Review

Capillary electrophoresis-based methods for the determination of lipids—A review

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ABSTRACT

Capillary electrophoresis (CE) is a high-resolution technique for the separation of complex biological and chemical mixtures. CE continues to emerge as a powerful tool in the determination of lipids. Here we review the analytical potential of CE for the determination of a wide range of lipids. The different classes of lipids are introduced, and the different modes of CE and optimization methods for the separation of lipids are described. The advantages and disadvantages of the different modes of CE compared to traditional methods like gas chromatography (GC) and liquid chromatography (LC) in the determination of lipids are discussed. Finally, the potential of CE in the determination of lipids in the future is illustrated.

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1. Introduction

Lipids have been loosely defined as biological substances that are generally hydrophobic in nature, and in many cases, soluble in organic substances [1–3]. Lipids may be categorized based on their chemically functional backbone as polyketides, acylglycerols, sphingolipids, prenols, or saccharolipids. However, in the latest classification system, it was chosen to separate fatty acyls from other polyketides, the glycerophospholipids (GPLs) from the other glycerolipids, and sterol lipids from other prenols, resulting in a total of eight primary categories, each containing distinct classes and subclasses of molecules [3]. Fig. 1 shows representative structures for each lipid category. Due to their wide occurrence in foods and their pro- and antioxidant effects, lipids have the potential to act as multifunctional additives in food, pharmaceutical, and industrial applications.

Several separation techniques have been used for the determination of lipids. For example, long-chain fatty acids have been determined by gas chromatography (GC) or liquid chromatography (LC). Supercritical fluid chromatography (SFC) [4] and thin layer chromatography (TLC) [5,6] have also been utilized for lipid separation. GC is an excellent technique for saturated fatty acids because of its high efficiency; however, it is very sensitive to compound polarity. In addition, the need for multiple derivatizations with oxidized species can make some analyses problematic, and run times are often 1 h or more for long-chain fatty compounds. LC suffers from lower efficiency but is generally more versatile for the separation of oxidized compounds [7]. The GC methods as well as most of the LC methods incorporate derivatization to obtain volatility and detectability, respectively. TLC lacks precision in detection and is time consuming while HPLC is costly because it requires large volumes of mobile phase and its separation columns are relatively expensive. The wide choice of mobile and stationary phase makes selectivity extremely powerful in HPLC. In recent years, interest in microcolumn LC has increased considerably. This is mainly due to the ability to work with small sample sizes, small volumetric flow rates, easy coupling to mass spectrometers and secondary chromatographic systems, and enhanced detection performance with the use of concentration sensitive detectors due to the reduced chromatographic dilution [8,9]. However, a limitation of using microcolumn LC is the lower sample capacity of the column, which is proportional to the surface area [8].

Additionally, the separation of lipids has been traditionally carried out on gels [10] or paper [11] using the classical electrophoresis technique. The advantages of this technique are that several samples can be run simultaneously and two-dimensional separations can easily be performed. However, this technique has several limitations. First, to avoid excess heating resulting in evaporation of the separation medium, only relatively low voltage gradients can be applied, leading to long determination times. Second, quantitative determination of lipids is only possible using reflection measurements and analytes often must be dyed before detection. Because of this limitations, there is no reliable relationship between the quantity of lipid and the quantity of dye taken up by the lipid [12].

Over the past three decades, capillary electrophoresis (CE) has emerged as a versatile analytical separation technique that can be applied to the determination of a wide variety of compounds. CE has also become a powerful technique for the separation of lipids. The fast and efficient determination of lipids is important to the environment and biological fields, as well as to synthetic chemists and the pharmaceutical industry. In contrast to HPLC, which is a pressure driven method of separation, CE is an electrically driven technique. The electro-osmotic plug flow profile of CE, as compared to the hydrodynamic flow profile in HPLC, results in higher number of theoretical plates. CE requires only nanoliters of sample, microliters of buffer, and minimal amounts of organic solvents or additives. Furthermore, short analysis times and the ability to interface CE on-line with other techniques like MS [13,14] are also often cited as advantages for some CE separations. However, the process of interfacing CE online with MS is not trivial.

In recent years, a large number of reviews have been provided on the developments and applications of CE. Most of these reviews have focused on the development of CE for proteins and peptides, chiral and achiral compounds, DNA analysis, food analysis, forensics, clinical diagnosis, and drug screening [15–24]. Although, various modes of CE exist and most of them have been used for the separation of lipids, there are only a few reviews with a limited number of references on the determination of lipids by CE [15,25]. However, the online lipid library maintains a list of references that have used electrophoretic separation methods for the determination of lipids [26]. In this article, we briefly describe lipids and the various CE methods used for lipid determination and provide a comprehensive survey of the application, advantages, and limitation of each CE mode as compared to other standard non-CE methods.

2. Classification of lipids

Of the eight categories of lipids, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, and sterol lipids have been routinely determined by CE. The fatty acyl structure represents the major lipid building block of complex lipids, and it is one of the most fundamental categories of biological lipids. The fatty acyl group is characterized by a repeating series of methylene groups that impart hydrophobic character to this category of lipids. Examples of the fatty acyls are the fatty acids, fatty acid esters, and the hydrocarbons.

