mixture, pH between 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: 1, C18:0; 2, C18:1; 3, C18:2.*

methyl group longer than the C18:0 migrated right before C18:0 with complete resolution as shown in Fig. 9 where the complete separation of the four standard FFA's (C19:0, C18:0, C18:1 and C18:2) is displayed. In all its aspects, the C19:0 was therefore a good choice as the internal standard for analyzing peanut oil

samples. Only the determination of the oleic acid and linoleic acid contents of the peanut seed under investigation were of a major interest to the present study. The same samples shown in Fig. 8 were then re-injected in the CE system using C19:0 as the internal standard (see Fig. 10).

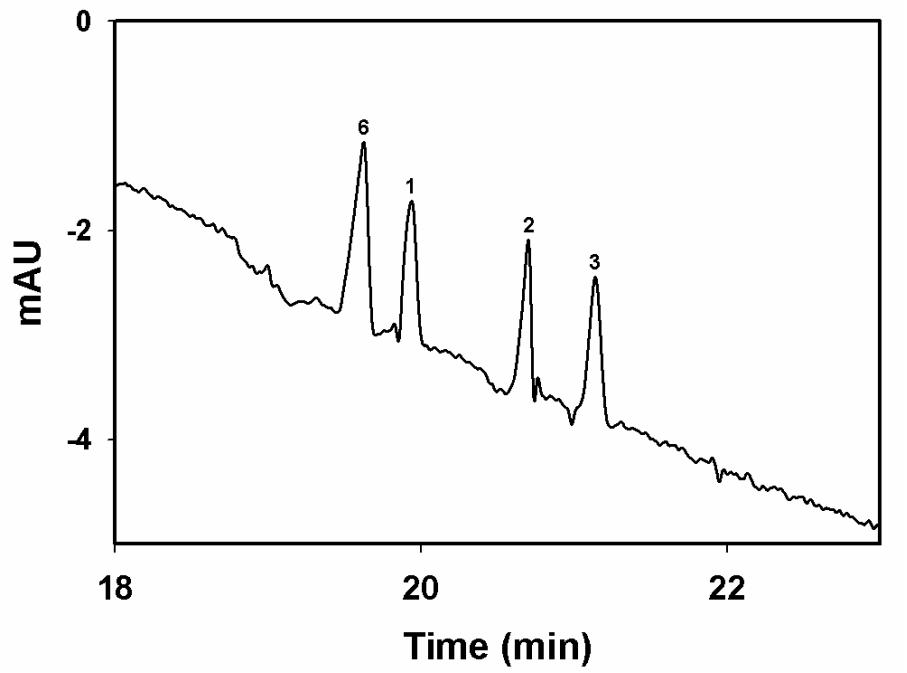

Figure 9. . *Electropherograms of four standard FFA's all having a concentration of 0.5 mM using a partially aqueous electrolyte consisting of 40 mM Tris, 2.5 mM AMP and 7 mM α–CD in NMF-dioxane-water (5:3:2, v/v) mixture, pH 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak Identification: 1, C18:0; 2, C18:1; 3, C18:2; 6, C19:0 (I.S.).*

To check the reproducibility of the extraction method employed, the peanut oil obtained from a single seed of each peanut line (Okrun and ARSOK-

R1) was split into three equal size oil samples, which then were extracted under the same set of conditions and subsequently analyzed by CE (Fig. 11). The quantitative data in terms of % oleic acid and % linoleic acid are summarized in Table 1. As can be seen In Table 1, the extraction is reproducible as expressed in terms of %RSD which are 0.79% and 0.44% for oleic acid from ARSOK-R1 and Okrun, respectively, and 1.22% and 4.96% for linoleic acid from ARSOK-R1 and Okrun, respectively.

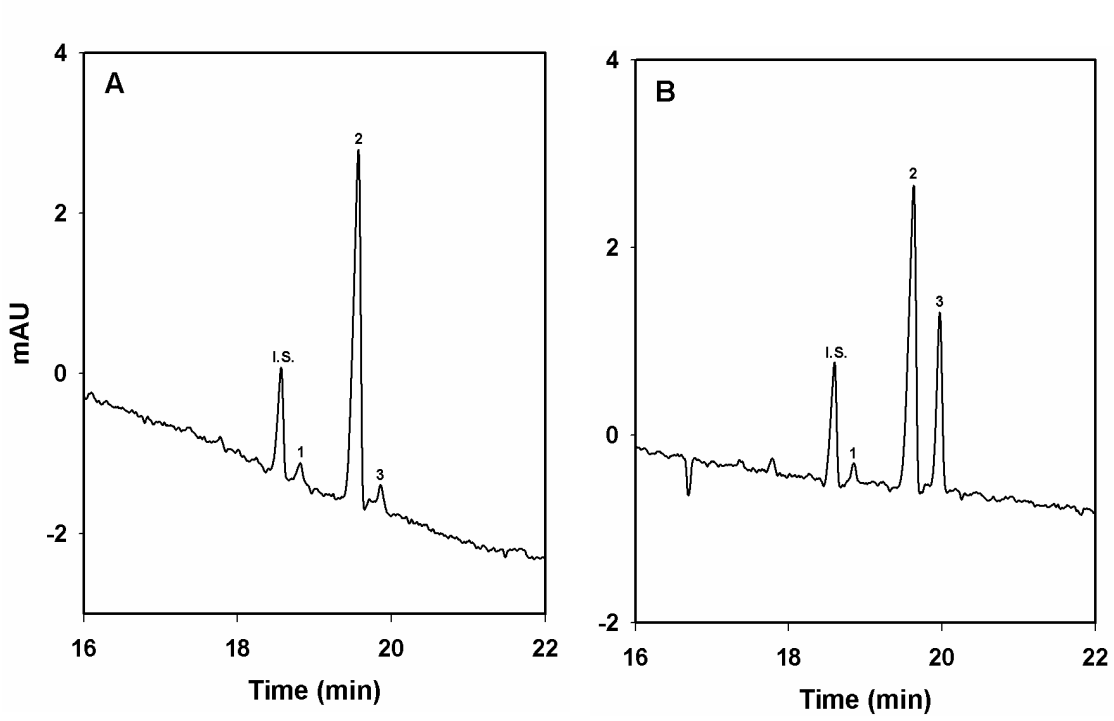

Figure 10. *Electropherograms of peanut oil samples from two small portions of two seeds of two lines, (A) ARSOK-R1 and (B) Okrun using a partially aqueous electrolyte consisting of 40 mM Tris, 2.5 mM AMP and 7 mM α–CD in NMF-dioxane-water (5:3:2, v/v) mixture, pH 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: I.S., C19:0; 1, C18:0; 2, C18:1; 3, C18:2.*

