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Chapter 2

Method Development by Use of Capillary Electrophoresis and Applications in Pharmaceutical, Biological and Natural Samples

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Additional information is available at the end of the chapter

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1. Introduction

Electrophoretic methods compose a family of related techniques that use narrow-bore fused-silica capillaries to perform high efficiency separations of both small and large molecules. These methods are commonly known as capillary electrophoretic methods. Capillary electrophoresis (CE) has, over the years, demonstrated its powerful separation ability in the area of chiral and achiral analysis. This is contributed to the advantages it offers when compared to chromatographic techniques: (1) low consumption of samples and solvents; (2) high separation efficiency and resolution; (3) versatility [1,2].

Two of the most important modes of CE, which will be discussed in this chapter, are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). CZE is the simplest and the most widely used mode of CE [1]. The separation mechanism of the analytes is based on their difference in charge-to-size ratios and their difference in electrophoretic mobilities, which, in turn, result in different velocities. However, due to the fact that neutral species do not possess an electrophoretic mobility, they cannot be separated by use of this mode.

In order to circumvent this problem, new modes of CE have been suggested as alternatives. MEKC, which combines the best features of both electrophoresis and chromatography, is considered an alternative mode because it can be used for the separation of charged as well as neutral compounds. It involves the introduction of a surfactant at a concentration above the critical micellar concentration (CMC), at which micelles are formed. MEKC was first introduced by Terabe *et al*. in 1984 [3]. Although it is a form of CE, its separation principle is more similar to HPLC than to CE. In this mode, analytes are separated according to their partitioning between the mobile and stationary phase and, when charged, their electrophoretic mobility. The driving force for the partitioning of analytes is hydrophobicity.

In addition, hydrogen bonding, dipole-dipole, and dispersive interactions can contribute to the solute partitioning between the two phases [4-6].

CE was originally considered as a powerful analytical tool for the analysis of biological macromolecules. It has though, over the years, been extensively used for the separation of other compounds, such as chiral drugs, food additives, pesticides, inorganic ions, organic acids, and others. In this chapter, the ability of CE, and particularly CZE and MEKC, to be used for the qualitative and quantitative determination of compounds in pharmaceutical, biological and natural samples is investigated. In each approach, a number of studies are reported and discussed. These studies involve establishment of optimum separation conditions, method validation, optimization of sample-preparation procedure and application for the determination of the analytes under study in real samples. The first part of this chapter involves the determination of polyphenolic compounds using CZE with UV-Vis detector in red and white wines, while the second part involves the determination of pharmaceutical compounds in biological samples, such as blood and urine, using the hyphenated technique CE-MS (mass spectrometry). The third and final part emphasizes the importance of MEKC in chiral analysis since it has been known that usually only one enantiomer is active, while the other may be less active, inactive or has adverse effects.

2. Determination of polyphenolic compounds in natural samples

Polyphenolic compounds exist in a variety of natural products, such as fruits, vegetables, beverages (tea, wine and juices), honey, cacao and herbs. They attract a lot of interest due to their beneficial implication in human health. They have been widely studied due to their antioxidant capacity and their association with several pathological conditions, such as hypertension, cardiovascular disease, dementia, and even cancer [7-9]. Therefore, due to their health significance, numerous analytical methods have, over the last decades, been developed for their separation, identification and quantitation in natural products [10-13].

According to literature, the simplest CE method, CZE, proved to be the best method for the determination of polyphenolic compounds in wine samples [14-17]. In such studies, and in each case, when the optimum CZE method was applied to different red and white wines, it was established that red wines have higher levels of polyphenolic compounds than white wines and that the polyphenolic composition varies among different wines.

2.1. Method development and validation

In this part of the chapter, a representative study performed recently in Cypriot wines is briefly described [17]. The influence of several experimental parameters is initially illustrated in order to obtain improved selectivity and resolution for the separation of seven flavonoids, which constitute the most important group of polyphenols, and trans-rasveratrol that are usually present in wine. This is accomplished by use of CZE and by examining different sample preparation procedures. Due to the low concentrations of flavonoids in wine and the high complexity of wine matrices, preconcentration methods are required, which can simplify the electropherograms. The optimized CZE and pre-treatment methods proved to be effective in characterizing flavonoids in red and white wine samples.

The effect of column temperature, and concentration and pH of background electrolyte (BGE) were investigated. These parameters, along with the applied voltage, are the most common parameters that are required to be examined in order to optimize a separation in CZE. Figure 1 illustrates the influence of the pH on the resolution and the analysis time.

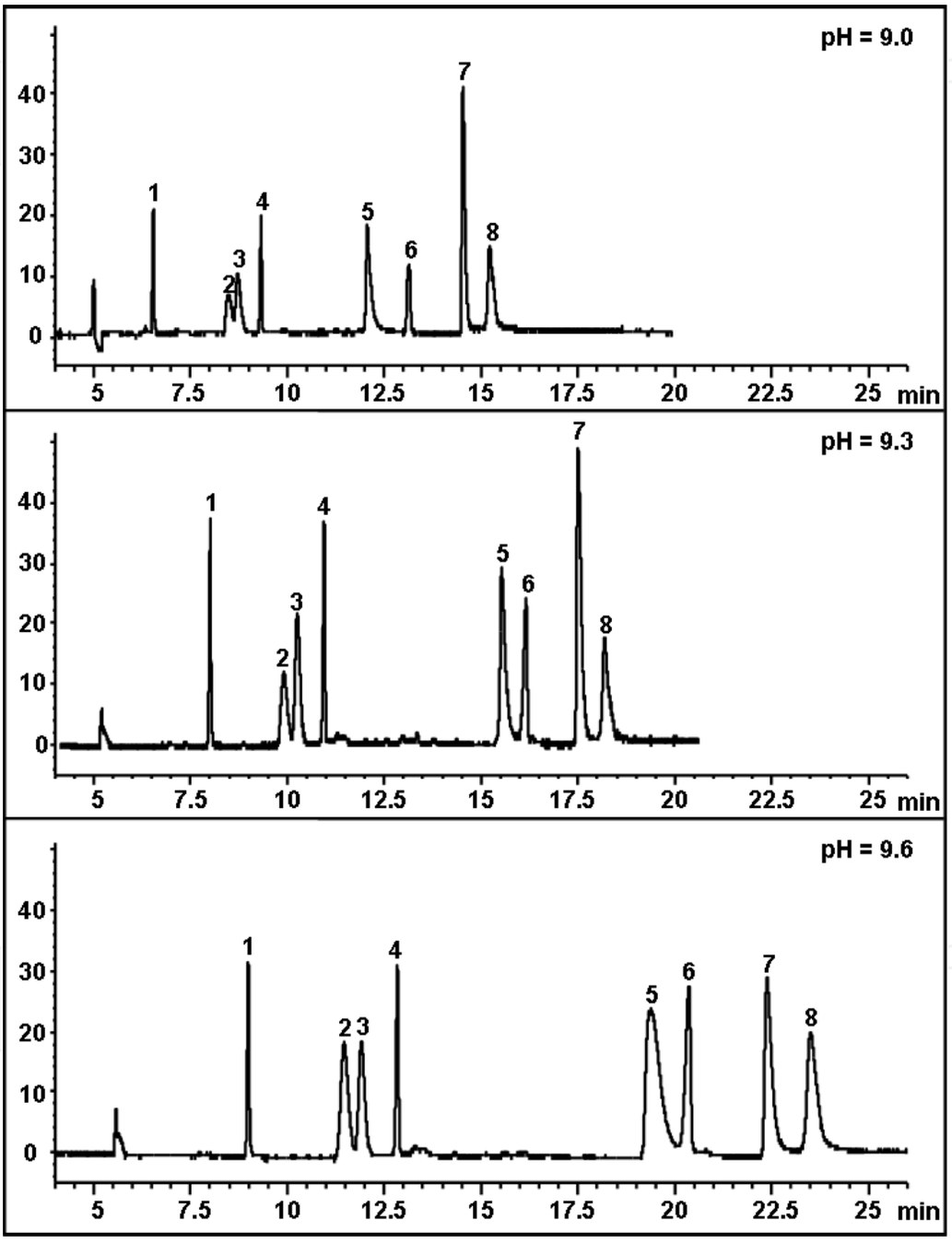

Figure 1. Effect of pH value on the separation of the eight polyphenols. (A) pH 9; (B) pH 9.3; (C) pH 9.6. Conditions: BGE 50 mM borate, 10 mM phosphate and 20 mM SDS; pressure injection, 30 mbar for 3 sec; applied voltage, 25 kV; temperature, 25 ºC; fused-silica capillary, 64 cm (55.5 cm effective length) x 50 μm i.d.; detection, 205 nm. Peak identification: trans-resveratrol (1), epicatechin (2), catechin (3), naringenin (4), kaempferol (5), apigenin (6), myricetin (7), quercetin (8) [17].