Briefly, fatty acids are divided into two groups depending on their degree of saturation, i.e., the saturated fatty acids (SAFAs) and those with different degrees of unsaturation, from monounsaturated fatty acids (MUFAs) to polyunsaturated fatty acids (PUFAs). The physical and chemical characteristics of fatty acids depend on the carbon chain number, double bonds, positions of double bonds, and cis–trans isomer conformations. A fatty acid is a carboxylic acid often with a long unbranched aliphatic tail, which is either saturated or unsaturated. Fatty acids are aliphatic monocarboxylic acids, derived from, or contained in esterified form in an animal or vegetable fat, oil or wax. Natural fatty acids commonly have a chain of 4–28 carbons which are usually unbranched and even num-
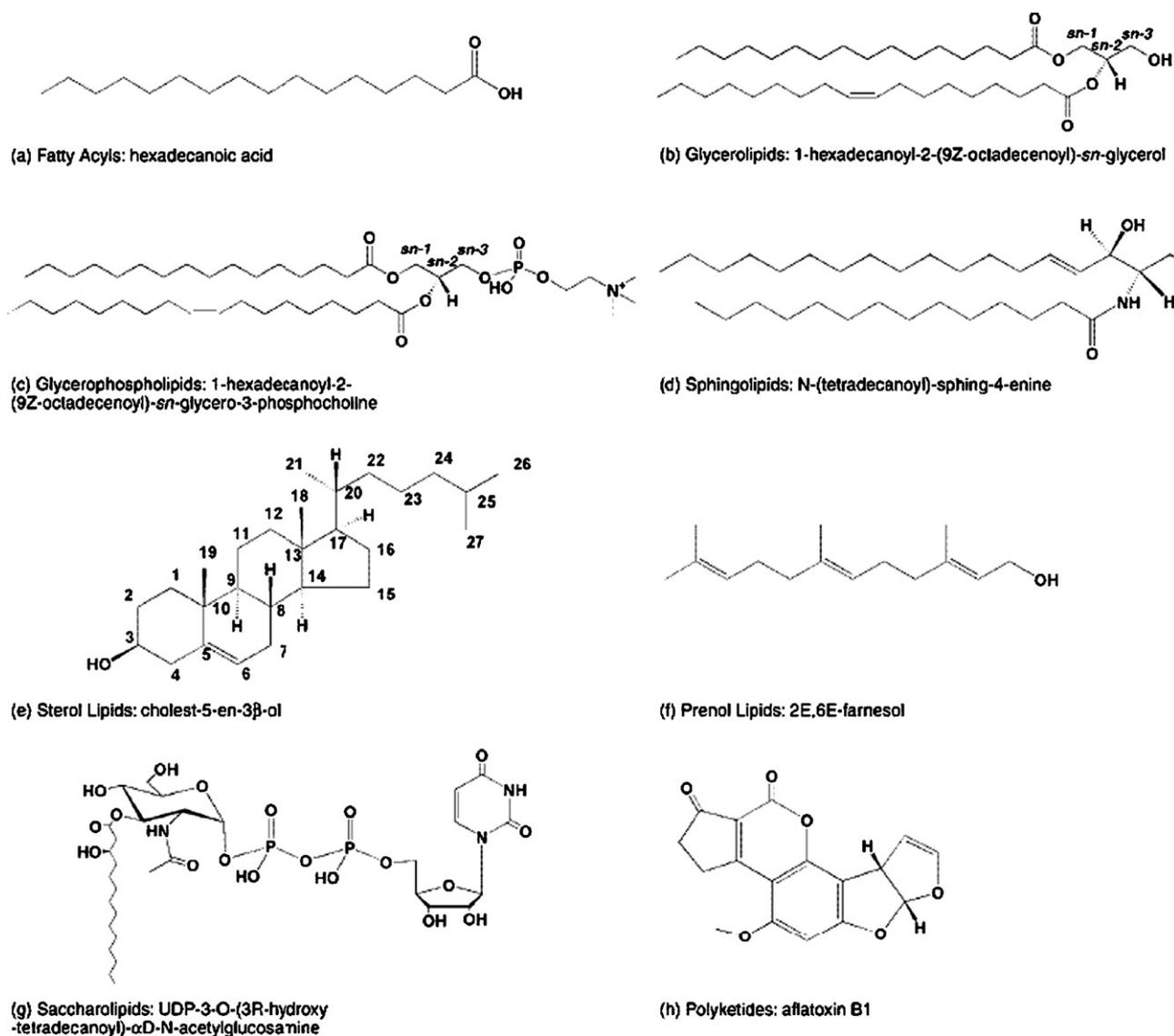
Fig. 1 – Representative structures for each lipid category. Adapted with permission from [3].

bered. By extension, the term is sometimes used to embrace all acyclic aliphatic carboxylic acids. Saturated fatty acids do not contain any double bonds or other functional groups along the chain. Unsaturated fatty acids are of similar form, except that one or more alkenyl functional groups exist along the chain, with each alkene substituting a single-bonded "—CH$_2$—CH$_2$—" part of the chain with a double-bonded "—CH=CH—" portion. Saturated fatty acids are the fats while unsaturated fatty acids are the oils.

Glycerolipids essentially encompass all glycerol containing lipids, and the most well known glycerolipids are the fatty acid esters of glycerol (acylglycerols) [27]. Other subclasses are the glycerolglycans [28]. In contrast, GPLs are derivatives of sn-glycero-3-phosphoric acid that contains at least one O-acyl, or O-alkyl or O-alk-1′-enyl residue attached to the glycerol moiety and a polar head made of a nitrogenous base, a glycerol, or an inositol unit. Examples are phosphatidyl choline and phosphatidyl ethanolamine. In addition to serving as the primary component of cellular membranes and binding sites for inter-

cellular and extracellular proteins, some GPLs in eukaryotic cells are either precursors of, or are themselves membrane derived second messengers [29]. The GPLs are ubiquitous in nature and are key components of the lipid bilayer of cells [30].

Sphingolipids are a class of lipids derived from the aliphatic amino alcohol sphingosine. The sphingosine backbone is O-linked to a (usually) charged head group such as ethanolamine, serine, or choline. The backbone is also amide-linked to an acyl group, such as a fatty acid. Sphingolipids are ceramide, phosphosphingolipids, glycosphingolipids, and other species, which include protein adducts [31]. Sphingolipids are often found in neural tissue, and play an important role in both signal transduction and cell recognition.

The sterol lipids, which include cholesterol and its derivatives, are a major component of membrane lipids. The sterols, have different biological roles as hormones and also as signaling molecules [32]. Examples of sterol lipids are the steroid

hormones. Prenol lipids are synthesized from the five carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate that are produced mainly via the mevalonic acid pathway [33]. Carotinoids and the quinones are examples of prenol lipids.

Saccharolipids are compounds in which fatty acids are linked directly to a sugar backbone, forming structures that are compatible with membrane bilayers. In this group, a sugar substitutes for the glycerol backbone that is present in glycerolipids and GPLs [34]. Included in this category are the acyl aminosugars of nitrogen fixing bacteria. Polyketides are synthesized by specialized multimodular enzymes that share mechanistic features with the fatty acid synthases, including the involvement of specialized acyl carrier proteins. The different classes of polyketide synthases produce a great diversity of natural product structures. Many commonly used antimicrobial, antiparasitic, and anticancer agents are polyketides or polyketide derivatives. Important examples of these drugs include erythromycins, tetracyclines, and antitumor epothilones [35].