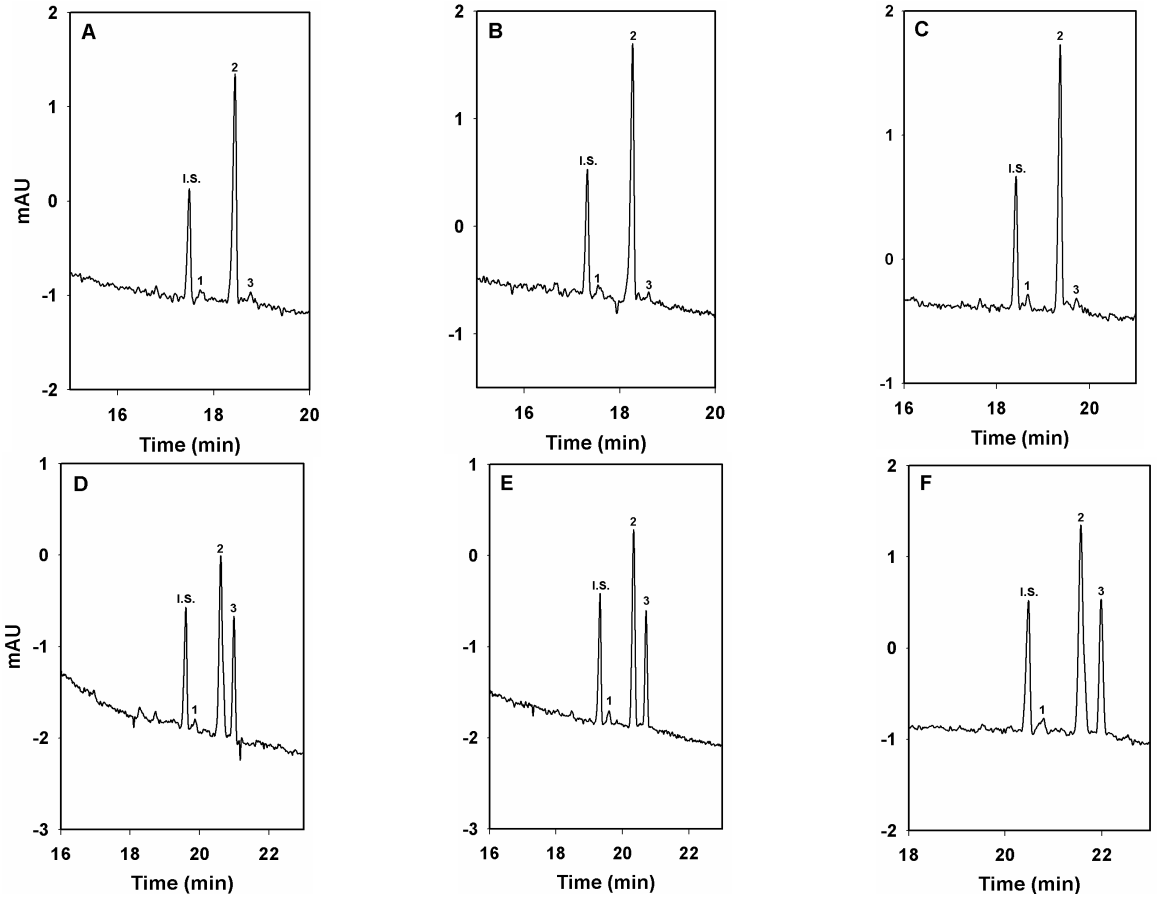

Figure 11. *Electropherograms of extractions from oil samples obtained from a single peanut seed. The samples were obtained from three extractions of three equal sizes oil samples obtained from a single peanut seed from each peanut line under the same set of conditions. (A), (B) and (C) shows three extractions of a sample from ARSOK-R1 line and (D), (E) and (F) from Okrun line. The partially aqueous electrolyte system consists of 40 mM Tris, 2.5 mM AMP, 7 mM α–CD in NMF-dioxane-water (5:3:2, v/v) mixture, pH 8-9. Hydrodynamic injection for 3 sec at 0.5 psi, applied voltage of 28 kV and detection at 254 nm. Peak identification: I.S., C19:0; 1, C18:0; 2, C18:1; 3, C18:2.*

Eight peanut oil samples derived from 8 small portions of 8 different peanut seeds each from Okrun and ARSOK-R1 were analyzed. The percent composition of oleic acid and linoleic acid in these 16 oil samples are given in Table 2. For Okrun, the oleic acid was found in the range of 39.9 – 59.3% and linoleic acid was in the range of 27 – 44.2%. For ARSOK-R1, the range for oleic acid was 60.8 – 85.9 % and the linoleic acid was present in the range of 4.8 – 6.7 %.

In all cases, the quantification of oleic acid and linoleic acid was achieved by comparing peak heights of the fatty acids in the sample with that of the standards from the calibration curve in the range of 0.2 mM to 1.4 mM. The calibration curves for oleic acid and linoleic acid were linear in the concentration range studied with R^2 equal to 0.9985 and 0.9961, respectively.

In all extractions aiming at determining the concentration of a given species in a given matrix, the major concern is the % recovery of the particular species form the matrix. In this regards some recovery measurements were undertaken and the results are summarized in Table 3. Here the recovery measurement should be taken using a species that can be added in known amount yet it is not present in the oil but similar in properties to the FFA´s. Under these conditions, the best choice was the nonadecanoic acid (C19:0).

In the recovery measurements, a known amount of C19:0 was added to a known amount of peanut oil from a small portion of a single seed so that the final concentration of C19:0 in the final extract and assuming that nothing is lost from it during the extraction process will be the same as that in a standard solution of

Table 1. *Determination of wt% oleic acid and wt% linoleic acid in peanut oil derived from a single peanut seed of ARSOK-R1 and Okrun peanut line. The oil fraction from each seed was divided into three equal portions each of which was subsequently treated and its fatty acids were extracted. Also, values of reproducibility expressed in terms of % relative standard deviation (%RSD) are listed.*

Oil portion number	ARSOK-R1		Okrun	
	% Oleic	% Linoleic	% Oleic	% Linoleic
1	85.86	5.25	51.98	36.92
2	87.04	5.31	52.08	33.43
3	85.87	5.38	52.42	35.23
Mean	86.26	5.31	52.16	35.19
SD	0.68	0.07	0.23	1.75
%RSD	0.79	1.22	0.44	4.96

Table 2. *Determination of wt% oleic acid and wt% linoleic acid in eight samples of peanut oil of ARSOK-R1 and Okrun peanut line. Each oil sample was derived from a small portion of a single peanut seed. The oil fraction from each seed was subsequently treated and its fatty acids were extracted.*

Peanut seed number	ARSOK-R1		Okrun	
	% Oleic	% Linoleic	% Oleic	% Linoleic
# 1	60.75	4.87	39.85	27.05
# 2	62.51	5.5	43.88	29.16
# 3	73.77	5.04	46.8	37.22
# 4	73.84	4.94	48.1	38.51
# 5	76.71	5.37	53.33	39.01
# 6	78.11	6.39	55.56	42.35
# 7	79.91	6.65	55.45	42.59
# 8	83.87	4.83	59.29	44.21

Table 3. *Percent recovery of nonadecanoic acid (C19:0) from peanut oil during the extraction process described in the Experimental section*

Extraction number	% Recovery	Absolute Error	Relative Error
# 1	102.11	5.52	5.71
# 2	93.44	3.15	3.26
# 3	94.22	2.37	2.45
Average	96.59	3.68	3.81

C19:0. Three extractions of three equally sized oil samples which were derived from a single peanut seed spiked with C19:0 were performed under the same conditions using the steps summarized in Fig. 1. Both the sample extracted from the spiked oil and containing C19:0 and the standard C19:0 solution were run in CE under otherwise identical running conditions. The peak heights of both the sample and the standard were compared thus yielding the % recovery listed in Table 3. As can be seen in Table 3, the % recovery was ~ 97% on the average. This % recovery is quite impressive given the number of steps involved in the extraction process, see Fig. 1.

Conclusions

This investigation has shown for the first time the suitability of CE with a partially aqueous electrolyte system for the analysis of free fatty acids (FFA´s) in small portions of single peanut seeds. The CE method developed here is based on a partially aqueous electrolyte system and allowed the separation of underivatized FFA´s using indirect UV detection. The extraction procedure of FFA´s from peanut oil was very reproducible with a ~97% recovery from the seed matrix. Furthermore, the CE method allowed the screening of high oil peanut seeds for breeding programs.

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CHAPTER III

CAPILLARY ELECTROPHORESIS OF PHOSPHOLIPIDS APPLICATION TO THE ANALYSIS OF PHOSPHOLIPIDS IN WATERMELON

Introduction

Phospholipids are a class of lipids, and a major component of all biological membranes, along with glycolipids, cholesterol and proteins. In its simplest form, a phospholipid is composed of one glycerol bonded to two fatty acids and one phosphate group. But phospholipids differ in terms of fatty acid chain length, degree of unsaturation (double bonds) in the fatty acids, and the polar (phosphate-containing) groups present. Thus, a phospholipid molecule consists of a polar hydrophilic (attracted to water) head group and a nonpolar hydrophobic (not attracted to water) tail attributing amphiphatic character to the molecule. This bipolar character of phospholipids is important in their biological activities in the cell membranes [1].

Additional chemical groups can be bonded to the phosphate head group (e.g. serine, inositol, glycerol, choline, etc.), and as a result the phospholipids can be classified depending on the nature of the attached chemical groups. Also, the

fatty acids can be any of a wide variety. For instance, cardiolipin is a diphoshatidyl glycerol in which two phosphatidic acids share a single glycerol. The structures of the phospholipids under study are shown in Fig. 1 [2].