The last two increased with increasing the pH, possibly due to an increase in the negative charge, which resulted in a greater affinity and a higher complexation between borate and phenols. Taking into consideration the migration times, the peak efficiency and the sufficient resolution, the following parameters provided a baseline separation of all polyphenolic compounds: BGE containing 50 mM borate and 10 mM phosphate at pH 9.6 and column temperature of 25 ºC (Figure 1C). The use of alkaline borate-based BGEs, in CZE, resulted in a sufficient separation of polyphenols due to the complex-formation ability of borate. In addition, an increase in the borate concentration from 25 to 50 mM and an increase in the pH value from 9 to 10 resulted in an increase in the migration times of all analytes, while the resolution was significantly improved. At pH 10 though, the analysis time was very long (~ 50 min) and joule heating effects, such as high current generation and peak broadering, were observed. An increase in pH increased the negative charge of the analytes, which, in turn, favored a greater affinity for the buffer and a higher complexation between borate and phenols [18].

The method was then validated by the terms of linearity, precision and LOD. Linearities for the eight analytes were very good, and precision, which was based on the relative standard deviation, was below 1%, indicating an excellent reproducibility. In addition, LODs, which were calculated as three times the standard deviation via the slope of the calibration curve, were between 0.03 and 5.05 μg/mL for all eight polyphenolic compounds.

2.2. Application

The qualitative and quantitative analysis of analytes in real samples is often difficult due to interruptions caused by different interfering substances found in the sample matrix. Therefore, a sample-preparation procedure is a necessary step prior to the electrophoretic analysis, in order to isolate the analytes under study from real samples. Different preconcentration methods have been used over the years, including solid-phase extraction (SPE) with C-18, silica, or other cartridges [14,16,19] and liquid-liquid extraction (LLE) with different organic solvents [10,20,21].

In the study performed in Cypriot wines, the sample preparation procedure was optimized in order to determine the one that was simple, fast and reliable [17]. Therefore, three LLE-procedures (C,D,E), a SPE-procedure (F), a procedure that involve evaporation and reconstitution of wine sample (B) and a direct injection of wine sample after dilution and filtration (A) were compared and the most effective method was applied to Cypriot wines. The electropherograms obtained by use of each sample preparation procedure are illustrated in Figure 2. When no extraction was performed, the electropherograms were complex, while SPE was found to be ineffective for the isolation of polyphenolic compounds from wine samples. LLE with diethyl ether, followed by evaporation of organic layer by nitrogen stream and reconstitution in ethanol proved to be the optimum sample pre-treatment method. When the optimum method was applied to Cypriot wine samples, the quantification of polyphenolic compounds was successfully achieved. It was observed that epicatechin and catechin exist in all wine samples in comparable concentrations, whereas myricetin and quercetin exist only in two of the three wine samples. Polyphenolic

composition varies among different wines, because it depends on several factors, such as the type of grapes used, the vivification process used, the type of yeast that participates in the fermentation, weather variations and other biological effects [22].

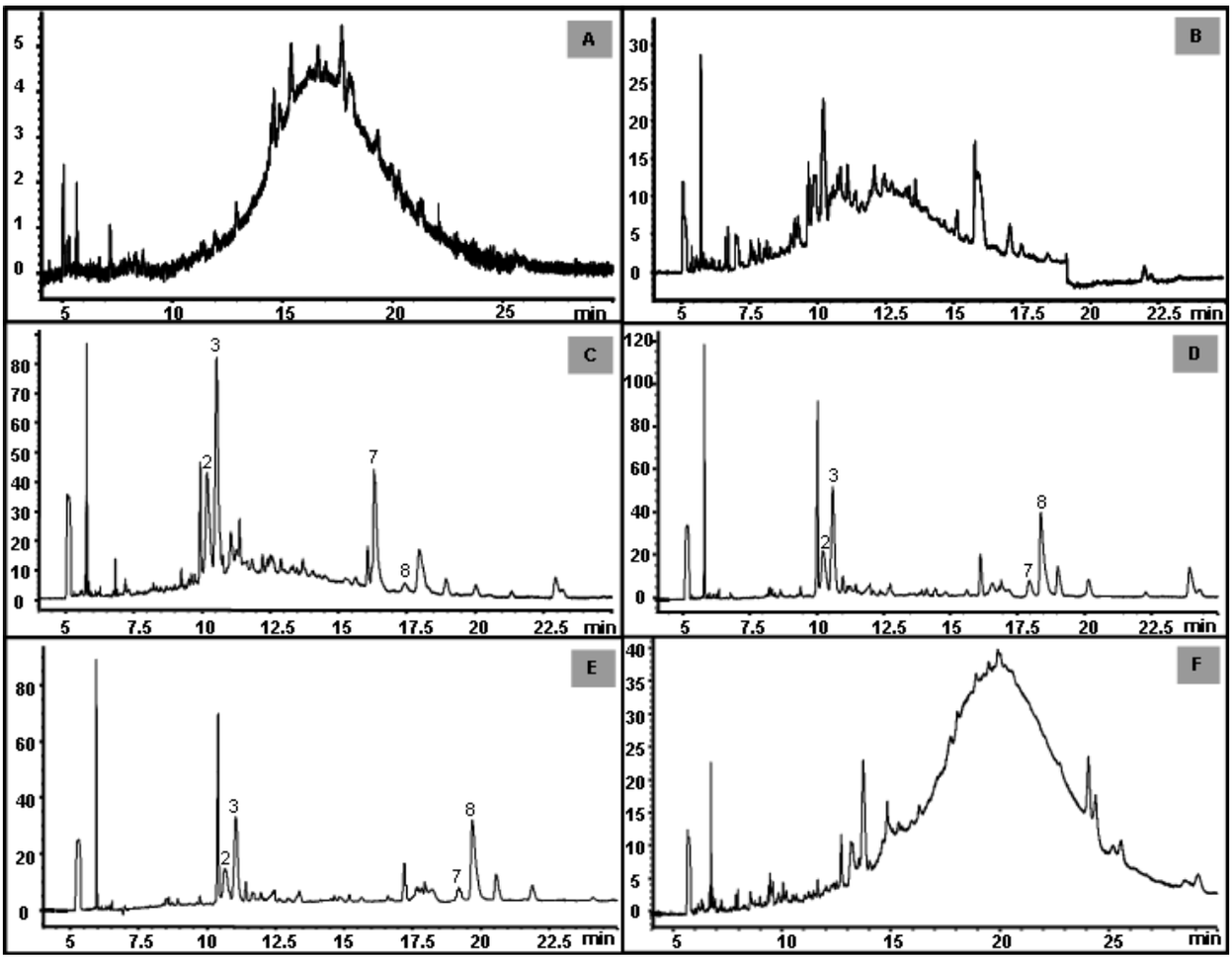

Figure 2. Electropherograms of the wine samples obtained using six different sample preparation procedures under optimum conditions. Conditions: BGE 50 mM borate, 10 mM phosphate and 20 mM SDS (pH 9.6); pressure injection, 30 mbar for 3 sec; applied voltage, 25 kV; temperature, 25 ºC; fused-silica capillary, 64 cm (55.5 cm effective length) x 50 μm i.d.; detection, 205 nm. Peak identification: epicatechin (2), catechin (3), myricetin (7), quercetin (8) [17].

Another important observation was that in white wine, the only flavonoid that was detected was catechin at a concentration of 7.3 μg/mL. This was not surprising since the majority of flavonoids in wine come from the extraction derived from grape's solids. White wine is made by pressing the juice away from the grape's solids, and then, by allowing it to ferment. So, red wines have higher levels of polyphenolic compounds [23].

3. Determination of pharmaceutical compounds in biological samples

Quantification of drugs in biological fluids, like plasma, has an important role in drug discovery and development. There are two main aspects that are taken into account in order

to make the identification of drugs in biological fluids possible. The first aspect is the development of an accurate analytical method, with high sensitivity, capable to identify desirable compounds in concentrations comparable to that in biological fluids. The second one is the exploration of the optimum extraction method that can effectively extract the drug from the biological matrix.