3. Determination of lipids by capillary electrophoresis

CE has several modes, the most common ones that have been used for lipid determination are capillary zone electrophoresis (CZE), capillary electrochromatography (CEC), electrokinetic chromatography (EKC) or micellar electrokinetic chromatography (MEKC), and microemulsion electrokinetic chromatography (MEEKC). Microchip CE has also been used for lipid determination. In depth details on CE modes, methods, and applications can be obtained from books on CE [36–39] or in some recent CE review articles [15–24], and references therein.

3.1. CZE in lipid determination

The use of CZE for lipid determination has been limited due to the lipids poor aqueous solubility and low UV absorbance. Aqueous electrolytes have been used to resolve lipids with C_2–C_{14} by CZE. However, most lipids with more than C_{17} need MEKC. FAs contain an acidic hydrogen because of their carboxylic acid functional groups. Thus, these compounds predominantly exist as anions in basic solutions. CZE can be used to separate both saturated [40–43], and unsaturated [43,44] FAs based on their differences in charge-to-mass ratios. However, saturated, FAs exhibit weak absorption in the region of 200 nm. CE with direct UV detection is problematic and results in limited sensitivity; therefore, CE with indirect UV is preferred [43]. Indirect UV detection exhibits greater sensitivity for lipids using appropriate chromophore additive [43,45–48]. Here, a chromophore is included in the background electrolyte (BGE) to generate high background absorption. For example, a UV absorbing additive such as adenosine monophosphate (AMP), which has a high molar absorptivity, large ratio of background absorbance to background noise, favorable transfer ratio, and closely matched mobility to the lipid being determined is commonly used. Non-aqueous capillary electrophoresis (NACE) has been used with indirect UV

detection for the determination of lipids [49,50]. Some electrolyte systems for FA determination use cyclodextrin and its derivatives as selector additives to improve resolution of difficult solute pairs, especially when PUFAs are present in the sample, Fig. 2 [41]. Cyclodextrins are also sometimes added to increase solubility and selectivity [41,51]. Gao and colleagues did an analysis of the separation and characterization of GPLs by NACE coupled to electrospray ionization–mass spectroscopy using ammonium acetate and acetic acid in the buffer and methanol and acetonitrile as additives [52]. The detection was better than UV because of the high sensitivity and information on molecular structure. Ho and coworkers, used a sodium phosphate buffer (5 mM, pH 7.40) containing high organic solvent concentration (80% methanol, and 10% acetonitrile) for the separation of *in vitro* oxidized GPLs [53].

CZE has been used in the complete separation of gangliosides [54–59]. Gangliosides are a conjugate of a ceramide and a sialoglycan. Many ceramides have microheterogeneity in the ceramide part of the molecule. The power of CE has been in resolving gangliosides having different glycans as well as gangliosides having the same glycans but different ceramides. The difficulty encountered has been the lack of chromophores to enable high sensitivity detection. Gangliosides have been shown to form micelles in aqueous media. Therefore, an organic solvent or additive capable of dispersing the gangliosides micelles is needed for separation of these compounds to be achieved. Cyclodextrins or acetonitrile were used to disperse the micelles in a borate buffer [55,56,59]. The biggest disadvantage of CZE in lipid determination is the inability to resolve extremely hydrophobic lipids that are difficult to dissolve in aqueous buffers. Mardones and colleagues showed that carnitine and five acylcarnitines can be separated by CE using either a quinine sulphate buffer or a buffer with methanol and copper sulphate with indirect photometric detection [60]. The method is rapid, simple, provides good resolution, and is sensitive enough for the compounds considered.

Qi et al. determined Cardiolipin (diphosphatidylglycerol) concentration in the inner mitochondrial membrane using on-line 10-N-nonyl acridine orange (NAO) dye interaction [61]. CZE and spectrophotometric detection with a sample throughput of 3 min were used. In addition to the presence of 0.1 mM NAO, the BGE composition was set at 80% methanol–10% acetonitrile–10% H_2O (all v/v) to provide good solubility and maximum absorbance enhancement. Brando et al. used CE with LIF detection for the characterization of fatty acids tagged by 4-aminofluorescein [62]. They separated palmitic, stearic, oleic, and tuberculostearic acids in less than 10 min using 25 mM sodium borate and 90% acetonitrile. This method was successfully used in the characterization of tuberculostearic acid in 1,000,000 mycobacteria. However, a simple and powerful assay for the diagnosis of tuberculosis and potentially other infectious agents would require the optimized method to detect the bacteria at a level of 1000 cells.

Li and Richards performed characterization of bacterial lipopolysaccharides (LPS) using CE–MS [13]. This hyphened methodology facilitates the determination of closely related LPS glycoform and isoform families by exploiting differences in their unique molecular conformations and ionic charge distributions by electrophoretic separation. On-line CE–MS also
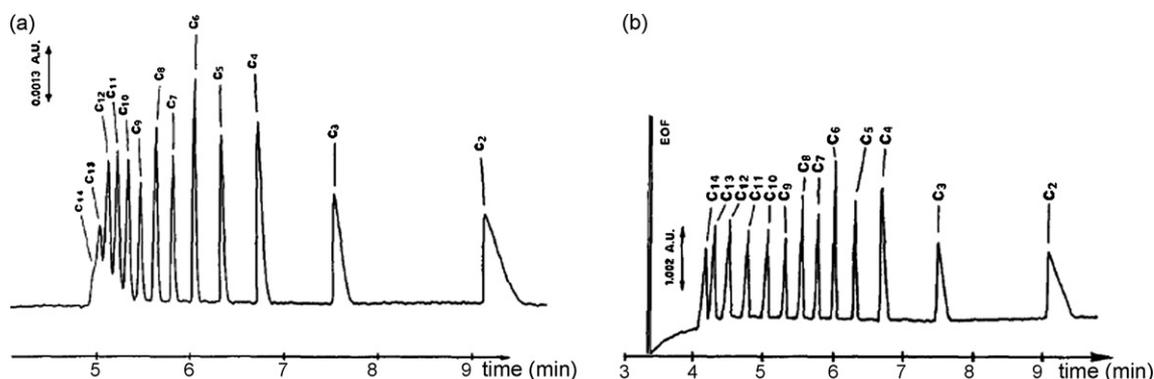
Fig. 2 – Effect of addition of a β-CD derivative on the separation of C_2–C_{14} linear FFA standard mixture (0.5 mmol L^{-1} each in methanol) in a purely aqueous electrolyte. Fused-silica capillary, 50 μm I.D. × 72 cm (50 cm to detector). Electrolyte, 20 mmol L^{-1} Tris-10 mmol L^{-1} p-anisate (pH 8.2): (a) without any additive; (b) with 0.75 mmol L^{-1} trimethyl-β-CD added. Applied voltage, 30 kV (I = 3 A). Temperature, 30 °C. Indirect absorbance detection at 270 nm. Hydrodynamic injection for 1 s. EOF = electro-osmotic flow. Adapted with permission from [41].