There are various techniques used for lipid analysis. Among these, thin layer chromatography (TLC) [3-5] and high performance liquid chromatography (HPLC) [6-9] are commonly used. However, in TLC there is lack of precision of the stained chromatograms because of inconsistency related to both the phospholipids and background. HPLC not only requires high consumption of organic solvent but also is very time-consuming. Compared to TLC and HPLC, capillary electrophoresis (CE) proved to be a powerful alternative to chromatographic separations [2, 10-18]. CE offers many unique features such as higher efficiency, shorter analysis time and minimal sample requirement.

Due to their amphiphatic character, phospholipids can form one of a number of lipid phases when placed in water, such as liposomes or small lipid vesicles. When lipids are mixed with water, their hydrophobic portions interact with each other and their hydrophilic domains interact with water resulting into sheet type packing called lipid bilayer. This bilayer is selectively permeable to polar solutes and ions [1]. This inter association among phospholipids prohibits their analysis in aqueous CE. To overcome this problem, nonaqueous or organic rich electrolyte systems must be substituted to plain aqueous electrolyte systems for the efficient analysis of phospholipids in CE [2, 12-14, 16, 17]. Furthermore, phospholipids lack chromophores in their structures a fact that does not allow

their sensitive detection in direct UV. Although the presence of unsaturated groups such carbonyl, carboxyl and phosphate in phospholipids allows their

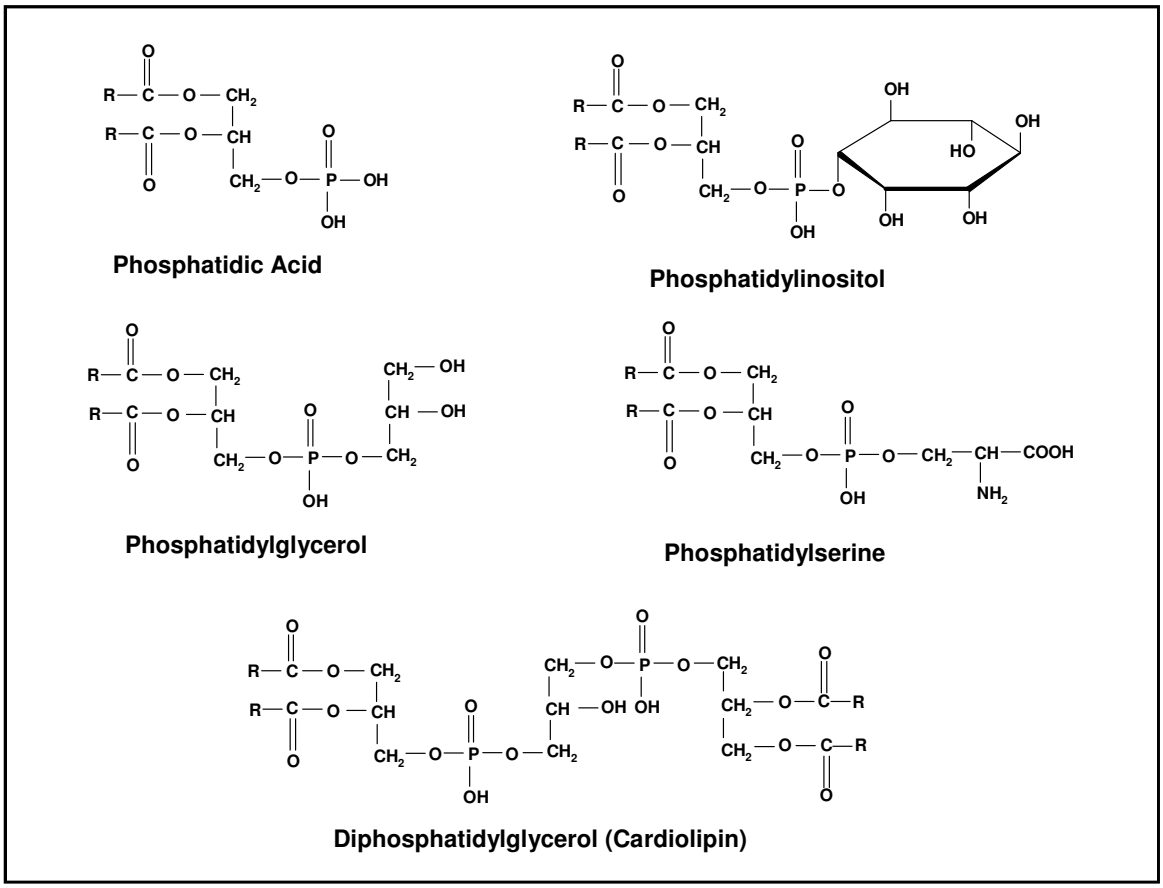

Figure 1. *General structure of the different classes of phospholipids studied where R is the hydrophobic tail of the attached fatty acid. R can primarily be palmitic acid and/or oleic for PA, stearic acid and/or arachidonic acid for PI, oleic acid and palmitic acid for PG, linoleic acid for PS and DG.*

direct UV detection at 200-214 nm such as in plant seeds [12], direct UV at low wavelengths had detection sensitivity problems in real samples. The lack of suitable chromophores can be overcome by indirect UV detection [10, 13].

The approach developed in Chapter II for the analysis of FFA´s, which involved the use of a partially aqueous electrolyte system and indirect UV at 254 nm, was slightly modified to accommodate the separation of phospholipids by CE. The method was then applied to phospholipid analysis in watermelon.

Study of phospholipids is often related to their biochemical and functional activities. For instance, limited shelf life of fresh cut pieces of watermelon is attributed to internal breakdown and cellular leakage. Over time, there is a fluid leakage from the watermelon chunks, and with time, there may be adverse loss in texture and the presence of nutrient-rich juice enhances microbial growth leading to product spoilage. This fluid, which is contained in the cell surrounded by the membrane leaks out due to membrane degradation thereby resulting in water soaking. The fluid leakage can be reduced or delayed by better maintenance of the cell membrane. Alterations in cellular membrane might be associated with decreasing cell leakage, thereby increasing shelf life. The analysis of phospholipids in watermelon by CE was conducted to evaluate these cellular alterations by measuring significant changes in the content of phospholipids, since they are the major structural component of the cell membranes. Samples of various breeding lines showing different fresh cut storability were utilized in the study. This project was conducted to detect

changes in membrane phospholipids and attribute these changes to cell leakage during fresh cut storage.

Experimental

Instrumentation

All lipid analyses were performed on a capillary electrophoresis system Model P/ACE 5010 from Beckman Instruments, Inc. (Fullerton, CA, USA). The instrument was equipped with a high voltage power supply giving voltage in the range from 1.0 to 30 kV and a fixed wavelength UV detector (deuterium lamp) having selectable filters at 200 nm, 214 nm, 254 nm and 280 nm. The electropherograms were recorded on a personal computer (Microsoft windows 95) equipped with Gold P/ACE (P/ACE station, version 1.0) system/software. The methods described by Gao *et al* [13] and Guo *et al* [12] were evaluated on a P/ACE MDQ CE instrument (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a photodiode array (PDA) detector and 0-30 kV high-voltage power supply. The data from P/ACE MDQ was collected on an IBM compatible personal computer configured with P/ACE MDQ (version 1.5) gold software.

Fused–silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an internal diameter of 50 μm and outer diameter of 363-359 μm were used. Analyses were performed in a capillary having total length of 37 cm and effective length of 30 cm and at a constant voltage of 28 kV. The temperature in all experiments was maintained at 25^{o}C. Standards and samples were introduced into the capillary by pressure injection at 0.5 psi for 5 sec and detected at a

wavelength of 280 nm by indirect UV detection using AMP as the chromophore in the running electrolyte.

Materials and Reagents

The standard phospholipids including L-α-phosphatidyl-L-serine (PS) and L-α-phosphatidylinositol (PI) ammonium salt, both from Glycine max (soybean), 3-sn-phosphatidic acid (PA) sodium salt from egg yolk, lecithin 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (PG) ammonium salt, cardiolipin (diphosphatidylglycerol DG) sodium salt from bovine heart, adenosine 5'-monophosphate (AMP) monohydrate from yeast and tris (hydroxymethyl) aminomethane were purchased from Sigma (St. Louis, MO, USA). Dioxane, HCl and acetic acid were from Fischer Scientific (Fairlawn, NJ, USA), whereas *N*-methylformamide (NMF) and 1-naphthoic acid (internal standard) were obtained from Aldrich (St. Louis, MO, USA). Methanol was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, Kentucky, USA). Ammonium acetate was obtained from Spectrum Quality Products, Inc. (Gardena, CA and New Brunswick, NJ, USA), 2-propanol from J.T. Baker Chemical Co., (Phillipsburg, NJ, USA) and acetonitrile from Pharmco Products Inc. (Brookfield, CT, USA).