Over the years, CE coupled to electrospray ionization-mass spectrometry (ESI-MS) has been utilized as a bioanalytical tool for the analysis of drug compounds in biological samples [24-29]. Even though the most common detector in CE is the UV detector due to its easy manageability and low cost, it has the drawback of low sensitivity due to the short optical path length. An alternative to this is the use of MS. The coupling of CE with MS is a well-established technique, which combines the high efficiency and resolution that are provided by CE and the detection sensitivity and selectivity and the identification potential that are provided by MS [25,30].

In recent years, a large number of publications have been provided on the general developments and biological applications of CE-ESI-MS [24-29]. Zheng *et al.* developed a CZE-ESI-MS method for monitoring the antiepileptic drug lamotrigine in human plasma [27]. The optimum conditions were obtained by varying a big number of BGE, sheath liquid and MS spray chamber parameters. In each case, both the CZE separation, as well as the MS detection sensitivity, were evaluated, and the parameter that provided a reasonable compromise between resolution and detection sensitivity was chosen as the optimum. The developed method was then applied to assay blank samples spiked with lamotrigine in order to set up the calibration curve and estimate the limit of detection (LOD). Both linearity of calibration curve and LOD (0.05 μg/mL) were good, and the optimum method was applied to 14 human plasma samples collected from a lamotrigine-treated subject over a period of 96 h after oral administration of 50 mg lamotrigine.

In a 2011 study, Elhamili *et al.* analyzed the anticancer drug Imatinib by use of CE coupled to ESI time-of-flight MS in human plasma [29]. The CE separation and ESI parameters were initially investigated and optimized in regard to peak efficiency, peak intensity and electrospray stability. The LOD and limit of quantitation (LOQ) were evaluated by injections of standard solutions of the drug compound, and they were determined to 5 and 20 ng/mL, respectively. In addition, the extraction recovery of Imatinib from human plasma using a common liquid-liquid extraction (LLE) method and a new strong cation exchange (SCX) solid-phase extraction (SPE) column was investigated and compared. The highest extraction recoveries were obtained by using the latter method. The SCX-SPE extraction followed by CE-ESI-TOF-MS analysis in patient plasma samples demonstrated good repeatability, linearity and sensitivity for possible therapeutic monitoring of Imatinib level. The authors, in this manuscript, also conclude that this method could be applied for the analysis, quantification, and clinical assessment of other drug compounds and their metabolites.

3.1. Method development

The performance and usefulness of CE-MS is also demonstrated here by providing a more in-depth analysis of a research work that was performed in a blood sample obtained from a

patient with Alzheimer´s Disease (AD) [24]. In this study, a CZE-ESI-MS method was developed for the analysis of the acetylcholinesterase inhibitor rivastigmine, using neostigmine bromide as an internal standard, which is highly recommended in order to avoid problems that are related to sample injection [31]. Rivastigmine is a pseudo-irreversible carbamate inhibitor of acetylcholinesterase, and it is clinically used for the symptomatic treatment of mild to moderate AD [32].

In a previous paper, MEKC coupled to a diode-array detector was used for the simultaneous separation of nine acetylcholinesterase inhibitors, including rivastigmine [33]. This method was validated and successfully applied to a real blood sample that was obtained from a patient who was not under any of this medication. The sample was spiked with rivastigmine in order to establish the ability of the method to separate the drug from other components that might exist in the blood sample. In this study, the blood sample was not directly injected into the capillary, because some components that exist in the sample can be absorbed to the capillary wall and deteriorate the performance of the column [34]. The blood sample was therefore diluted ten folds with the BGE [12.5 mM Na_2HPO_4 / 12.5 mM $Na_2B_4O_7$ / 20 mM SDS (pH 10)], and it was then spiked with 25 μg/mL of rivastigmine. However, due to the low sensitivity obtained by CE with on-column UV detection, the identification of rivastigmine in biological fluids using CE remained a challenge. In order for the technique to be used for the quantitation of an acetylcholinesterase inhibitor in body fluids, the sensitivity, and consequently the LOD had to be improved. The increased interest in exploring CE-MS and its potential to serve as an alternative method allowed further investigation for the determination of rivastigmine and related drugs in complex biological matrices.

When the CZE-UV method was compared with the CZE-MS, the first demonstrated a shorter analysis time of approximately 2 min due to the shorter effective length, while the S/N for the peak of rivastigmine at the SIM mode was estimated to be eight times bigger than with UV detection. This, in turn, indicated the high specificity and selectivity of the ESI-MS detector [24]. In the CZE-ESI-MS study, several electrophoretic and ESI-MS parameters were also examined, which were classified in three categories: the BGE parameters, such as the concentration, the pH and the use of organic modifier, sheath liquid parameters, such as the composition, the methanol (MeOH) content and the flow rate, and finally some spray chamber parameters, such as the temperature and the flow rate of the drying gas and the nebulizer gas pressure. The effect of each parameter on the S/N, and consequently the LOD, was examined and the optimum one was chosen for further optimization.

In the case of BGE parameters, it was observed that ammonium acetate provided the most reproducible migration times, a concentration of 40 mM ammonium acetate resulted in the highest S/N, while a higher concentration decreased the ratio, probably due to the Joule heating effect that increases the level of noise (Figures 3a & 4a). When the pH was examined, it was concluded that at pH 9, where rivastigmine starts to have a negative charge (pKa=8.6), both the analysis time and resolution increased, and a higher S/N was obtained (Figures 3b & 4b).

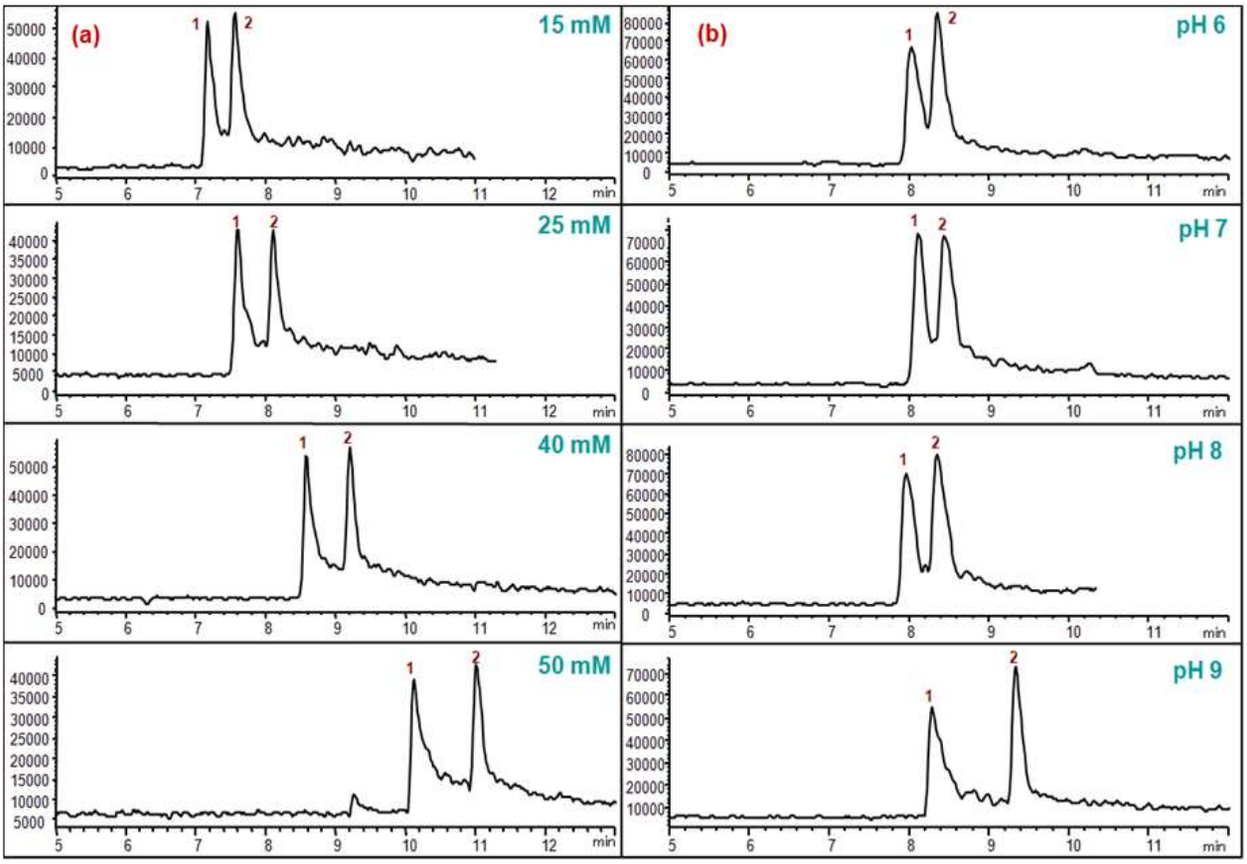

Figure 3. Effect of (a) ionic strength and (b) pH of the BGE on the separation of rivastigmine (2) and I.S (1). Conditions: BGE: ammonium acetate, sheath liquid 1 % acetic acid in water:MeOH (50:50 v/v) at a flow rate of 10 μL/min, analyte and I.S. concentrations 0.3 mg/mL. Drying gas flow rate 6 L/min and temperature 200 ºC, nebulizer gas pressure 20 psi [24].