provides an additional avenue to improve detection limits, which has been successfully applied to directly probe oligosaccharide LPS glycoform populations of bacteria isolated from infected animal models without the need for further passage.

Carnitine and carnitine esters in body fluids have been analyzed extensively using CZE [60,63–65]. Fig. 3 is an electropherogram of the separation of carnitine and acylcarnitine standards. Heinig and Henion developed a system for the detection of metabolic disorders through the detection of abnormal levels of carnitine esters in body fluids [64]. Many genetic disorders such as medium chain acyl-CoA dehydrogenase deficiency (MCAD), methylmalonic aciduria, propionic acidemia, and isovaleric acidemia, are characterized by abnormal production of carnitine and its acyl metabolites [66]. Thus,
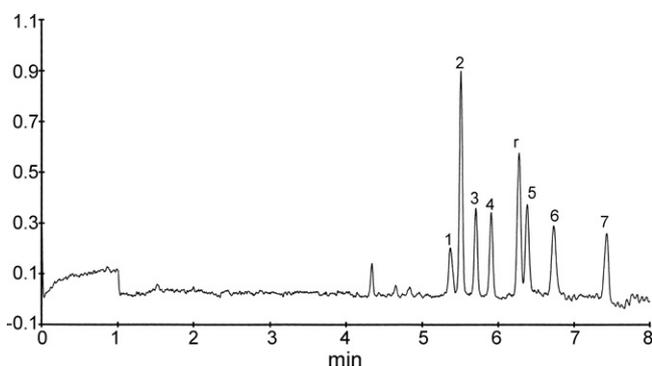

Fig. 3 – Typical electropherogram of carnitine and short-chain acylcarnitines standards. The concentrations are 100 mM for carnitine/acylcarnitines and 50 mM for the internal standard. The final preparation was diluted 10 times before injection. Capillary electrophoresis buffer and additives were 20 mM NaH_2PO_4, 20 mM H_3PO_4 and 20 mM SDS in acetonitrile–water (50:50, v/v). The peaks are: (1) internal standard, (2) carnitine, (3) acetylcarnitine, (4) propionylcarnitine, (5) isovalerylcarnitine, (6) hexanoylcarnitine, (7) octanoylcarnitine and (r) peak from the derivatizing agent. UV detection at 260 nm. Adapted with permission from [63].

carnitine and acylcarnitines can be used as biomarkers for genetic inborn errors during the routine neonatal screening of blood.

Recently, a quantitative assay relying on capillary electrophoresis with laser-induced fluorescence detection was developed to measure the inter-conversion of sphingosine and sphingosine-1-phosphate. The assay was demonstrated to be capable of determining the in vitro activity of both kinase and phosphatase using purified enzymes. The fluorescent substrate was shown to be readily taken up by mammalian cells making it possible to study the endogenous activity of sphingosine kinase activity in living cells [67]. Dovichi and Hu have pioneered the field of chemical cytometry [68] where modern analytical separation tools are used to characterize the composition of single cells. This group has also done impressive work in metabolic cytometry, which is the use of a fluorescently labeled substrate and CE with LIF to monitor metabolic pathways in single cells [69,70]. They have used this very powerful technique to monitor the uptake and metabolism of glycosphingolipids in single cells.

3.2. MEKC

MEKC uses the same instrumental setup as CE, except that charged organized media, such as micelles or molecular micelles (polymeric surfactants), are added to the buffer as the separation medium for neutral solutes. The charged pseudo-stationary phase moves through the capillary under an applied voltage at an electrophoretic velocity that is proportional to the charge-to-size ratio. The separation of charged solutes is based on their charge-to-size ratio, while that of neutral solutes is based on their differential partitioning into the micellar phase.

Lipids that have short alkyl chains are relatively less hydrophobic and are easier to separate. Generally, it is possible to achieve baseline resolution of lipids differing by a single carbon atom in their alkyl chain that have less than 8–10 carbon atoms. However, as the length of this chain increases, the relative difference in chain length between two consecutive homologues rapidly declines, and their separa-

tion becomes difficult. Concomitantly, their hydrophobicity increases and solubility of such heavier lipids in purely aqueous electrolytes eventually becomes a limiting factor. The solubility of such lipids cannot be satisfactorily circumvented by resorting only to organic solvents, since the determination time would increase dramatically. Thus, other additives such as the organized media mentioned above are needed. Other problems related to resolving highly hydrophobic lipids are unstable electric currents and capillary clogging. For the determination of free fatty acids (FFAs), the addition of neutral cyclodextrins to the separating electrolyte constitutes a valuable alternative to the use of hydroorganic media, which allows one to fully resolve a linear saturated C_{12}–C_{24} FFA mixture [41,71]. This approach was successfully used to separate a similar C_{12}–C_{24} mixture while keeping the methanol content in the electrolyte to a minimum. The heaviest FFAs are better solubilized by micellar systems than by cyclodextrins [71]. Haddadian and coworkers demonstrated the power of polyoxyethylene lauryl ether (Brij 35) for the separation of C_{12}–C_{31} saturated FFAs differing by one carbon, in a buffer containing 40 mM Tris, N-methylformide-dioxane (3:2), 0.5% (w/v) Brij 35 and 2.5 mM AMP [43]. For the separation of a saturated C_{12}–C_{24} FFA mixture, Erim et al., and de Oliveira et al., adopted a mixed micellar system comprised of Brij 35 and an anionic surfactant, sodium dodecyl benzenesulfonate (SDBS), in an hydroorganic medium containing 50% acetonitrile [72,73]. Brij 35 was selected for its high level of purity, high UV transparency, and its very low critical micellar concentration. Acetonitrile was chosen as it reduces the EOF and causes reduced retention and an improvement in peak sharpness. Acetonitrile also allows for the analysis of chromatographic selectivity, which is a major issue for the assessment of alternate separation methods. Thus, the selectivity of Brij 35 micelles for the C_{12}–C_{24} FFA pair was quite similar to that currently obtained in gas chromatography. The impact of the two main parameters; methanol content and Brij 35 concentration in the electrolyte, was investigated in depth by de Oliveira and coworkers [73]. They showed that these parameters govern the resolution, the determination time, as well as the signal-to-noise ratio for the indirect absorbance conditions that were employed. Brij 35 was also used for the separation of isomeric hydroperoxides from the reaction of oleic, linoleic, and linolenic acids with singlet oxygen in a single run by MEKC [74].