The running electrolyte was sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonic Corp., Danbury, CT, USA) and the pH of the buffer was measured using a pH meter from Jenco Electronics, Ltd. (San Diego, CA, USA). The standards and samples were stirred in a Vortex from Baxter Scientific Products.

The watermelon samples were prepared and kindly provided by Dr. Niels Maness from Horticulture Department at Oklahoma State University.

Preparation of Sample and Electrolyte Solutions

Stock solutions of 10 mM standard phospholipids (i.e., PI, PS, PG, PA and DG) were prepared in NMF-dioxane (4:1 v/v). The standards were then prepared by diluting aliquots of stock solutions in the running electrolyte to obtain solutions for calibrations in the concentration range from 0.025 mM to 3.5 mM. The internal standard (1-naphthoic acid) was dissolved in NMF-dioxane (4:1 v/v) to give a stock solution at a concentration of 10 mM. From this standard solution an aliquot was transferred to the phospholipids solutions used in the calibration curve.

The running electrolyte consisted of 40 mM tris and 2.5 mM AMP in a solvent mixture of NMF-dioxane-water (5:3:2 v/v) and was degassed prior to injection into the capillary.

The dried watermelon samples were diluted in the running electrolyte before injection. The stock and standard solutions along with the diluted samples were stored in refrigerator after use.

For the method described by Gao *et al* [13], the running electrolyte was composed of 5 mM AMP in methanol-water (9:1 v/v) solvent. The buffer was adjusted to pH 9.5 with a 1 M NaOH solution. The stock solutions were prepared in neat methanol and diluted to the appropriate concentration in the running

electrolyte. Prior to injection, the electrolyte solutions and standard solutions were sonicated.

For the second method studied [12], the running electrolyte consisted of 0.3 % acetic acid and 60 mM ammonium acetate in acetonitrile-2-propanol (3:2 v/v). The standard phospholipids were dissolved in the running electrolyte and injected.

Procedures

Electrophoretic Procedure. Before use, the new capillaries were rinsed successively and manually with a micro syringe with 1 M NaOH for 20 min, deionised water for 5 min, 0.1 M HCl for 20 min, deionised water for 5 min and at least 5 min with the separation electrolyte. As a daily routine and before sample analysis, the capillary was sequentially flushed with 1 M NaOH for 5 min, deionised water for 5 min followed by the running electrolyte for 5 min at a pressure of 20 psi. Between injections, capillary was pressure rinsed for 2 min with the separation electrolyte. Prior to sample analysis, the capillary was equilibrated daily with the CE running electrolyte for 20-30 min.

Standards and samples were injected hydrodynamically for 5 s at 0.5 psi. The temperature of the capillary was maintained at 25 ^{o}C. The CE separations were carried out in normal polarity at a wavelength of 280 nm and at a voltage of 28 kV. The running electrolyte of the inlet vial was changed several times a day and of the outlet vial changed daily. During weekends and at nights, the capillaries were stored in deionised water.

Extraction of Phospholipids from Watermelon. Lipids were extracted from watermelon by following the method described by Mao *et al* [19] with slight modifications. In a waring blender, ca. 20 g of watermelon was grinded and the pulp was separated from the juice using a Buckner funnel. The pulp along with the internal standard (i.e., 1-naphthoic acid) was then stirred with ca. 40 mL of chloroform-methanol (2:1 v/v) solvent followed by filtration. The filtrate was washed with ca. 10 mL of 0.5 M aqueous KCl and centrifuged for about 10-15 min. After centrifugation, the lower/bottom layer (organic phase) was withdrawn/recovered in a suitable vial and dried under nitrogen at room temperature. To the dried sample, 200 μL of running electrolyte was added, vortexed and centrifuged. The lower layer of sample was collected after centrifugation, diluted further and subjected to CE analysis under the given set of conditions.

Results and Discussion

Separations of Phospholipids

As first attempts to separate the phospholipids under investigation, two previously described methods were tested in this study. In one case, the standard phospholipids were analyzed by CE using the method described by Gao *et al.* [13]. According to this method, the running electrolyte consisted of 5 mM AMP in methanol: water (9:1 v/v) solvent mixture using a Beckman's P/ACE

MDQ instrument equipped with a photodiode array (PDA) detector. The baseline was monitored by indirect UV detection at 259 nm employing AMP as the background chromophore. The running voltage was set at 30 kV in one set of experiments and also at 25 kV in another set of experiments. The standard phospholipids were injected hydrodynamically by applying a positive pressure of 50 mbar (i.e., 0.5 psi) for 5 sec. This system could not detect all phospholipids under investigation and the migration time of the phospholipids that were detected (PG and PS) was not reproducible. This system was used in the pH range of 9 to 10.

The second electrolyte system investigated was referred from the work done by Guo *et al*. [12]. It consisted of a nonaqueous medium composed of acetonitrile- 2-propanol (3:2 v/v), 0.3% v/v acetic acid and 60 mM ammonium acetate. In this electrolyte system direct UV detection was utilized by setting the PDA detector at 200 nm. The separation voltage used was set at 30 kV, and the standard phospholipids were pressure injected for 5 sec at 0.5 psi on a P/ACE MDQ. A broad peak was seen suggesting that all standards co-migrated. The system was irreproducible and also suffered from baseline instability.

Based on the above results, the two electrolyte systems described in the literature proved unsuitable for the separation of phospholipids under investigation. These two electrolyte systems were abandoned and were not further considered for the analysis of phospholipids in watermelon.