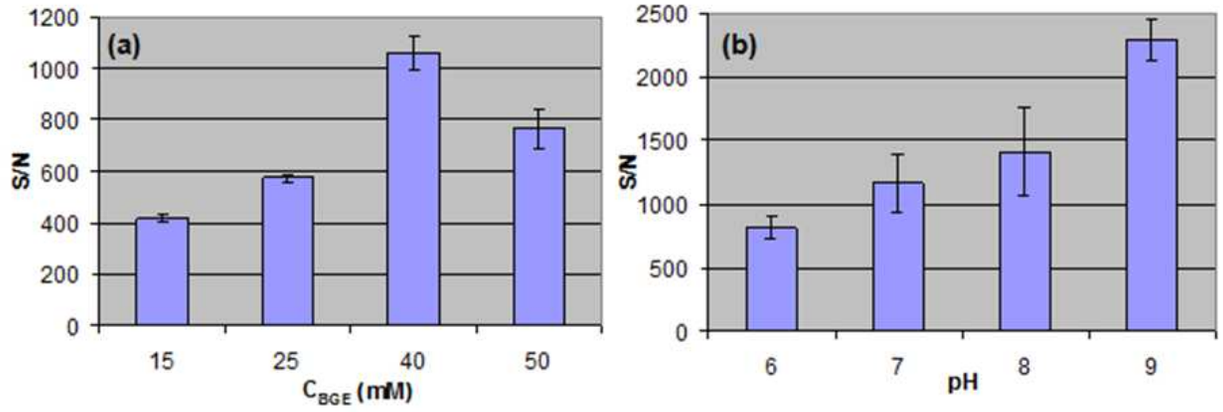

Figure 4. Effect of (a) ionic strength of the BGE and (b) pH of the BGE upon S/N ratio. Conditions: BGE: ammonium acetate, sheath liquid 1 % acetic acid in water:MeOH (50:50 v/v) at a flow rate of 10 μL/min, analyte and I.S. concentrations 0.3 mg/mL. Drying gas flow rate 6 L/min and temperature 200 ºC, nebulizer gas pressure 20 psi [24].

As far as the sheath liquid parameters are concerned, it was observed that its composition and its flow rate affected the ESI-MS sensitivity significantly. This was not a surprising

observation since the sheath liquid plays an important role in the CE-MS system. The sheath liquid is used as the make-up liquid that can solve the flow-rate incompatibility problems between CE and MS [35]. These problems are encountered because the flow rate through the CE column is very low (nL/min), and it cannot support a stable electrospray, whose flow rate is typically a few μL/min. In addition, the sheath liquid is used for establishing an electrical connection at the cathode end of the CE capillary, and it provides the suitable solvent conditions for the electrospray, which does not depend on the CE BGE [36].

When different sheath liquids were evaluated, the one that was able to support the formation of positively charged ions, and consequently provide the highest S/N, was acetic acid (1%) (Figure 5a). The influence of methanol as an organic modifier in the sheath liquid was also examined, because the use of such solvents allows an easier protonation of the analytes, which results in a higher signal [28]. By varying the percentage of methanol, it was concluded that 50% was the optimum since the noise level was the lowest (Figure 5b). Finally, the flow rate of the sheath liquid was set at 10 μL/min (Figure 5c). Other values were either too low to establish an electric contact that is required to achieve separation, or they affected the spray stability negatively, which, in turn, lead to higher noise levels.

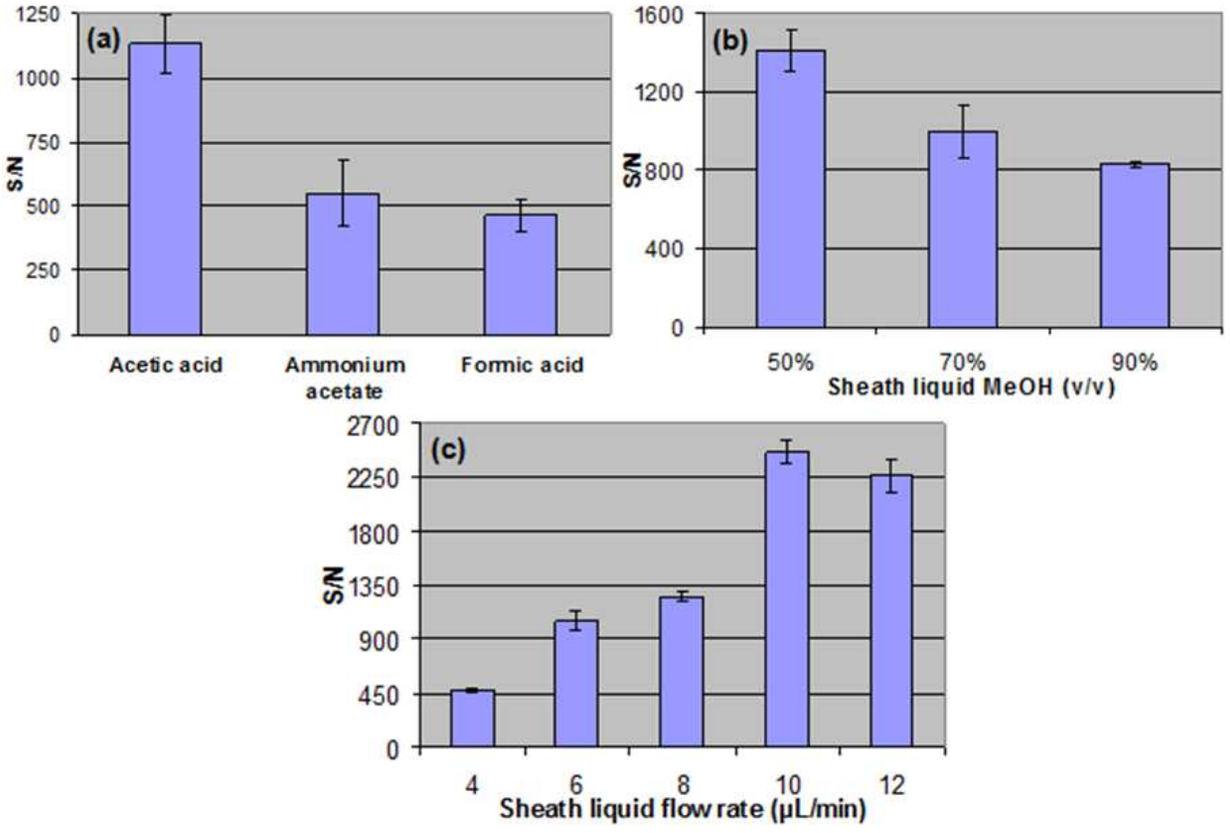

Figure 5. Effect of (a) sheath liquid composition, (b) sheath liquid organic modifier and (c) sheath liquid flow rate upon S/N ratio. Conditions: BGE: ammonium acetate 40 mM, at pH 9.0; analyte and I.S. concentrations 0.3 mg/mL. Drying gas flow rate 6 L/min and temperature 200 ºC, nebulizer gas pressure 20 psi [24].

The spray chamber parameters, which are the last parameters examined in this study, have an important effect on the response of the MS system. One of these parameters involves the drying gas, which is used for accelerating the buffer desolvation, increasing the MS sensitivity, and eliminating any undesirable ions from entering into the MS system. It was observed that the drying gas flow rate has an effect on the stability of the electrospray, and consequently, the levels of the noise. The flow rate was set at 6 L/min, because at this flow rate an increased number of ions come closer to the liquid-gas interface, and this increases the desolvation velocity [37] (Figure 6a). In addition, other flow rates that were examined in this study either caused an unstable electrospray or lowered the S/N. The drying gas temperature was varied from 150 °C to 350 °C, and the highest S/N was obtained at 200 °C, which was considered as the optimum (Figure 6b). The nebulizer gas pressure was the last parameter examined in this category, and based on the stability of the electrospray and the S/N, 20 psi was selected as the optimum. At 20 psi, the electrospray is more efficient, probably due to an improved ion evaporation process because smaller initial droplets are obtained with higher nebulizer gas pressure (Figure 6c).

