APPLICATIONS OF SYNTHETIC MONOLITHIC MATERIAL IN CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTROCHROMATOGRAPHY

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entitled

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APPLICATIONS OF SYNTHETIC MONOLITHIC MATERIAL IN CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTROCHROMATOGRAPHY

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ABSTRACT

Monolithic material is one of the newest chromatographic supports for chromatography. The advantage of monolith is its high porosity resulting in low back pressure. Monoliths have been widely used for both separation and sample pre-concentration/extraction purposes. Two investigations of monoliths were carried out in this thesis. The first was the use of monolith as an in-line solid phase extraction for capillary electrophoresis (CE). The second part was an application of monolith for separation of vitamin E in forms of tocopherols and tocopherol acetate by capillary electrochromatography (CEC).

In the first part, two monoliths were examined as in-line pre-concentrator column or solid phase extraction (SPE). Performance test of the monolithic SPEs was carried out using three model compounds, terbutaline, 4-hydroxy-3-methoxy-methamphetamine and benzyl alcohol. Irreproducible results were observed when passing pure aqueous medium through the monolithic SPEs. Better repeatability was observed when the solutions contained at least 10 % (v/v) organic solvent such as acetonitrile or methanol. However coupling the SPE with non-aqueous CE mode still could not give good repeatability. In-line coupling of a short packed silica based C18 SPE with CE was also developed. Very good repeatability with a large improvement in sensitivity for this material was demonstrated.

In the second part, a pentaerythritol diacrylate monostearate with ethylene dimethacrylate monolith (PEDAS-EDMA), was investigated in order to separate the tocopherol isomers, α-, β-, γ-, and δ-tocopherols, and α-tocopherol acetate as well as the antioxidant butylated hydroxytoluene, by CEC. Retention studies indicated a reversed-phase mechanism was involved in the separation on the PEDAS-EDMA column, but polar interactions with the underlying ester and hydroxyl groups enhanced the separation of the problematic β- and γ-isomers. The column showed better selectivity compared to that for conventional packed C18 column. An adequate separation was obtained in 25 min by using 3:10:87 (v/v/v) 100 mM Tris buffer pH 9.3 : methanol : acetonitrile as the mobile phase. The developed method was successfully applied to pharmaceutical samples with good agreement with the labelled value and good repeatability with % recovery between 93 and 99.

KEY WORDS: MONOLITH / CAPILLARY ELECTROPHORESIS / CAPILLARY ELECTROCHROMATOGRAPHY / PRE-CONCENTRATION / VITAMIN E

135 pp.

การประยุกต์วัสดุสังเคราะห์ที่มีโครงสร้างพรุนแบบต่อเนื่องในการวิเคราะห์ด้วยเทคนิคแคปปิลารีอิเล็กโทรโฟรีซิสและแคปปิลารีอิเล็กโทรโครมาโทกราฟี (APPLICATIONS OF SYNTHETIC MONOLITHIC MATERIAL IN CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTROCHROMATOGRAPHY)

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บทคัดย่อ

โมโนลิธเป็นวัสดุสังเคราะห์ที่มีโครงพรุนแบบต่อเนื่องที่กำลังเป็นที่นิยมสำหรับงานทางด้านโครมาโทกราฟีเนื่องจากมีความต้านทานการไหลต่ำ ในงานวิจัยนี้ได้ทำการศึกษาการใช้โมโนลิธสำหรับการทำให้สารเข้มข้นขึ้นก่อนทำการแยกด้วยเทคนิคแคปปิลารีอิเล็กโทรโฟรีซิส ในส่วนที่สองของงานวิจัยเป็นการประยุกต์โมโนลิธสำหรับการวิเคราะห์วิตามินอีโดยเทคนิคแคปปิลารีอิเล็กโทรโครมาโทกราฟี