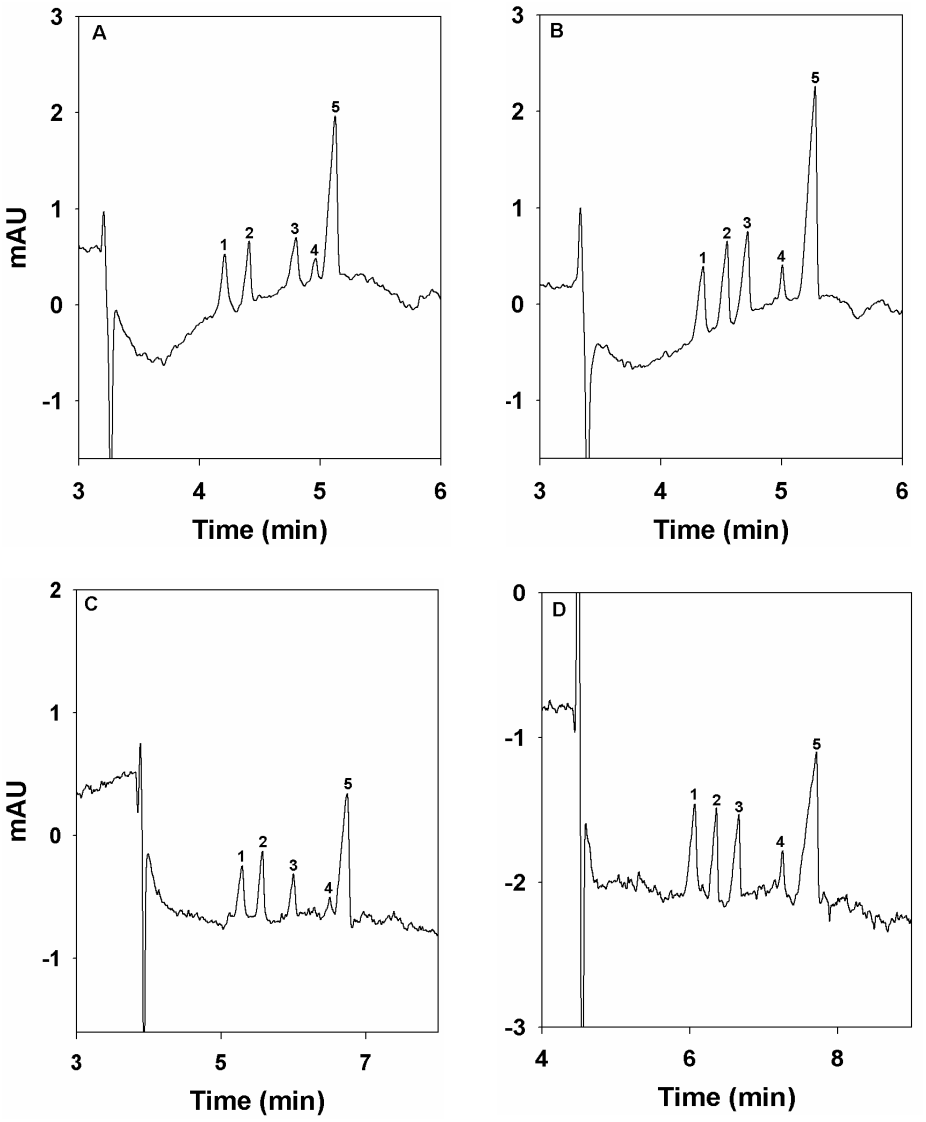

Figure 2. *Effect of the water content of the electrolyte system on the separation selectivity of the standard phospholipids. Electrolyte, 40 mM tris and 2.5 mM AMP in (A) NMF-dioxane-water (6.5:3:0.5 v/v), (B) NMF-dioxane-water (6:3:1 v/v), (C) NMF-dioxane-water (5.5:3:1.5 v/v (D) NMF-dioxane-water (5:3:2 v/v)) mixture, pH in the range 8-9. Hydrodynamic injection for 5 sec at 0.5 psi. Applied voltage of 28 kV. Indirect UV detection at 280 nm. Solutes: 1, PI; 2, PG; 3, PS; 4, PA and 5, DG.*

In another set of experiments, the system developed earlier for fatty acids analyses (see Chapter II) was applied in this study with some modifications. The system was composed of 40 mM tris and 2.5 mM AMP in a NMF-dioxane-water (5:3:2, v/v) solvent mixture. The running electrolyte was high in organic phase ensuring complete dissolution of phospholipids. As can be seen in Fig. 2D separation of all five phospholipid standards were successfully achieved.

Different anionic classes of phospholipids were detected by indirect UV detection at 280 nm (see Fig. 2). Indirect detection is a universal detection technique for species that lack chromophore or have low molar absorptivity. AMP was used as the chromophore in the running electrolyte as AMP has a high molar absorptivity, large ratio of background absorbance to background noise and closely matched electrophoretic mobility with that of the phospholipids [20].

Spiking individual phospholipids and comparing peak height before and after spiking confirmed the migration order of phospholipids (see Fig. 2). Due to the basic environment (pH ca. 8.8) of the running electrolyte, the phospholipids had permanent negative charges resulting from the deprotonation of the phosphate groups. PI, PG and PS have each a net negative charge of -1 and charge-to-mass ratios of 1.18×10^{-3}, 1.30×10^{-3} and 1.28×10^{-3}, respectively, migrated in the order of increasing charge-to-mass ratio (see Fig. 2). The fact that PS migrated slightly later than PG despite that the latter has about the same charge-to-mass ratio as the former may be attributed to the difference in their hydrodynamic radius. The fact that PA has a -2 charge and a charge-to-mass ratio of 2.87×10^{-3} explain its migration later that the PI, PG and PS. Although

DG has a -2 charge, its high molecular weight of ~1500 makes its charge-to-mass ratio of 1.34 x 10^{-3} to be almost half of that of PA and about the same as that of PG and PS. Despite the fact that the charge-to-mass ratio of DG is about the same as that of PG and PS and half of that of PA, DG migrated last (see Fig. 2). This may indicate that DG has a smaller hydrodynamic radius in the organic medium than all the other phospholipids under investigation.

Figure 3A represents an electropherogram given by a watermelon sample in the presence of 20 % water in the running electrolyte. As can be seen in Fig. 3A, a group of unresolved peaks (or cluster of peaks) is detected around 6.5 to 7.5 min. After comparing this result to the standard mixture of phospholipids shown in Fig. 2D, it was concluded that some of the phospholipids might be in that cluster of peaks. To separate the various phospholipids components of the watermelon samples, tuning of the separation selectivity was needed. Optimization of the separation selectivity was first pursued by including cyclodextrins (CD) in the running electrolyte. Various CD's such α-CD, β-CD, γ-CD and hydroxypropyl β-CD were examined in varying concentrations. Inclusion of CD's gave a sharp increase in t_0 and as a result of this, peaks were either not eluted or became significantly broad due to increase in their migration time. Adsorption of CD's on to the capillary walls might have been the reason for such behavior, see Chapter for explanation.

Another approach to modify selectivity was by varying the composition of the mobile phase. It has been reported that at high concentration of water, the

phospholipids tend to aggregate to form small lipid vesicles/bilayer due to their amphiphatic nature. Different percentages of water in the mobile phase were

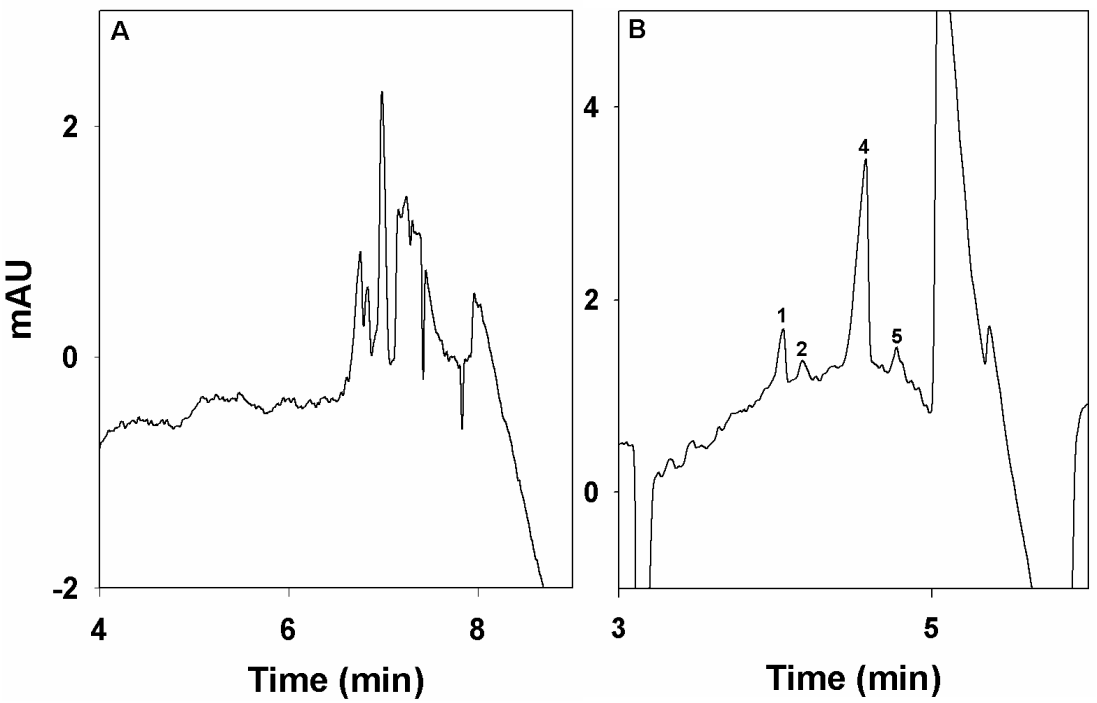

Figure 3. *Electropherograms of a watermelon sample. Electrolytes, partially aqueous systems consisting of 40 mM tris and 2.5 mM AMP in (A) NMF-dioxane-water (5:3:2, v/v) and in (B) NMF-dioxane-water (6:3:1,v/v) solvent, pH ~ 8.8. Hydrodynamic injection for 5 sec at 0.5 psi. Applied voltage, 28 kV. Indirect UV detection at 280 nm. Peak labeling: 1, PI; 2,PG; 3, PS; 4, PA; 5, DG.*

examined by compensating with the percentage of NMF. Dioxane content was kept the same, i.e. 30% v/v. Figure 2 features electropherograms obtained with electrolytes at varying water content. At 5% v/v water and 65% v/v NMF (Fig.