MEKC can be used to separate long-chain fatty acid isomers that are non-chiral and relatively non-polar [75]. Ohman and colleagues separated conjugated linoleic acid isomers and parinaric fatty acid isomers in a buffer containing a chiral surfactant (R) dodecoxycarbonylvaline ((R)-N-DOCV), and a mixture of two cyclodextrins, heptakis-(2,3-dimethyl-6-sulfo)-β-CD (charged) and β-CD (uncharged) with a pseudo-stationary phase of sodium dodecyl sulfate (SDS) or sodium cholate [76,77]. An illustration of this separation is shown in Fig. 4. Their studies showed that even long-chain polyunsaturated fatty acid isomers differing only in the geometry and position of double bonds as well as cis–trans isomers can be resolved using MEKC.

The neutral micelle forming surfactant, Brij 35, has been used in MEKC to separate compounds like unsaturated fatty acids and related isomeric hydroperoxides either alone or
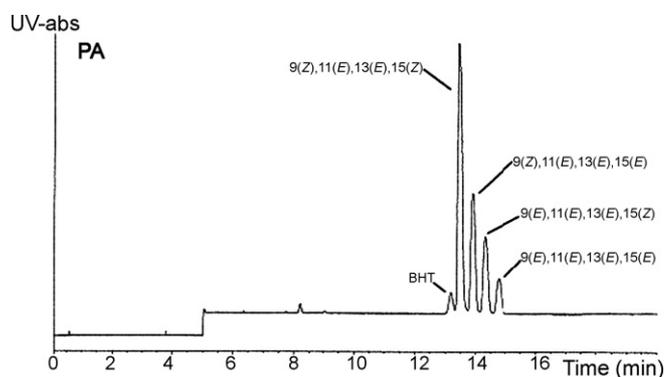
Fig. 4 – Separation of underivatized 9,11,13,15-octadecadienoate isomers (α-parinaric acid and its geometrical isomers). Conditions: capillary, fused silica 50 μm I.D., effective length 50 cm (total length 58 cm); 30 kV; 20 μA; 15 °C. Background electrolyte, 40 mM borate (pH 9.20), 4 M urea, 15 mM SDS, 20% MeOH (v/v); detection: UV at 305 nm. BHT = butylated hydroxytoluene. Adapted with permission from [76].

together with SDS as mixed micelles [71,78]. The mixture of SDS and CDs is very efficient for the separation of lipids because this mixture allows differential partitioning between the CDs and the micelles in the BGE, and the analyte during separation. Thus, the analyte which would otherwise be too strongly attached to the micelles can now be separated [77,79]. Zhang et al. did a determination of lipids using MEKC with LIF detection [80]. The advantage of LIF is that it is extremely sensitive as compared to UV detection. However, this sensitivity is slightly lowered by the presence of micelles in the BGE because the micelles increase the signal-to-noise ratio.

MEKC separations of fatty acids up to C_{18} have been achieved using 60% acetonitrile [7] or 60% methanol with cyclodextrin added to increase solubility and selectivity [41]. A mixture of fatty acids up to C_{20} was separated in a water–acetonitrile medium containing sodium dodecyl benzenesulfonate and Brij 35 [72]. Therefore, it appears that a separation medium containing a high organic solvent content is required for electrophoretic separation of long-chain fatty acids. Many organic solvents have been investigated as electrophoresis media. Formamide, having a higher dielectric constant than water, has been shown to provide higher efficiencies and shorter determination times than aqueous media [81]. N-methylformamide (NMF) has an even higher dielectric constant and was reported to be a suitable separation medium in CE [43,82]. Separation of C_{12}–C_{22} fatty acids was achieved in less than 15 min in a NMF medium. However, the solubility of the long-chained fatty acids (C > 22) was poor, making it necessary to add a modifier. Dioxane was chosen due to its proton acceptor properties [43,83]. To minimize peak distortion, BGE with a similar mobility as that of the analytes was used with maximum UV absorbance. However, the BGE should not comigrate with analyte. For some analytes, reversed-flow MEKC (RF-MEKC) can provide better resolution and efficiency than normal flow MEKC under conditions adjusted to provide comparable separation time [84]. Using RF-MEKC in capillaries coated with polydimethylacrylamide, separation time of

anacardic acids from cashew nut shell liquid was shorter than in non-coated capillaries. The polarity of the potential applied on the capillary was reversed with respect to the conventional MEKC in non-coated capillaries. The micelles of the anionic SDS move to the anode at a higher velocity than anacardic acids, which partition between the SDS micelles and a free aqueous solution that does not move in the absence of electro-osmotic flow. Their partition coefficients and hence the migration velocities increase according to increasing hydrophobicity. Therefore, the migration order of samples is opposite with respect to the non-coated capillaries. To improve the separation selectivity of anacardic acids, cyclodextrins were examined as additives to the SDS buffer. Cyclodextrins are excellent selectors for chiral and positional isomers, which enhance the separation selectivity by formation of inclusion complexes [79,80]. Cyclodextrins are mostly used as chiral selectors to resolve enantiomeric mixtures [51]. Melchior and Gab used MEKC with borax–SDS or meglumin–SDS buffer and obtained a rapid separation of hydroperoxy and hydroxy fatty acids and the non-oxidized unsaturated fatty acids from which they are derived [85]. Nearly all the isomers of the hydroperoxides and hydroxy fatty acids derived from oleic, linoleic, linolenic, and arachidonic acids can be determined both qualitatively and quantitatively within 10 min. Trans fatty acids in hydrogenated oils have been determined using CE with indirect UV detection [73,86]. C_{16} and C_{18} PUFAs were separated using SDBS, Brij 35, 1-octanol, and 45% acetonitrile. The experiment monitored the formation of trans fatty acids during the hydrogenation of brazilnut oil.