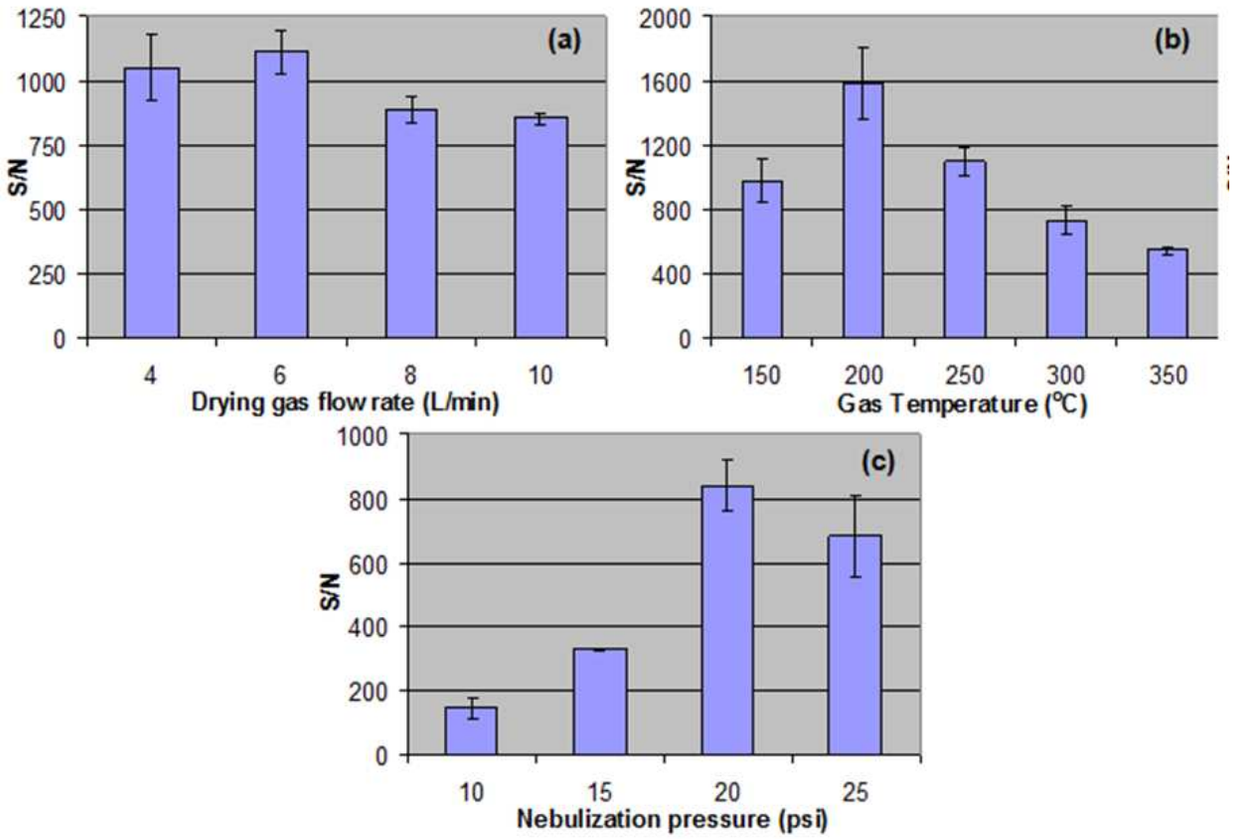

Figure 6. Effect of (a) drying gas flow rate, (b) drying gas temperature and (c) nebulizer gas pressure upon S/N ratio. Conditions: BGE: ammonium acetate 40 mM at pH 9.0, sheath liquid 1 % acetic acid in water:MeOH (50:50 v/v) at a flow rate of 10 μL/min, analyte and I.S. concentrations 0.3 mg/mL [24].

3.2. Method validation

All the parameters mentioned above are the common parameters that need to be examined in a method development process that involves a CE-MS system. These parameters affect the analysis time, resolution, response of the analyte under study, noise level, and sensitivity of the system. All these are important if the developed method is expected to be applied to biological samples for the detection and the quantification of drug and other compounds.

When the optimum conditions for the analysis of rivastigmine were determined, the method was validated in terms of linearity, precision, stability, recovery, LOD and LOQ. Two calibration curves were constructed, in human plasma and in standard solutions, and linearity was good in both cases. The precision, which was evaluated based on migration times and peak areas, was excellent, and particularly in the case where the peak area of the internal standard was also taken into consideration. The LOD and the LOQ were determined based on the standard deviation of the peak area and the slope of the calibration curve. The LOD and the LOQ were calculated as 3 and 10 times the above correlation, respectively. In the plasma sample, the LOD and the LOQ were found to be 2.8 ng/mL and 8.4 ng/mL, respectively, while in standard solutions they were 1.6 ng/mL and 5.0 ng/mL, respectively. These values are considered satisfactory for the accurate and precise quantification of rivastigmine in AD patients treated with the particular drug compound, and this is based on clinical studies that were performed in such patients [38,39].

3.3. Application

Biological matrices are among the most difficult samples to analyze because of the big number of components they contain that they may be adsorbed onto the capillary wall or interfere in the detection and/or separation process. Therefore, before plasma analysis, it is important and necessary to perform a sample preparation procedure. In addition to this, the concentration of most of the analytes in biological samples is low; so, a preconcentration step before the detection and quantitation is required. In many cases, different sample pre-treatment methods are used and compared in order to determine the most effective one, in regard to analyte recovery, difficulty, time and reproducibility. In this study, one LLE and two different SPE procedures were examined. In the case of SPE, two different SPE cartridges were used, a C18 cartridge and an Oasis HLB cartridge. LLE proved to be inefficient for rivastigmine assay, and it was time consuming because the extraction step was followed by additional steps that involved evaporation and reconstitution of the residue in an organic solvent. When the two SPE methods were compared, the C18-SPE cartridge proved to be the optimum, because the S/N was three times higher (S/N=154) than when Oasis HLB cartridge was used (S/N=52), and it provided better recoveries.

The optimum CZE-ESI-MS parameters and the optimum sample preparation procedure were finally applied for the determination of rivastigmine in a plasma sample obtained from an AD patient following rivastigmine patch administration (dose of 9.5 mg/mL rivastigmine/24-h). Figure 7 demonstrates the SIM electropherograms of C18-SPE extract of

plasma sample collected 2.0 hours post-application, at m/z 223 and 251, for I.S. and rivastigmine, respectively. The mean (± S.D.) plasma concentration obtained for rivastigmine was 14.6 (± 1.7) ng/mL.