2A), all standard phospholipids are separated within 6 min whereas at 15% v/v water and 55% v/v NMF (Fig. 2C) the separation was achieved within 8 min. The migration time for the EOF marker (t_0) at 5% v/v water and 10% v/v water did not show significant difference but at 15% v/v water it increased to around 4 min. Since the watermelon fruits contain PA and PI in significant amounts, therefore it was necessary that these phospholipids show good separation selectivity and resolution. Comparing all electropherograms shown in Fig. 2, the 10% v/v water content (Fig. 2B) showed not only well resolved separation of all phospholipids, but also the peaks were sharp in comparison with the ones obtained at 5% and 15% v/v water. On the other hand, the water content did not affect the migration order of phospholipids but it did affect the migration time of PS significantly. At 5% v/v water (Fig.2A), the PS migrated close to PA whereas at 15% v/v water (Fig. 2C), its migration time shifted away from PA. Hence, the new running electrolyte consisting of NMF-dioxane-water at 6:3:1, v/v was chosen for the analysis of phospholipids in watermelon samples.

Quantitative Analysis of Phospholipids in Watermelon

A sample of watermelon was analyzed in the optimal running electrolyte system described above and the results are shown in Fig. 3B. The effect of water is clearly evident in this electropherogram. The sample shows the presence of PI and PA in considerable amount whereas PG, PS and DG are present in low amounts. The confirmation of phospholipids was achieved by spiking the watermelon sample with standard phospholipids. As shown in Fig. 3B at around

5 min, a tall, broad peak was observed. This peak could be from other nonpolar compounds including some kind of lipids present in the watermelon. However, no attempts were made to identify or resolve the components of this peak as this project deals only with analysis of phospholipids.

Having determined the optimal composition of running electrolyte, the next step in the analysis was to find a suitable internal standard having similar properties to the phospholipids. Various FFA's such as heptanoic acid (C7:0), dodecanoic acid (C12:0), hexadecanoic acid (C16:0), heptadecanoic acid (C17:0), octadecanoic acid (C18:0), eicosanoic acid (C20:0), docosanoic acid (C22:0) and tetrasanoic acid (C24:0) were examined. The majority of these fatty acids migrated at about the same time as the broad unknown peak. An internal standard that migrates immediately after the DG was needed. C22:0 and C24:0 were the potential candidates that would satisfy this requirement, but C22:0 co-migrated with DG and C24:0 migrated between PA and DG showing partial separation from DG (see Fig.4). The next internal standard that was examined was 1-naphthoic acid, which has two aromatic rings making it a bulky molecule and hence, making it similar in nonpolar character to the phospholipids under investigation. In addition, 1-naphthoic acid migrated at around 7-8 min far from the analytes and from the broad unknown peak in the watermelon sample. As 1-naphthoic acid had no interference with the phospholipids, it was used as the internal standard. Electropherograms of standard phospholipids and watermelon sample with 1-naphthoic acid as the internal standard are shown in the Fig. 5.

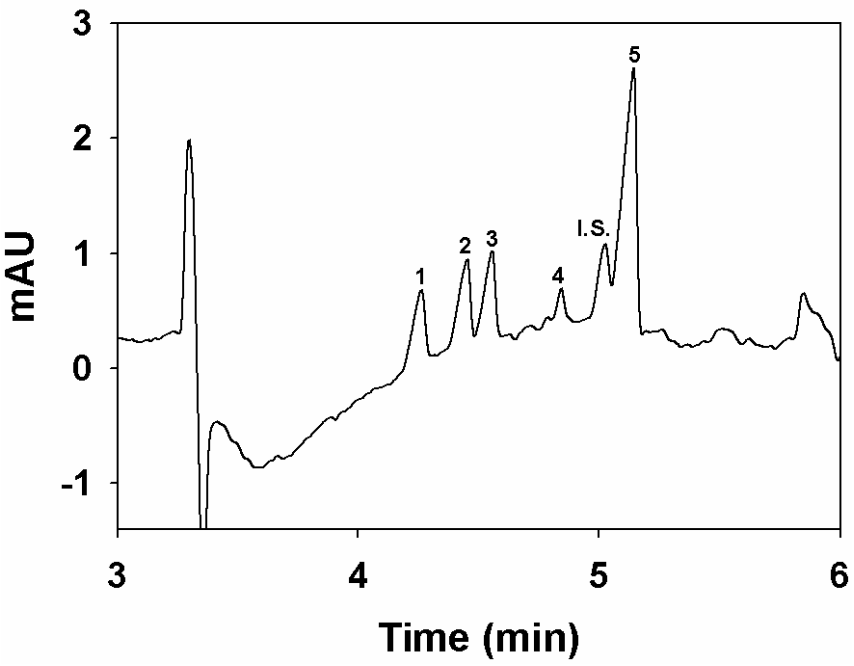

Figure 4. *Electropherogram showing five standard phospholipids along with C24:0 as the internal standard (I.S). Electrolyte, partially aqueous electrolyte system consisting of 40 mM tris and 2.5 mM AMP in NMF-dioxane-water (5:3:1, v/v) solvent, pH ~ 8.8. Hydrodynamic injection for 5 sec at 0.5 psi. Applied voltage of 28 kV. Indirect UV detection at 280 nm. Peak labeling: 1, PI; 2, PG; 3, PS; 4, PA; 5, DG; I.S., C24:0.*

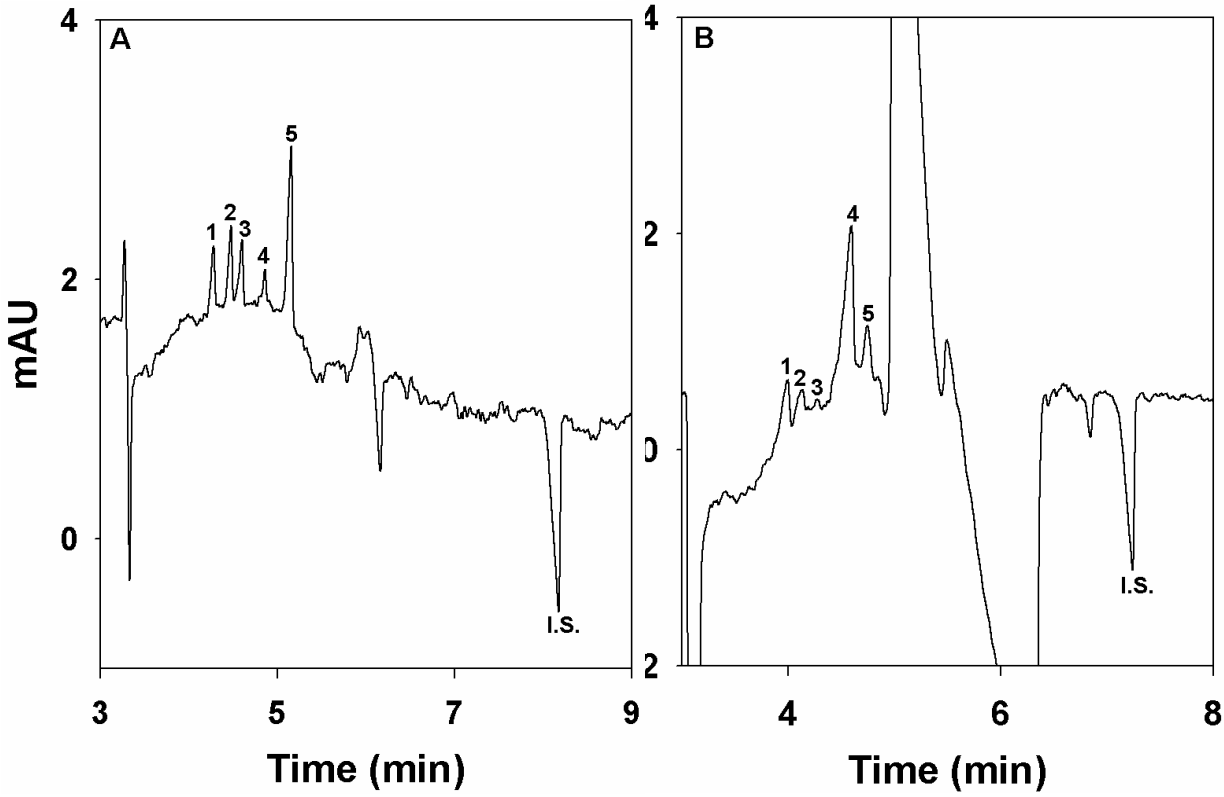

Figure 5. *Electropherograms showing 1-naphthoic acid as the internal standard. (A), standard phospholipids mixtures and (B), watermelon sample (log #31, short storage time). Electrolyte, an organic rich electrolyte system consisting of 40 mM tris and 2.5 mM AMP in NMF-dioxane-water (5:3:1, v/v) solvent, pH ~ 8.8. Hydrodynamic injection for 5 sec at 0.5 psi. Applied voltage of 28 kV. Indirect UV detection at 280 nm. Peak labeling: 1, PI; 2, PG; 3, PS; 4, PA; 5, DG; I.S., 1-naphthoic acid.*

Establishing the Calibration Curves. For pursuing the quantitative determination of phospholipids in watermelon samples, a standard calibration curve with fixed concentration of internal standard had to be developed. Calibration curves were established using different concentration levels ranging from 0.025 mM to 1.0 mM for the four standard phospholipids, namely PI, PG, PS, and DG (Fig. 6A) and from 0.2 mM to 3.5 mM for PA (Fig. 6B) against the ratios of peak height of the phospholipids to that of internal standard. As shown in Fig. 6, the calibration curves for all phospholipids were considerably linear. Also, given in Fig. 6 are the equations and R^2 values for the phospholipids. These calibration lines were then used in the determination of individual phospholipids in the samples of watermelon.