ในงานส่วนแรกได้ศึกษาการใช้โมโนลิธสองชนิดสำหรับการทำให้สารเข้มข้นขึ้นเพื่อเพิ่มความไวในการแยกด้วยเทคนิคแคปปิลารีอิเล็กโทรโฟรีซิส โดยประยุกต์ใช้สำหรับสารเทอร์บูทาลีน (TER), 4-ไฮดรอกซี-3-เมทอกซี-เมทแอมเฟทตามีน (HMMA) และเบนซิลแอลกอฮอล พบว่าการเชื่อมต่อโมโนลิธกับเทคนิคแคปปิลารีอิเล็กโทรโฟรีซิสเพื่อทำให้สารเข้มข้นขึ้นแบบอินไลน์ให้ผลที่ไม่สามารถทำซ้ำได้ ได้ทำการศึกษาการใช้วัสดุแบบดั้งเดิมที่มีลักษณะเป็นเม็ด พบว่าวิธีมีความสามารถในการทำซ้ำสูง และสามารถเพิ่มความไวของการวิเคราะห์สารทั้งสามได้อย่างมาก มี pre-concentration factor สำหรับ HMMA และ TER เป็น 1000 และ 333 ตามลำดับ

ในส่วนที่สองได้ศึกษาการใช้ เพนตะอิริธริทอล ไดอะไครเลต โมโนสเตียเรต โมโนลิธิคคอลัมน์สำหรับแยกวิตามินอีในรูปของโทโคเฟอร์รอล ไอโซเมอร์ (แอลฟา, เบต้า, แกมม่า, และเดลต้า) และแอลฟา-โทโคเฟอร์รอล อะซีเตรต รวมทั้งบิวทิลเลเตตไฮดรอกซีโทลูอีน (ป้องกันการสลายตัวของวิตามินอี) โดยเทคนิคแคปปิลารีอิเล็กโทรโครมาโทกราฟี พบว่ากลไกการแยกหลักเป็นแบบรีเวิร์สเฟสและมีอันตรกิริยาระหว่างสารกับหมู่ที่มีขั้วบนโมโนลิธเกิดขึ้นทำให้การแยกเกิดขึ้นได้ดีกว่าการใช้ C18 คอลัมน์แบบเก่า การแยกใช้เวลา 25 นาทีโดยใช้วัฏภาคเคลื่อนที่ที่เป็นของผสมระหว่าง 100 มิลลิโมลาร์ ทริสบัปเฟอร์ pH 9.3, เมทานอล และอะซีโทรไนไตร์ล อัตราส่วน 3:10:87 โดยปริมาตร วิธีที่พัฒนาขึ้นได้ถูกนำไปใช้ในการวิเคราะห์ตัวอย่างวิตามินอีเสริมและให้ผลที่ใกล้เคียงกับค่าที่ระบุบนฉลาก โดยมีเปอร์เซ็นต์การกู้คืนอยู่ระหว่าง 93 และ 99 เปอร์เซ็นต์

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LIST OF ABBREVIATIONS

SEM	Scanning electron microscopy
SEC	Size exclusion chromatography
BET	Brunauer-Emmett-Teller method
K	Permeability
HPLC	High performance liquid chromatography
CEC	Capillary electrochromatography
CE	Capillary electrophoresis
LC	Liquid chromatography
EOF	Electroosmotic flow
UV	Ultra-violet
SPE	Solid phase extraction
OT	Open-tubular column
EDMA	Ethylene dimethacrylate
AIBN	Azobisisobutyronitrile
TMOS	Tetramethoxysilane
TEOS	Tetraethoxysilane
PEO	Poly(ethylene oxide)
γ-MAPS	3-(Trimethoxysilyl) propyl methacrylate
PEEK	Polyether ether ketone
PTFE	Poly tetra fluoroethene
MAA	Methacrylic acid
TOH	Tocopherol
PEPS	Pentafluorophenylsilica
ULTIMA C18	Polar embedded C18 stationary phase
PEDAS	Pentaerythritol diacrylate monostearate
BHT	Butylated hydroxytoluene

LIST OF ABBREVIATIONS (CONT.)