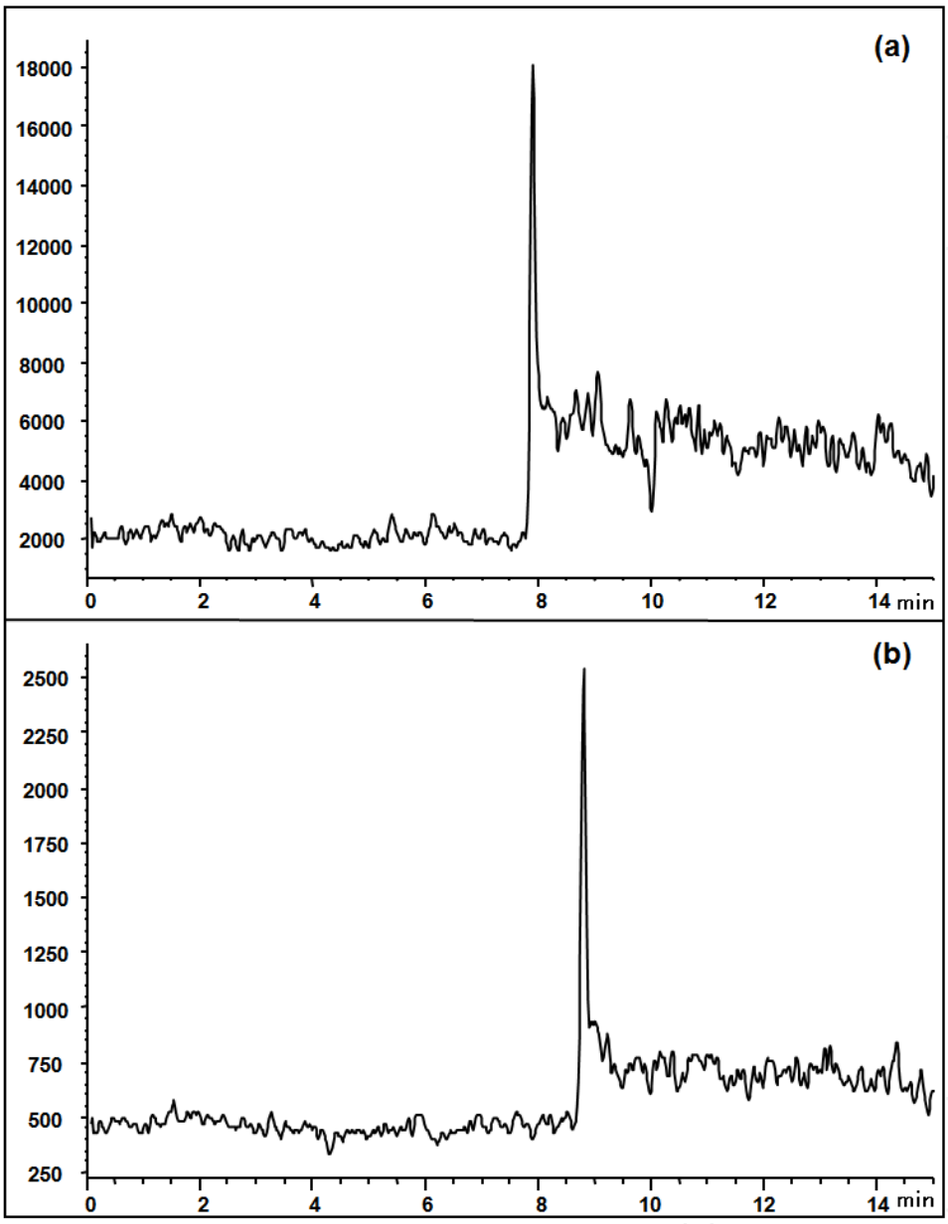

Figure 7. Electropherograms of C18-SPE extracts of plasma from an AD patient following rivastigmine patch administration in a dose of 9.5 mg/mL / 24-h in the SIM-mode at (a) m/z 223 (I.S.) and (b) m/z 251 (rivastigmine). Conditions: BGE: 40 mM ammonium acetate at pH 9, sheath liquid 1 % acetic acid in water:MeOH (50:50 v/v) at a flow rate of 10 μL/min, analyte and I.S. concentrations 0.3 mg/mL. Drying gas flow rate 6 L/min and temperature 200 ºC, nebulizer gas pressure 20 psi [24].

Based on the studies mentioned above, the CZE-ESI-MS method proved to be a promising technique in drug and pharmaceutical analysis. The development of such a method has several advantages over HPLC-MS and GC-MS. The most important ones are the reduction of the reagents cost, the low injection volume requirements, and the avoidance of disposing

large volumes of organic waste. In particular, when the study described here is compared to previous studies, where HPLC-MS and GC-MS were used for the analysis of rivastigmine, the required injection volume of plasma for a single analysis is reduced from microliters [40-43] to nanoliters.

4. Determination of enantiomers in pharmaceutical samples

In the last three decades, there has been a growing interest in the separation, detection and quantification of enantiomers in pharmaceutical, clinical, environmental and food analysis. It has been known that usually only one enantiomer is active while the other may be less active, inactive or has adverse effects. Among the separation techniques, HPLC [44-48] GC [44,45,49] and CE [50-55] are most often applied in chiral analysis. Temperature and derivatization are major problems encountered in GC, and poor separation efficiency is observed in HPLC. CE has proven to be a powerful separation technique in the area of chiral analysis, since it has the major advantage of low consumption of samples and solvents.

The most common modes of chiral CE are electrokinetic chromatography (EKC) in the presence of a chiral selector, MEKC, capillary electrochromatography (CEC), where the chiral selector can be either used as a coating (OT-CEC), a packing (P-CEC) or a monolithic material (M-CEC) in the capillary, and others [55-63]. The prerequisite for separation of enantiomers in CE, as in every chromatographic system, is the formation of either stable diastereoisomers by the use of a chiral derivatization agent or reversible diastereoisomeric complexes with the addition of a chiral substance, (chiral selector). In the first case, the two enantiomers are separated based on their different physicochemical properties, while in the second case, they are separated based on their different mobilities. In general, the "three point rule," illustrated by Easson and Stedman [64], describes the interactions that are necessary for chiral discrimination. A minimum of three simultaneous interactions have to occur between the chiral selector and one of the enantiomers so that chiral separation is achieved. The other enantiomer, due to spatial restrictions, should have at least two types of interactions, which can be hydrophobic interactions between the hydrophobic core of the polymer and the analyte, electrostatic interactions between the polar head group of the polymer and the analyte, dipole-dipole forces, such as hydrogen bonding between the polar group of the chiral selector and the analyte, and secondary interactions, such as π-π interactions, ion-dipole bonds, and Van der Waals forces. This difference in the number and type of interactions between the enantiomers and the chiral selector generates a mobility difference between the enantiomer-chiral selector complexes, which is necessary for the achievement of a chiral separation.

A big number of chiral selectors have been widely used, over the years, in CE for improved chiral separations of various classes of analytes. These chiral selectors include cyclodextrins, polymeric surfactants, cyclofructans, macrocyclic antibiotics, crown ethers, and others. Cyclodextrins are molecules with large ring-like structures composed of α-(1,4)-linked D-(+)-glucopyranose units. Native cyclodextrins are cyclic oligosaccharides consisting of six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units. The chiral recognition ability of cyclodextrins can be improved by their derivatization with different functional groups, such

as methyl-, sulfate-, acetyl- and prolyl-, and with the modification of the hydroxyl groups, which are present on the rim of the CD. The mechanism of enantiomeric discrimination is the inclusion of the hydrophobic group of the analyte into the cavity and interactions of the hydroxyl groups of the C2 and C3 at the upper rim of the CD, such as hydrogen bonds and dipole-dipole interactions.

Navarro *et al.* [65] developed a CZE method for the analysis of lansoprazole enantiomers in three different pharmaceutical preparations (Davur, Alter and Cinfa). β-CD was used as a chiral selector and sodium sulphite was used as an additive. Recoveries of 91-102% of the label content were obtained, and this demonstrated the potential of the method for the routine quality control of lansoprazole enantiomers in pharmaceutical formulations.

Chai *et al.* [66] used the chiral selector hydroxypropyl-γ-cyclodextrin in order to separate the antifungal drug iodiconazole and the structurally related triadimenol analogues. This chiral selector provided the best results in regard to resolution due to its large cavity and the hydrogen bonding between the analytes and the cyclodextrin. The mechanism for the chiral discrimination of hydroxypropyl cyclodextrins possibly involves the development of secondary interactions between the chiral analyte and the hydroxypropyl groups on the cyclodextrin rim after the inclusion of the analyte into the cavity. The degree of substitution and the type of the hydroxyalkyl group on the cyclodextrin rim, which influences the depth of the cavity, can therefore change the enantiorecognition ability of the cyclodextrin [67,68].

4.1. Method development and validation

The use of CE, and particularly MEKC, in chiral analysis is demonstrated further here by providing a more in-depth analysis of a research work that was performed in a pharmaceutical formulation that contained one of the enantiomers of Huperzine A [55]. Huperzine A is considered to be a potent, highly specific and reversible inhibitor of acetylcholinesterase with high efficiency and low toxicity. The mechanism of complexation of Huperzine A with acetylcholinesterase is similar to that of other pharmaceutical drugs that are used for the treatment of AD [69]. The (-)-enantiomer of Huperzine A is three times more biologically active than the synthetically racemic mixture, and only this form behaves as a potential acetylcholinesterase inhibitor. Therefore, the development of an analytical method for the enantiomeric separation of the synthetic Huperzine A is of greatest importance.

It is important here to mention that the type of the chiral selector used in this study was the polymeric surfactant. The use of polymeric surfactants in both chiral and achiral CE has attracted considerable attention. In 1994, Wang and Warner [70] were the first to report the use of a polymeric surfactant added to the BGE in MEKC. Polymeric surfactants offer several distinct advantages over conventional micelles [63,71-73]. Firstly, polymerization of the surfactant eliminates the dynamic equilibrium due to the formation of covalent bonds between the surfactant aggregates. This, in turn, enhances stability and improves resolution. Secondly, polymeric surfactants can be used at low concentrations because they do not depend on the CMC. This usually provides higher efficiencies and rapid analysis. They have, over the years, been extensively used in a BGE [74-80], in a polyelectrolyte multilayer coating [63,74,81-83], and in a CE-MS system [84-86].