Analysis of Phospholipids in Watermelon. The phospholipids from watermelon were extracted by the method described in the experimental section. Analysis of watermelons from 4 different varieties that vary in suitability for the fresh cut market was carried out using the optimal, organic rich electrolyte system mentioned above. The watermelons were provided by a vegetable seed company, which is involved in watermelon breeding program to produce melons having better fresh cut storage life. The watermelon samples analyzed were stored for varying length of time. Figure 7 shows typical electropherograms of some watermelon samples, which shows pronounced changes in the phospholipids content. As seen from the electropherograms, PA and PI are

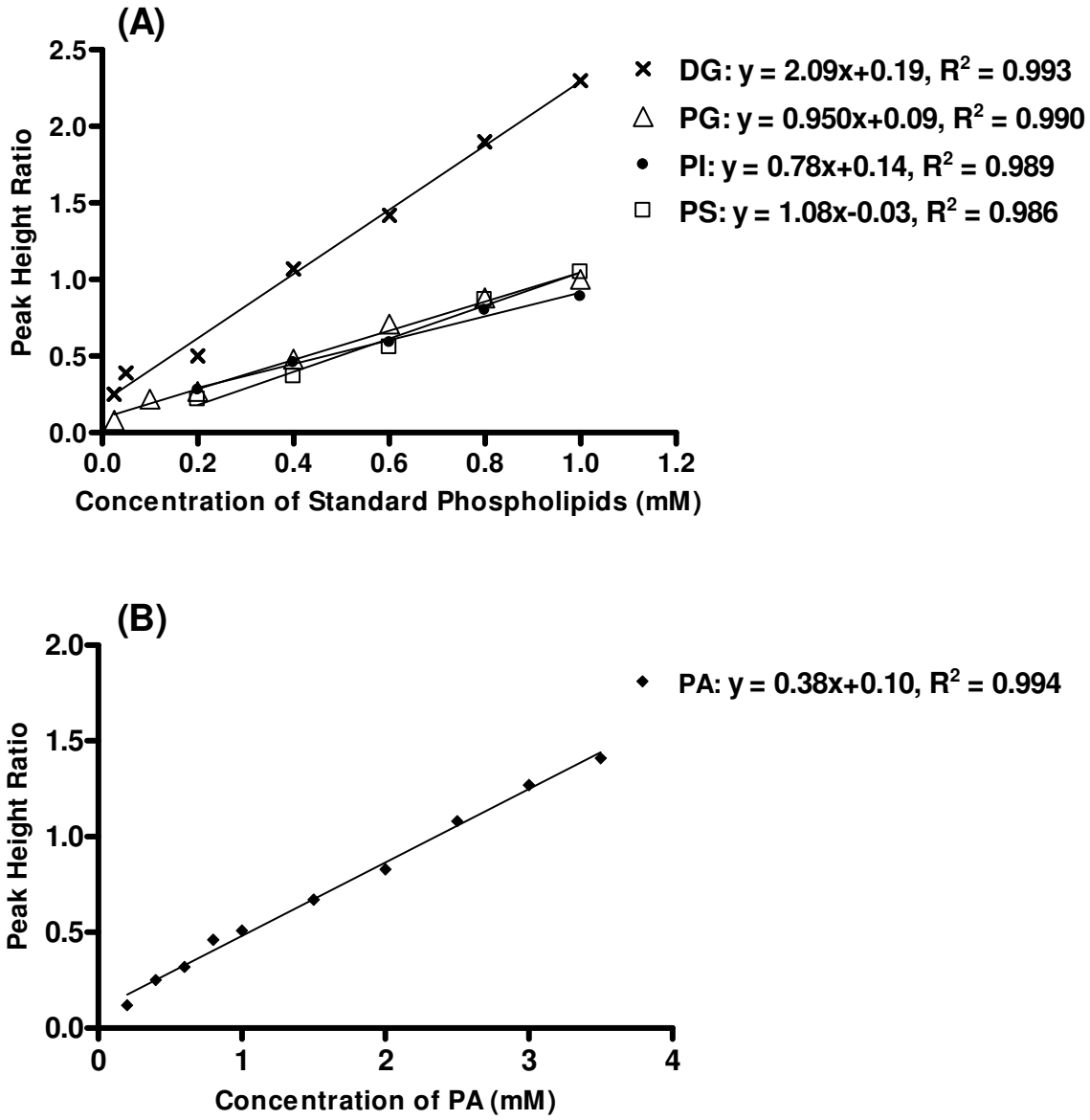

Figure 6. *Standard calibration curves for the standard phospholipids under investigation. DG, PG, PI and PS in (A) and PA in (B).*

present in relatively considerable amounts in all samples whereas the amounts of PG, PS and DG vary from sample to sample.

According to the work of Mao *et al* water-soaking is related to changes in the composition of the phospholipids in the cell membrane [19]. Eighteen watermelon fresh cut samples of four different varieties were analyzed and the data obtained are shown in Table 1. These fresh cut samples were stored for short and long duration of time. Thirteen of the fresh cuts exhibited low leakage, three cuts showed high leakage and two cuts medium leakage. While low juice leakage was observed with both short and long storage time, high and medium leakages were obtained in cases of long storage time. From the table, and with a few exceptions, it is observed that the wt% of PG and DG are relatively lower than the wt% of PA and to a lesser extent for the wt% of PI. While the wt% of PI was lower for long storage duration as compared to that for short storage duration, the wt% of PG, PA and DG did not follow a fixed trend among the four watermelon varieties. In the case of PS, its presence varied from sample to sample and when present, it was in trace amounts. In general, high and medium juice leakages correspond to fresh cuts having total wt% phospholipids lower than the total wt% phospholipids found for fresh cuts with low leakage.