TAc	Tocopherol acetate
Bz,	Benzyl alcohol
HMMA	4-Hydroxy-3-methoxy-methamphetamine
TER	Terbutaline
BGE	Background electrolyte
MeOH	Methanol
ACN	Acetonitrile
BMA	Butyl methacrylate
mm	Milli-meter
μm	Micro-meter
nm	Nano-meter
L	Liter
% (v/v)	Percent volume by volume
%(w/w)	Percent weight by weight
M	Molar
mM	Mili-molar
hr	Hour
^{o} C	Degree celsius
kV	Kilo-volt
OD	Outer diameter
ID	Inner diametr
Min	Minute
Ppm	Part per million
Ppb	Part per billion
RSD	Relative standard deviation
LOD	Limit of detection

THE RELEVANCY OF THE RESEARCH WORK TO THAILAND

Chromatography is a powerful separation technique, and is extensively used in many fields for analysis of various compounds in complex or high matrix samples. Basically, the technique consists of stationary and mobile phases. The stationary phase is one of the most important keys for method development. In conventional method, stationary phase is usually packed-based particulates which raise a limitation in high back pressure due to high flow resistance of the small packing stationary phase particles.

Recently, an innovative chromatographic stationary phase, monolithic material has been shown advantages over the conventional packed particle stationary phase in terms of low back pressure with equivalent efficiency, easy to prepare and provides wider choices for stationary phase selection.

In order to explore knowledge and experience for this interesting material, two applications of monoliths were investigated in this work. The applications were for sample pre-concentration for capillary electrophoresis and separation of vitamin E by capillary electrochromatography. The preparations of monolithic materials are simple and can be readily *in-situ* prepared in a capillary without need of advance apparatus. The technology in synthesis of monolithic materials would be useful in the future for Thailand, since the monolithic technology can save cost of imported chromatographic columns. With this technology, one can synthesize the column to suit his/her own purposes without having to import chromatographic columns into the country. Also the developed technology in making monolithic column can be transferred to Thai manufacturer in the future for export the good.

PUBLICATIONS

1. **Chaisuwan P, Nacapricha D, Wilairat P, Jiang Z, Smith NW. Separation of α-, β-, γ-, δ-tocopherols and α-tocopherol acetate on a pentaerythritol diacrylate monostearate - ethylene dimethacrylate monolith by capillary electrochromatography. Electrophoresis (accepted) 2008.**

2. **Chaisuwan P, Nacapricha D, Wilairat P, Jiang Z, Smith NW. Monolithic and packed particle materials for in-line pre-concentration in capillary electrophoresis for 4-hydroxy-3-methoxy-methamphetamine and terbutaline. Electrophoresis (submitted, under revision) 2008.**

CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

In this chapter, four topics concerning the research work are introduced. The chapter consists of four parts. The first part is introduction to monolithic material. In this part, important parameters such as structure, properties, and preparation of monolith are explained. In the second and the third parts, two related separation techniques, capillary electrophoresis and capillary electrochromatography are described, respectively. The last part focuses on the importance and literature review for the analysis of vitamin E.

1.1 Monolithic materials

Monolithic material is the newest and innovative chromatographic stationary phase introduced in the 1970's by Kubin *et al.*[1]. The first monolith was prepared from open-pore polyurethane foams and applied to size exclusion chromatography. Although the monolith could be used to separate proteins, the permeability of the column was very low. Methods for preparation have been further developed by many research groups with the aim of increasing the porosity and efficiency [2-5]. In 1991, Nakanishi and Soga described a new approach for preparation of high permeable silica monolith [6]. However the method was still relatively difficult. After much research and improvement in the preparation method [7-9], monolithic material has been increasingly applied since 1997, as shown by the increasingly number of publications in monolithic research (Figure 1.1). Many reviews for monolith on both preparation and applications have been reported [10-15].