In this study, the optimal conditions, in regard to resolution, efficiency and analysis time, were initially established by varying different electrophoretic parameters. The BGE type, concentration and pH are usually the first parameters to be examined in a method development procedure. Sodium acetate at acidic and neutral pHs, where the analyte exhibits cationic behavior, was chosen as the optimum. BGEs with basic pHs did not exhibit any enantiomeric discrimination, and the analysis time was very long. The optimum pH was 5.0 because it provided slightly better peak shapes, and the optimum concentration was 50 mM because it provided higher resolution (Figure 8). The very low peak efficiency, which needs to be improved, is clearly illustrated in this figure.

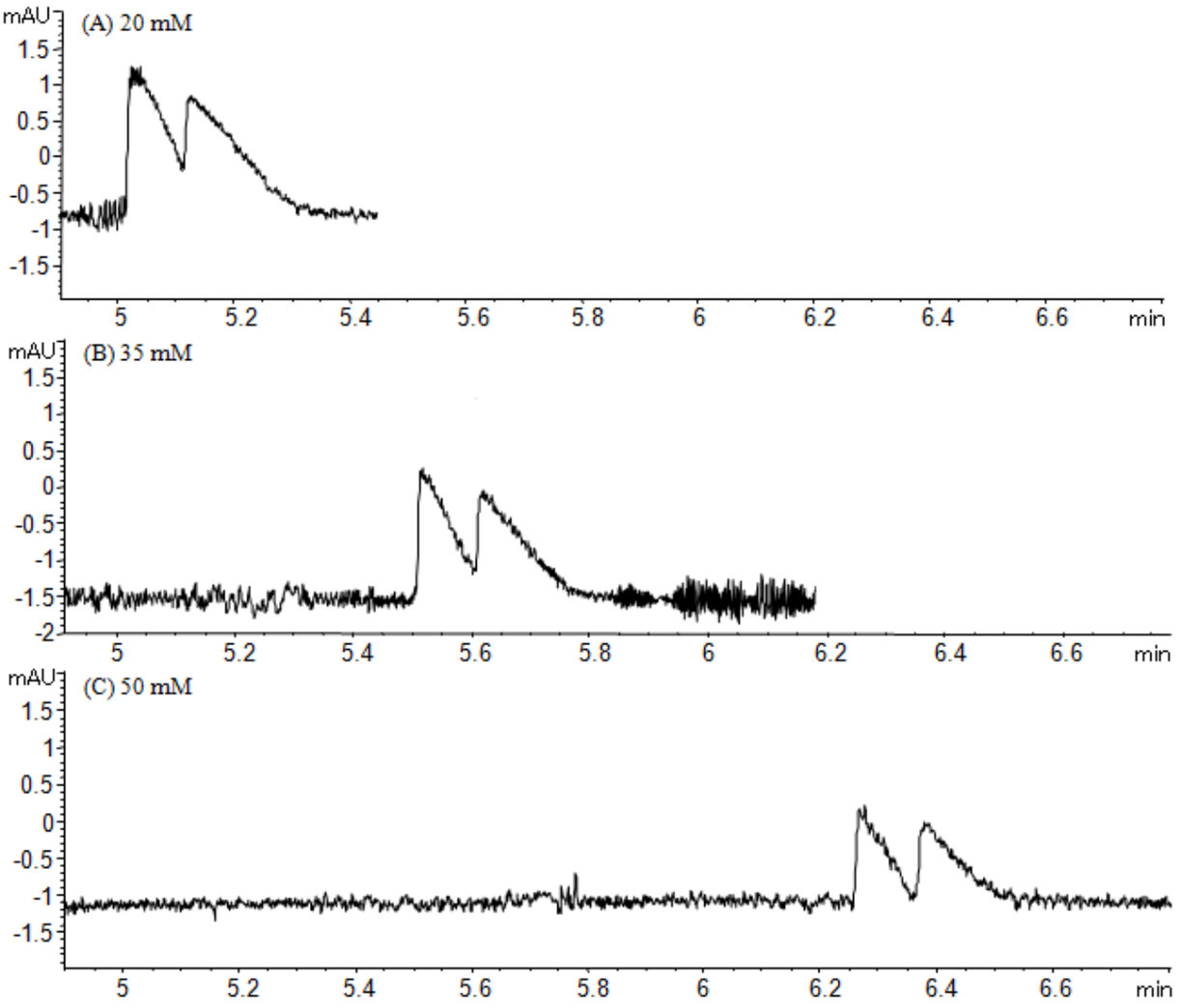

Figure 8. Effect of BGE concentration on the separation of the enantiomers of Huperzine A: (A) 20 mM, (B) 35 mM and (C) 50 mM. Separation conditions: BGE: sodium acetate (pH 5.0), 0.075% (w/v) poly-LL-SULV; pressure injection, 30 mbar for 3 s; applied voltage, 20 kV; temperature, 25 °C; fused-silica capillary, 64 cm (55.5 cm effective length) x 50 μm i.d.; detection, 230 nm [55].

As far as the chiral selector is concerned, different polymeric surfactants were examined, such as poly(sodium *N*-undecanoyl-L-leucinate) (poly-L-SUL), poly(sodium *N*-undecanoyl-LL-leucyl-leucinate) (poly-LL-SULL), poly(sodium *N*-undecanoyl-LL-leucyl-valinate) (poly-LL-SULV), poly(sodium *N*-undecanoyl-L-valinate) (poly-L-SUV), poly(sodium *N*-undecanoyl-L-valyl-glycinate) (poly-L-SUVG), poly(sodium *N*-undecanoyl-LL-alanyl-valinate) (poly-LL-SUAV), poly(sodium *N*-undecanoyl-LL-leucyl-alanate) (poly-LL-SULA), and poly(sodium *N*-undecanoyl-LL-valyl-valinate) (poly-LL-SUVV). The polymeric surfacant poly-LL-SULV, which has shown the best chiral discrimination ability for a number of pharmaceutical compounds [80], was the first to be examined in different concentrations. The concentration of 0.075% w/v was chosen as the optimum, based on analysis time, efficiency and resolution. This concentration though did not provide baseline resolution.

Another parameter examined in order to improve peak efficiency and resolution was the addition of modifiers. None of the organic solvents at different concentrations were able to improve the separation. An alternative to this was the addition of a salt, such as D- and L-alanine tert-butyl ester hydrochloride (D- and L-AlaC$_4$Cl). poly-LL-SULV became insoluble when the salt was added into the BGE. Therefore, the other polymeric surfactants mentioned above were examined at different concentrations. In each case, D- and L-AlaC$_4$Cl were used individually as additives, the electropherograms were obtained, and resolution and efficiency were estimated. Based on this, the combination of poly-LL-SUAV at a concentration of 0.20% (w/v) with L-AlaC$_4$Cl provided the best results.

However, the use of L-AlaC$_4$Cl did not provide satisfactory reproducibility of the migration time and efficiency. This is probably due to the hydrolysis of the salt in an aqueous BGE solution. An alternative involved the use of tert-butanol, one of the hydrolysis products, at different concentrations. Figure 9 clearly demonstrates the improved peak efficiency, in comparison with Figure 8. Each electropherogram was obtained at a different concentration of tert-butanol. A concentration of 10% (v/v) was the optimum, because it provided the highest resolution (1.45) and the highest peak efficiency (Figure 10).

The validation of the method demonstrated good linearities and very low relative standard deviation values, indicating excellent run-to-run and day-to-day reproducibilities. In addition, the LOD and LOQ were determined to be 4.17 μg/mL and 13.92 μg/mL, respectively.

4.2. Application

As previously shown, after method development and validation, the optimum separation conditions are applied to a real sample. In this case, the optimum parameters were applied to a pharmaceutical formulation in order to detect and quantitate the acetylcholinesterase inhibitor (-)-Huperzine A. The extraction procedure followed for extracting Huperzine A from the pharmaceutical formulation proved to be effective because the enantiomer determined in the sample was in a relatively good agreement with the amount that was stated on the bottle. Therefore, the developed MEKC-UV method is able to control the purity of (-)-Huperzine A in pharmaceutical formulations.