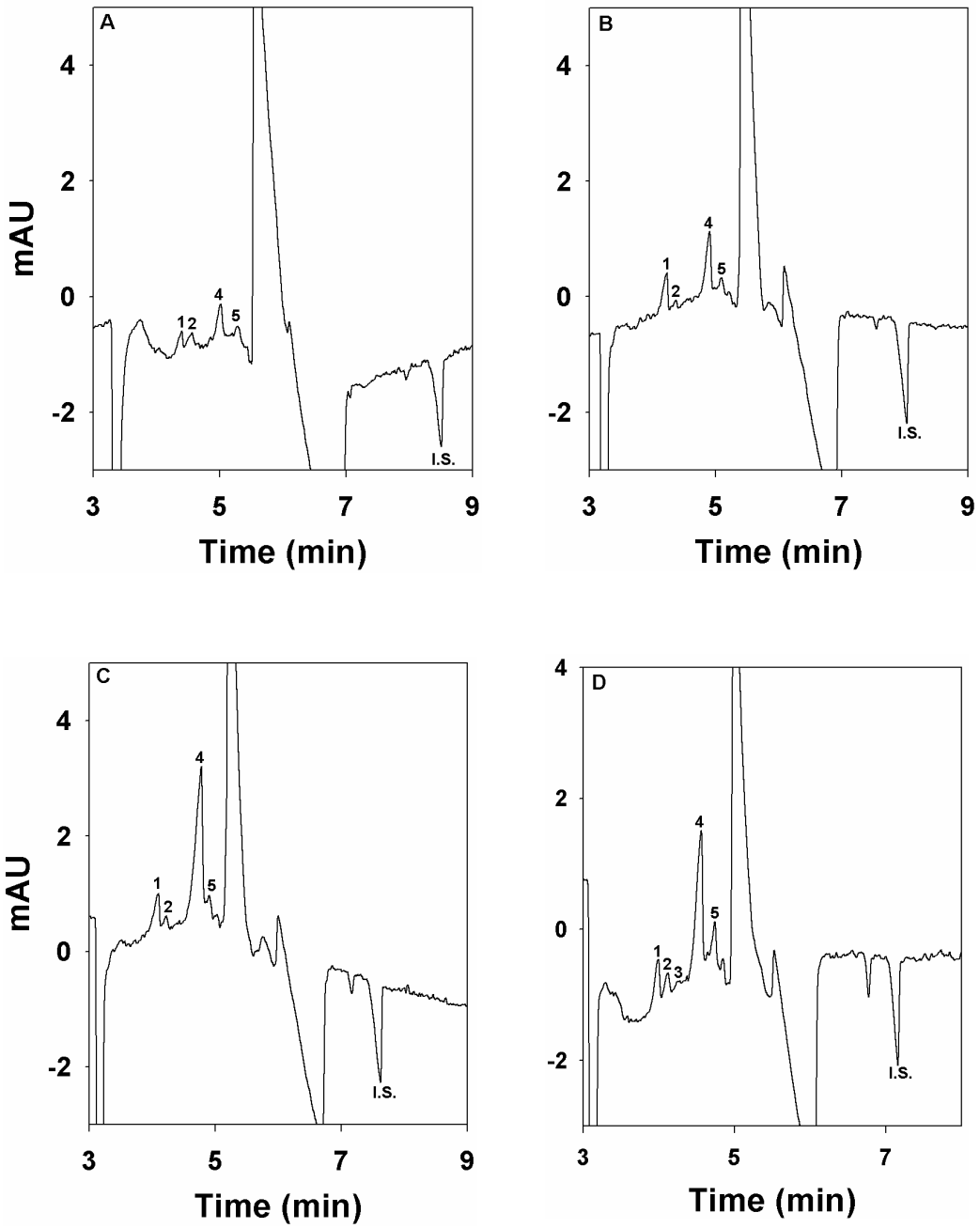

Figure 7. *Electropherograms of some watermelon samples from different varieties showing varying concentration of phospholipids. Conditions and peak labeling as in Fig. 5. Samples: (A) #20, long storage, low leakage; (B) #6, short storage, low leakage; (C) #3, long storage, medium leakage; (D) #13, short storage, low leakage.*

Table 1. *Weight percent of PI, PG, PA and DG in samples of watermelons obtained from four varieties. NM, not measurable. "Short" refers to a storage time from 0 to 1 day while "Long" refers to a storage time of 10 to 14 days. The % juice leakage for low leakage ranged 0-5%, for medium leakage ranged 6-15% and for high leakage is >16%.*

Sample log number	% PI	% PG	% PS	% PA	% DG	Storage time (Leakage)
Variety 1						
1	NM	3.98×10^{-4}	NM	110×10^{-4}	NM	Long (Low)
18	11×10^{-4}	NM	NM	91.2×10^{-4}	NM	Short (Low)
3	6.24×10^{-4}	NM	NM	78.4×10^{-4}	NM	Long (Medium)
24	15.2×10^{-4}	1.88×10^{-4}	NM	150×10^{-4}	NM	Short (Low)
Variety 2						
2	7.81×10^{-4}	2.21×10^{-4}	NM	34.9×10^{-4}	NM	Short (Low)
32	5.04×10^{-4}	1.89×10^{-4}	NM	46.1×10^{-4}	1.04×10^{-4}	Long (High)
13	12.9×10^{-4}	3.36×10^{-4}	NM	89.8×10^{-4}	6.74×10^{-4}	Short (Low)
33	6.84×10^{-4}	1.09×10^{-4}	NM	53.8×10^{-4}	1.93×10^{-4}	Long (Medium)
17	6.04×10^{-4}	1.75×10^{-4}	NM	59.9×10^{-4}	0.99×10^{-4}	Long (High)
19	10×10^{-4}	2.30×10^{-4}	NM	66×10^{-4}	NM	Short (Low)
Variety 3						
8	7.31×10^{-4}	0.48×10^{-4}	NM	48.4×10^{-4}	NM	Short (Low)
20	4.35×10^{-4}	2.48×10^{-4}	NM	13.3×10^{-4}	NM	Long (Low)
10	2.41×10^{-4}	2.61×10^{-4}	NM	25.3×10^{-4}	NM	Long (High)

30	11.7×10^{-4}	1.87×10^{-4}	NM	90×10^{-4}	NM	Short (Low)
16	11.2×10^{-4}	2.83×10^{-4}	NM	93.4×10^{-4}	6.52×10^{-4}	Short (Low)
31	7.6×10^{-4}	2.52×10^{-4}	NM	43.8×10^{-4}	2.19×10^{-4}	Long (Low)
Variety 4						
23	6.03×10^{-4}	1.22×10^{-4}	NM	31.2×10^{-4}	NM	Long (Low)
35	7.78×10^{-4}	2.21×10^{-4}	NM	59×10^{-4}	1.78×10^{-4}	Short (Low)

Conclusions

This investigation allowed the separation of phospholipids in an organic rich electrolyte system by CE coupled with indirect UV detection at room temperature using AMP as the background absorber. The composition of the running electrolyte played an important role in controlling the separation selectivity. The electrolyte consisted of 90 % organic and 10 % water. This method was successfully applied to the profiling of five phospholipids (i.e., PI, PG, PS, PA and DG) in watermelon extracts. Changes in the phospholipid content in the watermelon fruit were monitored, and these changes are believed to be associated with cellular leakage, which results in limited shelf life of the fresh cut watermelon fruit. However, further systematic studies will have to be conducted in the future in order to accurately correlate the phospholipids content of watermelon with leakage and spoilage of watermelon fresh cut and also to discern which phospholipids are associated with fluid leakage from fresh cuts.

Nevertheless, the CE method developed here is expected to be of general usage and could be applied to the analysis of phospholipids present in other food matrices.

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VITA

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Candidate for the Degree of

Master of Science

Thesis: CAPPILARY ELECTROPHORESIS OF LIPIDS DERIVED FROM SOME NATURAL PRODUCTS

Major Field: Chemistry

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Name: Yogita Bannore Date of Degree: May, 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CAPPILARY ELECTROPHORESIS OF LIPIDS DERIVED FROM SOME NATURAL PRODUCTS

Pages in Study: 84 Candidate for the Degree of Master of Science

Major Field: Chemistry

Scope and Method of Study: The purpose of this study was to develop capillary electrophoresis methods for the analysis of some lipids, namely free fatty acids and phospholipids in food produces such as peanut seeds and watermelon, respectively. In the case of peanuts, the free fatty acids from single peanut seeds were extracted and then analyzed for their content of oleic acid and linoleic acid using a partially aqueous electrolyte based on *N*-methylformamide, dioxane and low water content in the presence of small amounts of α-cyclodextrin as the selectivity modulator. The capillary electrophoresis system, thus developed was coupled with an indirect UV detection mode using adenosine monophosphate as the UV absorber. In the case of watermelon, the slightly modified and partially aqueous capillary electrophoresis system developed for free fatty acid analysis was successfully applied for the analysis of phospholipids extracted from watermelon fruit.

Findings and Conclusions: This investigation showed that a partially aqueous capillary electrophoresis method coupled with an indirect UV detection could be readily applied for the separation and determination of some lipids in food matrices. Free fatty acids analysis of several samples from two major peanut breeding lines was successfully achieved. This was facilitated by incorporating α–cyclodextrin, a cyclic oligosaccharide, in the running electrolyte, which modified the separation selectivity of the fatty acids, thus allowing their full separation within 25 min. The capillary electrophoresis method allowed the rapid screening of high oleic acid peanuts from low oleic acid peanuts. Using a slightly modified and partially aqueous electrolyte system at lower water content devoid of cyclodextrin allowed the separation and determination of some phospholipids in watermelon within 8 min. These capillary electrophoresis methods are expected to be of general use in the analysis of free fatty acids and phospholipids extracted from other natural products.

ADVISOR'S APPROVAL: Dr. Ziad El Rassi

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