Most separations of GPLs by CE employed MEKC [80,87–90]. With MEKC, highly hydrophobic compounds such as GPLs are often difficult to separate due to their high solubility in the micellar phase [91,92]. Short size *n*-alkyl alcohols, e.g., methanol, ethanol, and 1-propanol, are frequently used in MEKC as a background electrolyte modifier or additive. These modifiers increase the solubility of the more hydrophobic compounds in the aqueous phase, permitting the hydrophobic molecules to spend more time in the aqueous phase and decreasing retention times [87,93]. 1-Propanol is the most frequently used organic modifier for GPL separations by MEKC [80,87–90,94,95]. The most common surfactants for the separation of GPLs are the bile salts, sodium cholate and sodium deoxycholate. These bile salts are lower in hydrophobicity than detergents such as SDS [87,96]. Consequently, hydrophobic analytes have lower micellar solubilities in the bile salts relative to those in other detergent micelles. The bile salts are also thought to minimize GPL aggregation and enhance GPL solubility. Zhang and colleagues performed the determination of aminoglycerophospholipid molecular species using methyl-β-cyclodextrin with LIF detection. They achieved baseline resolution of phosphatidylethanolamine (PE), phosphatidylserine (PS), and lysophosphatidylethanolamine (LPE) molecular species within 7 min. The separation of these GPLs is shown in Fig. 5. Many of the MEKC methods developed for the separation of GPLs are carried out in the 40–55 °C temperature range [80,88–90,94,87]. However, in some CE applications, it is difficult to maintain this elevated temperature throughout the capillary. An example is the lysis and loading of a cell into a capillary followed by separation of the cellular contents [97].
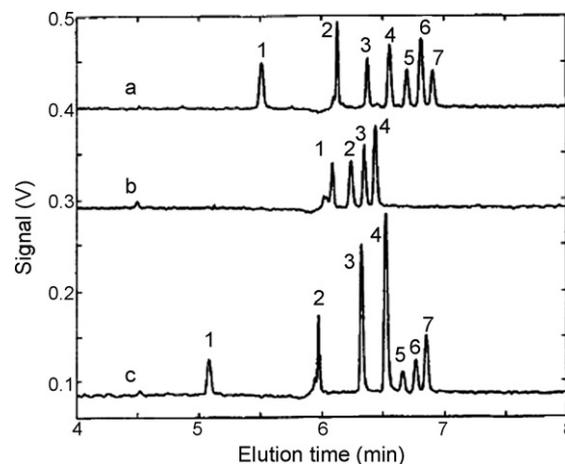

Fig. 5 – Electropherograms of FQ-labeled phospholipid molecular species in nanomolar concentrations. Running buffer, 10 mM borax + 35 mM SDC + 5 mM M-β-CD. (a) Phosphatidylethanolamine (PE) species: (1) C6:0, 2.05×10^{-8} M; (2) C8:0, 1.80×10^{-8} M; (3) C10:0, 1.61×10^{-8} M; (4) C12:0, 5.80×10^{-8} M; (5) C14:0, 1.77×10^{-7} M; (6) C16:0, 1.10×10^{-7} M; (7) C18:0, 1.14×10^{-7} M. (b) Lysophosphatidylethanolamine (LPE) species: (1) C12:0, 1.88×10^{-8} M; (2) C14:0, 1.76×10^{-8} M; (3) C16:0, 4.40×10^{-8} M; (4) C18:0, 5.72×10^{-8} M. (c) Phosphatidylserine (PS) species: (1) C6:0, 4.37×10^{-7} M; (2) C8:0, 4.10×10^{-7} M; (3) C10:0, 5.80×10^{-7} M; (4) C12:0, 5.90×10^{-7} M; (5) C14:0, 2.99×10^{-7} M; (6) C16:0, 2.38×10^{-7} M; (7) C18:0, 3.47×10^{-7} M. LIF excitation wavelength was 488 nm. Figure adapted with permission from [95].

Thus, the development of CE methods for GPLs separation at ambient temperature would be of high utility.

Most recently, we separated phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) labeled with BODIPY FL at the hydrophobic tail or near the hydrophilic head group by CE-LIF [98]. The optimal separation buffer at a pH of 8.5 was 100 mM Tris, 5 mM SDC, 1 mM MgCl$_2$, 30% 1-propanol, and 5% EOTrol^{TM} low reverse. In addition to developing a capillary-based separation for GPLs, we also investigated the mechanism of separation of the GPLs. Our results were quite surprising. The existing school of thought is that GPLs separation in an aqueous media in a capillary requires MEKC [90,87]. We found that the tested GPLs did not separate by MEKC and indeed that surfactants were not even required for resolution of these GPLs [98]. This finding is significant and merits further investigation and would be of great interest to the CE community, and especially to those investigators working in the area of lipid separations.

NACE was used to dissolve extremely hydrophobic lipids mostly by using methanol and acetonitrile in a buffer of ammonium acetate with the addition of cetyltrimethylammonium bromide (CTAB) and myristyltrimethylammonium bromide (MTAB) for viscosity [99–101]. The selectivity of NACE is often realized by manipulating the separation medium [102], the additives and their concentrations [103], and electrolyte

concentrations [104]. Acetonitrile was shown to decrease the electro-osmotic flow (EOF), improve peak sharpness, and reduce analyte retention [52].

In GPL determination, because the different substituted groups on the phosphate head group have different proton donating–accepting capabilities, NACE could be a good alternative to MEKC. NACE has been used for the determination of GPLs [99,50]. One advantage of the NACE methods over other CE-based methods is that the NACE methods can be performed in a buffer appropriate for full dissolution of the lipids. However, many biological samples are dissolved in aqueous solutions that may not be compatible with the organic solvents used in NACE [50].