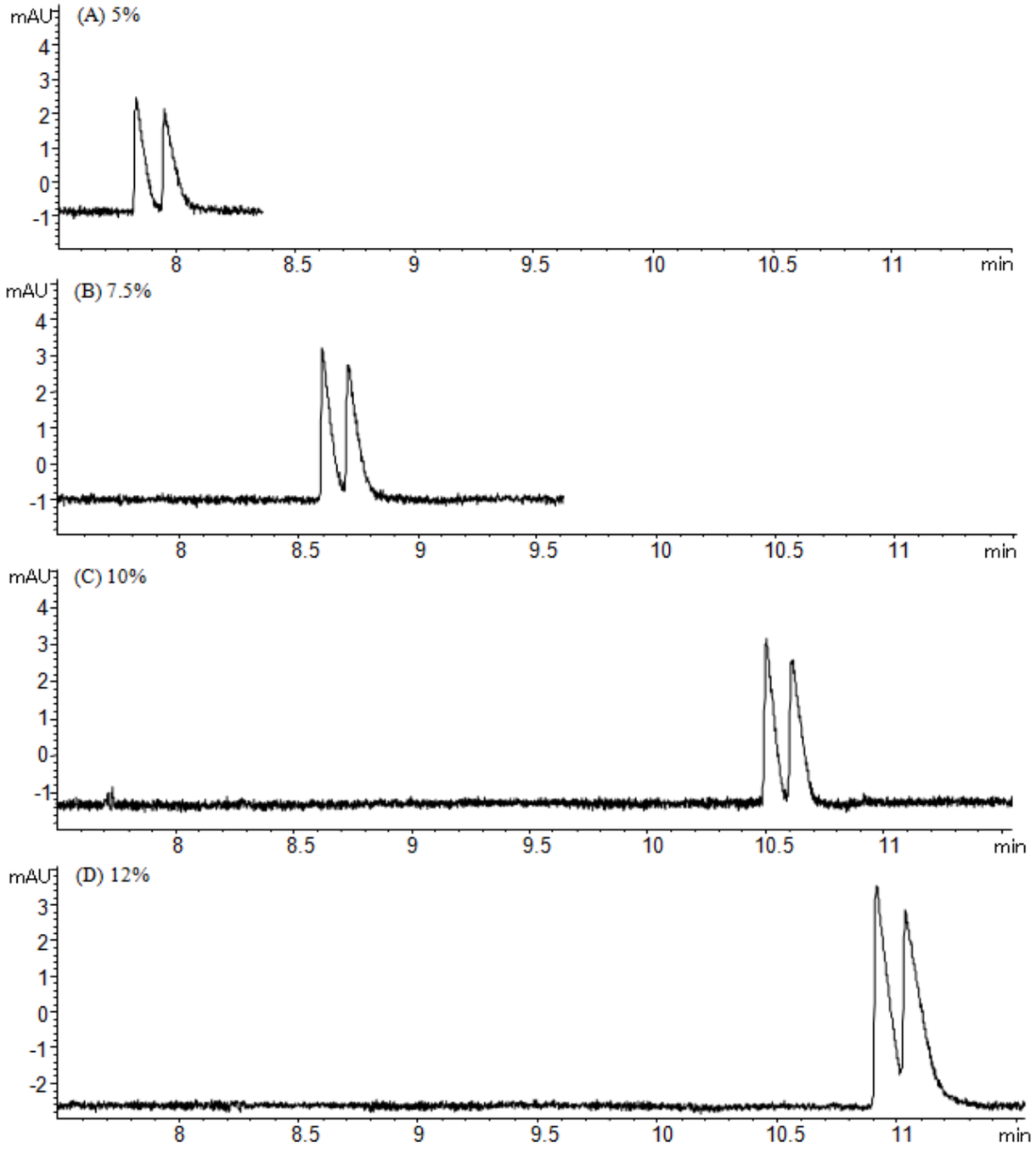

Figure 9. Effect of the concentration of tert-butanol on the separation of the enantiomers of Huperzine A: (A) 5%, (B) 7.5%, (C) 10% and (D) 12% (v/v). Separation conditions: BGE: 50 mM sodium acetate (pH 5.0), 0.2% (w/v) poly-LL-SUAV; pressure injection, 30 mbar for 3 s; applied voltage, 20 kV; temperature, 25 °C; fused-silica capillary, 64 cm (55.5 cm effective length) x 50 μm i.d.; detection, 230 nm [55].

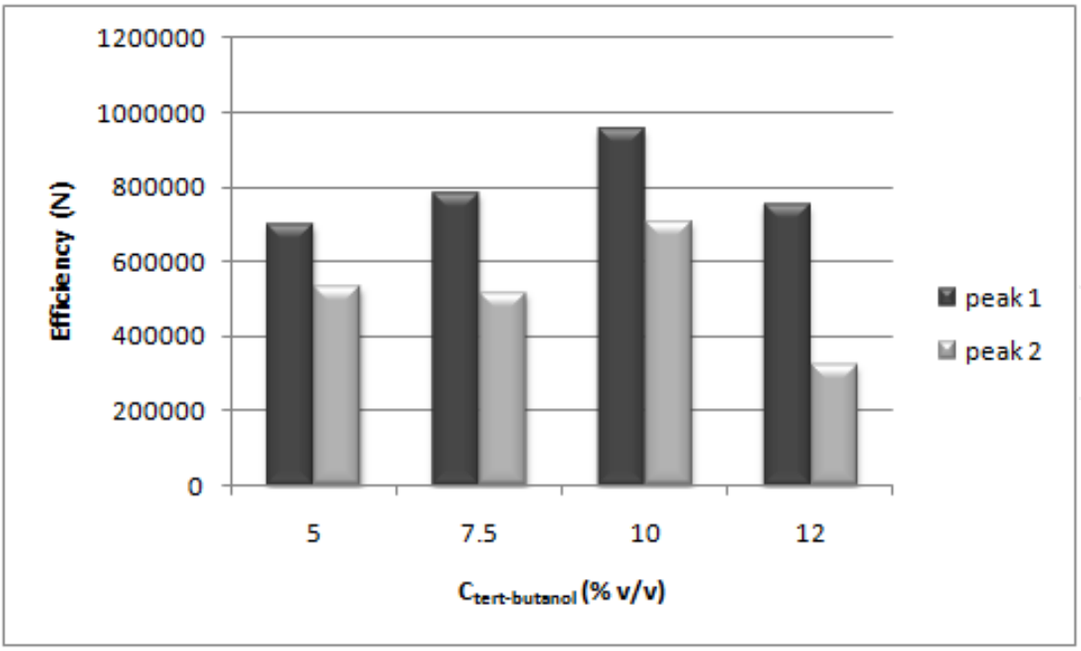

Figure 10. Effect of the concentration of tert-butanol on the efficiency. Separation conditions: Same as Fig. 4. BGE: 50 mM sodium acetate (pH 5.0), 0.2% (w/v) poly-LL-SUAV; pressure injection, 30 mbar for 3 s; applied voltage, 20 kV; temperature, 25 °C; fused-silica capillary, 64 cm (55.5 cm effective length) x 50 μm i.d.; detection, 230 nm [55].

5. Concluding remarks

Analysis of chiral and achiral analytes in natural, pharmaceutical and biological samples can be extremely difficult. Co-migration may occur, which can cause problems in detection, and the electropherograms obtained can be very complex. In addition, the analytes of interest are usually present in the matrices at very low concentrations. Therefore, all the analytical steps, including method development, detection and sample preparation, which is an essential stage in any analysis process, have to be optimized in order to obtain the desirable sensitivity, resolution, robustness and analysis time.

Among the separation techniques that have so far been used for pharmaceutical, clinical and food analysis, CE has been established as a powerful analytical tool, which has rapidly been developed and matured since its introduction. CE and its related techniques offer a number of advantages, including low consumption of sample and solvents, high separation efficiency, rapid method development, fast migration times, versatility, and simple instrumentation. Another important aspect involves its ability to separate small and large molecules, charged and neutral species, inorganic and organic molecules, synthetic and natural compounds, along with proteins and peptides.

The coupling of CE to MS provides nowadays a promising alternative to UV detection. The combination of high sensitivity, high selectivity, and high specificity provided by MS with high resolution, and high efficiency provided by CE makes it an attractive technique in different fields, such as clinical, forensic, pharmaceutical, and others. However, chiral analysis by use of CE-MS still needs some improvement, in regard to resolution and peak

capacity. In addition, contamination of the ionization source induced by the chiral selector added in the BGE is still considered a main problem, even though different procedures have, in recent years, been developed in order to overcome this limitation [87].

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