Lipopolysaccharides have been characterized from *H. influenzae* [105] and *T. paurometabola* [106], and other complex lipopolysaccharides [107]. Amadzadeh and colleagues used 10-nonyl acridine orange to form a fluorescent complex with cardiolipin, a GPL found only in mitochondria, to characterize individual mitochondria sampled directly from muscle tissue [108].

Microemulsion electrokinetic chromatography (MEEKC) has emerged as a powerful tool to separate difficult mixtures [109–111]. In general, the microemulsions (oil-in-water, o/w) are formed from oil (named the core phase, usually a hydrocarbon or other hydrophobic substance), water (buffer), and a surfactant and co-surfactant (such as a medium alkyl-chain alcohol). It appears that the structure of the microemulsion which is similar to the structure of the micelle-oil droplet, is stabilized by the surfactant and co-surfactant located on the droplet surface [112]. A novel microemulsion based on a mixture of diethyl ʟ-tartrate (DET) and SDS was developed for the MEEKC determination of structurally related steroids. The system consisted of 0.5% w/w DET, 1.7% w/w SDS, 1.2% w/w 1-butanol, 89.6% w/w phosphate buffer (40 mM, pH 7.0), and 7% w/w acetonitrile. MEEKC showed obvious advantages over MEKC for the separation of highly hydrophobic substances. A mixture of saturated fatty acids containing even number of carbon atoms was successfully separated as phenacyl esters by MEEKC. Although, MEKC employing SDS was unsuccessful in separating all long-chain fatty acids, it appears that the use of more hydrophobic derivatives with microemulsion pseudophases can generally result in good separations of mixtures that are difficult to separate by MEKC [9,113].

3.3. CEC

Capillary electrochromatography (CEC) is a liquid chromatographic technique that utilizes electro-drive to significantly improve chromatographic performance. This has significant advantages since the velocity flow profile in the capillary corresponds approximately to plug flow for an electrically driven system compared to parabolic for pressure driven flow. In CEC, liquid is moved through the column by EOF, which is movement of the bulk of the liquid except the surface layers. CZE contrasts to CZE in which separation is predominantly based on the differential migration of charged species under the influence of an applied electric field. This provides a large increase in chromatographic efficiency. Originally, CEC column packing was performed using standard HPLC stationary phases, they commonly had pore sizes of

8–10 μm with octadecylsilane being the most commonly used bonded stationary phase. The use of polyacrylamide gels in columns without frits was described by Fujimoto [114]. Abidi et al. used pentafluorophenylsilica (PFPS), triacontylsilica (TCS), and octadecylsilica as stationary phases for the separation of sterols [115]. In addition to packed columns, there are open-tubular columns of which the inner surface is modified by ligands or coatings [116], and monolithic columns made by on-column copolymerization of various monomers which produce gels [117]. Mobile bile phases for the sterols separation by CEC include methanol, acetonitrile, dimethylformamide, and tetrahydrofuran. CEC provides superior analyte selectivity and is ideally suitable for the determination of thermally labile compounds. CEC features high efficiency, high-resolution, and high-speed microscale separations with minimal solvent consumption and is a combination of CE and HPLC. CEC is more convenient compared to GC because GC requires the use of thermally stable columns and chemical derivatization before sample determination. Octadecyl sulfonated silica (ODSS), consisting of octadecyl functions bonded to a negatively charged layer containing sulfonic acid groups was used as the stationary phase in the separation of neutral and acidic glycosphingolipids (GSLs) [118]. The mobile phase contained 10 mM aqueous sodium borate and a total of 80% methanol and acetonitrile. Acetonitrile allowed the elution of the glycosphingolipids and eliminated their aggregation, whereas methanol was used to adjust the selectivity of the system. Triglycerides have been separated in different vegetable oils using CEC packed with 3 μm Hypersil ODS [119–121]. The separation buffer was made of acetonitrile/isopropanol/*n*-hexane in the ratio 57/38/5 and 50 mM ammonium acetate. The results showed better efficiencies than liquid chromatography. CEC separations of plant sterols and related esters were evaluated under various conditions. Stationary phases included octadecylsilica (C_{18}) and triacontylsilica (C_{30}). Mobile phases comprised acetonitrile, tetrahydrofuran, and tris(hydroxymethyl) aminomethane buffers in aqueous or non-aqueous systems. Apart from notable differences in component resolution, both C_{18} and C_{30} phases had dramatic influence on the elution behavior of the title compounds. Generally, C_{18} had greater selectivity for most components with elution patterns consistent with the hydrophobicity of side chain structures, while no predictable trend of analyte elution was observed in CEC with C_{30}. In the latter column systems, analyte separations appeared to be improved by conversion to benzoates or ferulates. Twenty-four-epimers of campesterol acetate and 7-campestenol acetate as well as the campesterol–stigmasterol pair were readily resolved by CEC with either phase. However, the cholesterol–stigmasterol pair was barely resolved and had an elution order opposite to that of their acetates or benzoates. In addition, the potential applicability of CEC in the determination of sterols and sterol ferulates in vegetable oil [122], and the separation of retinyl esters (lipidic vitamins) using aqueous [123], and non-aqueous solvents was demonstrated [124].

3.4. Microchip CE

Microchip capillary electrophoresis is an emerging technology that promises to lead the next revolution in chemical anal-

ysis. Microfluidic research has expanded tremendously over the last decade, although the field is still in its developing stage [125,126]. It has the potential to simultaneously assay hundreds of samples in a matter of minutes or less. Multiple channels in parallel and multiple assay steps can be integrated into one single device, allowing high throughput and complex processes in compact, easy-to-handle devices. Microchips typically consume only picoliters of sample. These samples may potentially be prepared on-board for a complete integration of sample preparation and determination functions. CE on microchips is based upon microfabrication techniques where microchannels are fabricated in microchips using photolithography or micromolding to form channels for sample injection and CE separation [127]. The small injection plugs, high fields, and short separation lengths produce separation times measured in seconds or minutes. Microfluidic devices for CE have been very successful for a wide range of bioanalytical measurements [128,129]. Lin et al. separated phosphatidylinositol, phosphatidylinositol 3-phosphate, and phosphatidylinositol 3,4-bisphosphate on a microfluidic chip at room temperature [95]. These lipids were linked to BODIPY FL via their acyl chain. The separation buffer contained 20 mM SDC, 35% 1-propanol, 0.1% coating-3 reagent, 100 mM Tris (pH 8.5), 1 mM EDTA, 1 mM Hepes (pH 7.0), 1 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.4% glycerol. In addition, Lin and coworkers exploited the separation method to develop an assay to monitor enzyme activities of lipid-modifying enzymes. A disadvantage of this method is that it used a complex buffer system. While microfluidics

has many assets, it is currently not a useful format for many applications particularly the analysis of the contents of cells attached to a solid surface. Thus, further research is highly desired.

4. Discussion

CE, has the potential to combine the best features of both GC and LC; however, due to low solubility of lipids in aqueous buffer, high amounts of organic solvent in the separation medium are often required. Conversely, organic solvents decrease micelle size, and in certain circumstances, they even depress micelle formation [72]. In HPLC, mobile phase gradients are easily established and the collection of fractions for further examination is possible unlike in CE where the analyte loadability is small. CE has been explored as an alternative to GC and LC for the separation of lipids [40,41,45].

Difficulties of separating lipids by CE include increasing analyte aggregation, especially at concentrations above their critical micellar concentrations and decreasing separation selectivity between successive homologs. Most CE methods use an aqueous electrolyte separation medium. Thus, the determination of lipids by CE is difficult because lipids are sparingly soluble in aqueous buffers. Compared to aqueous solutions, organic solvents offer unique physical and chemical properties such as relative permittivity, viscosity, auto proteolysis constant, polarity, and volatility that can be successfully

CE mode	Lipid class	Common additives	References
Table 1 – Summary of the different CE modes used to analyze lipids and a cross-section of buffer additives used			
CZE	Fatty acids	Acetonitrile, N-methylformide-dioxane	[40,41,43,49,62]
	Glycerophospholipids	Acetonitrile	[45–47,50,52,53,61,100]
		Methanol	
		1Propanol	
	Gangliosides	Cyclodextrin	[54,55]
	Carnitines	Acetonitrile	[60,63,64]
		Methanol	
MEKC	Saturated fatty acids	Sodium dodecylsulfate	[86]
		Dimethyl-β-cyclodextrins	
		Acetonitrile	
	Unsaturated fatty acids	P-anisate	[71,72,74,78]
		Polyoxyethylene-23-dodecyl ether (Brij 35)	
		Methanol	
		Sodium dodecyl benzene sulfonate	
	Glycerophospholipids	Methanol, Acetonitrile, Brij 35, Sodium deoxycholate	[45,80]
		Methyl-α-cyclodextrins	
MEEKC	Fatty acids	Cholate	[117]
		Heptane	
		N-butanol	
CEC	Triglycerides	Acetonitrile	[123,125]
		Isopropanol	
		N-hexane	
	Sterols	Acetonitrile	[126]
		Tetrahydrofuran	
		Hydroxymethyl aminomethane	
	Retinyl esters	Lithium acetate	[127,128]
		-N,N-dimethylformamide	
		Methanol	

exploited to achieve excellent separations in CE. Therefore, the use of a mixture of aqueous and organic buffers or exclusively organic buffers may result in the separation and quantitative determination of lipids that have almost identical or similar structures.

Definitely, the research towards the migration behavior of analytes in mixed solvent system is of great importance, because the mixed solvents are more often used compared to one component solvent in the determination of lipids using CE. Although the lack of chromophores is a problem for identification and quantification of lipids, ultraviolet (UV) detection of GPLs at 200–214 nm is frequently applied to the determination of GPLs due to the presence of unsaturated groups, such as carbonyl, carboxyl and phosphate [87]. Absorption at these wavelengths is weak and the typical limits of detection (LOD) are 50 ppm (50 μM). In addition, many solvents, buffer additives, and other analytes absorb in the region of 190–220 nm. These properties present a major challenge for the determination and quantitation of lipids. Many lipids are difficult to detect due to the lack of conjugated double bonds and unreactive aliphatic functional groups in the structure. Although laser-induced fluorescence can give high sensitivity for lipids, the derivatization process is complex and non-stoichiometric. Another commonly used method is CZE with indirect UV detection due to the low UV absorbance of lipids [80]. Many of the lipid CE determination systems employ indirect UV absorbance detection, which suffers mainly from a moderate dynamic range and detection limits in the micromolar range. Determination of fatty acids from biological samples often requires much lower detection limits. The coupling of CE with mass spectrometry provides a powerful approach for rapid identification of target analytes present at trace levels in biological matrices, and for structural characterization of complex biomolecules [13,14]. Non-aqueous separation media are gaining popularity as a means of expanding the range of mixtures separable by CE [49,50,81]. Acid–base dissociation constants, capillary wall potentials, and compound solubilities, among others, can be altered dramatically in non-aqueous solutions. Separation of the so-called neutral lipid compounds (which include fatty acids) requires non-aqueous solutions to solubilize compounds with chain lengths beyond approximately C_{12}, depending on the degree of unsaturation, oxidation, and ionization of the head group.

Microchip CE is still undergoing development and in future it will find great application in the determination of complex matrices such as blood, natural fluids and cells which is currently a challenge for these microchannels [130,131]. Table 1 is a summary of the different CE modes used to analyze lipids and a cross-section of buffer additives used.

5. Conclusion

It is evident that CE and CE-based analytical tools have great potential in the determination of lipids. The speed of CE and its high resolving power make lipid determination very efficient. The difficulty remains to identify a universally optimized buffer system that would dissolve the entire cross-section of lipids. This, together with the identification of a simple, highly sensitive detection mechanism are the two areas that have

curtailed the widespread use of CE in quality control of foods, pharmaceuticals, and chemical industry as well as extremely sensitive diagnostic protocols in medicine. For medical diagnosis, CE can find much use in atherosclerosis diagnosis as well as diagnosis of genetic disorders. The emergence of microchip CE technology with its speed and potential high throughput would further increase the impact of CE as an analytical tool. The use of UV detection is still commonplace but is still not sensitive enough. LIF and the derivatization process are too involving which makes it difficult to work with physiological samples like blood and other body fluids. CE–ESI–MS is the way forward with the potential of on-line automation of an array of CE microchips for collecting and analyzing bulk data.

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