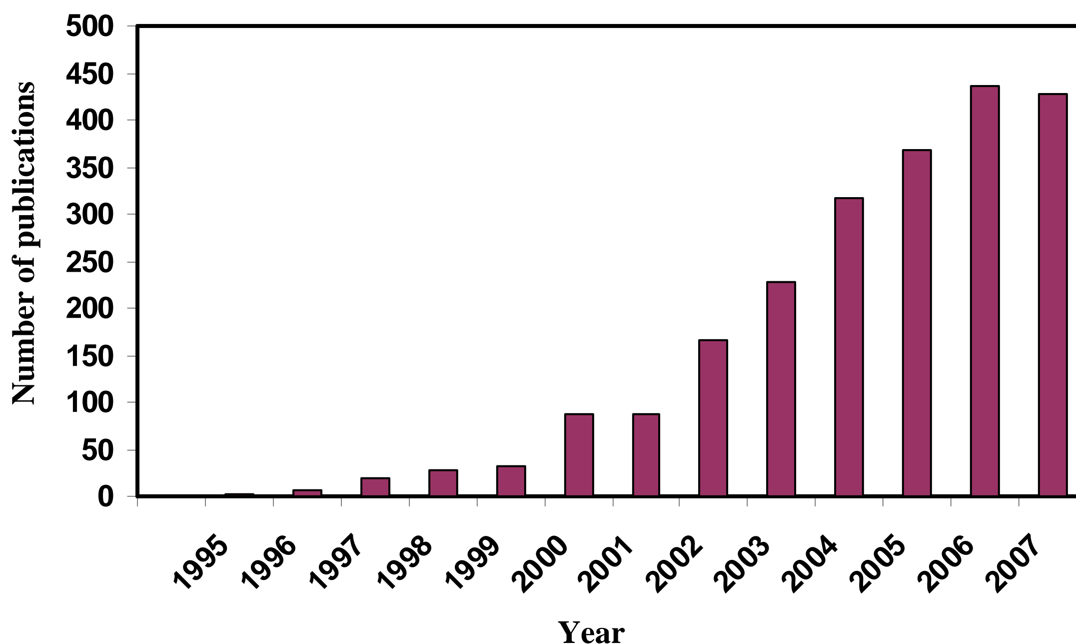
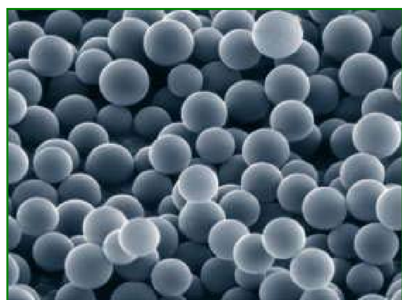


Figure 1.1 Publications for monolithic materials since 1995 to 2007.
[SciFinder Scholar Database, using keyword 'monolithic column']

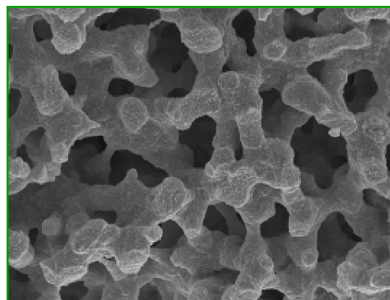
The material has been shown to have remarkable advantages over conventional packed particle phase or microparticulate column in terms of high porosity with high chromatographic efficiency [7, 16-18]. The higher porosity allows operating at higher flow rate in order to obtain rapid separation, high sample throughput and also more flexibility for coupling to other systems [19-20]. In addition, the monolith can be prepared *in situ* and strongly bonds with the glass surface of fused silica capillary and therefore no retaining frit is needed for this type of material.

1.1.1 Structure of monolithic material

In contrast to conventional particle packed column, monolithic column is a single structure with interconnected skeletons (see Figure 1.2). Its shape is sponge like [7]. The structure is a continuous skeleton with bimodal pores, the macropores and mesopores, as shown in Figure 1.3. The macropores provides a high flow rate due to low resistance. These pores determine the permeability properties of the column. The mesopores are located on the skeletons and provides the surface area for chromatographic separation.



Microparticulate stationary phase



Monolithic stationary phase

Figure 1.2. Scanning electron microscope picture of microparticulate and monolithic stationary phases [21].

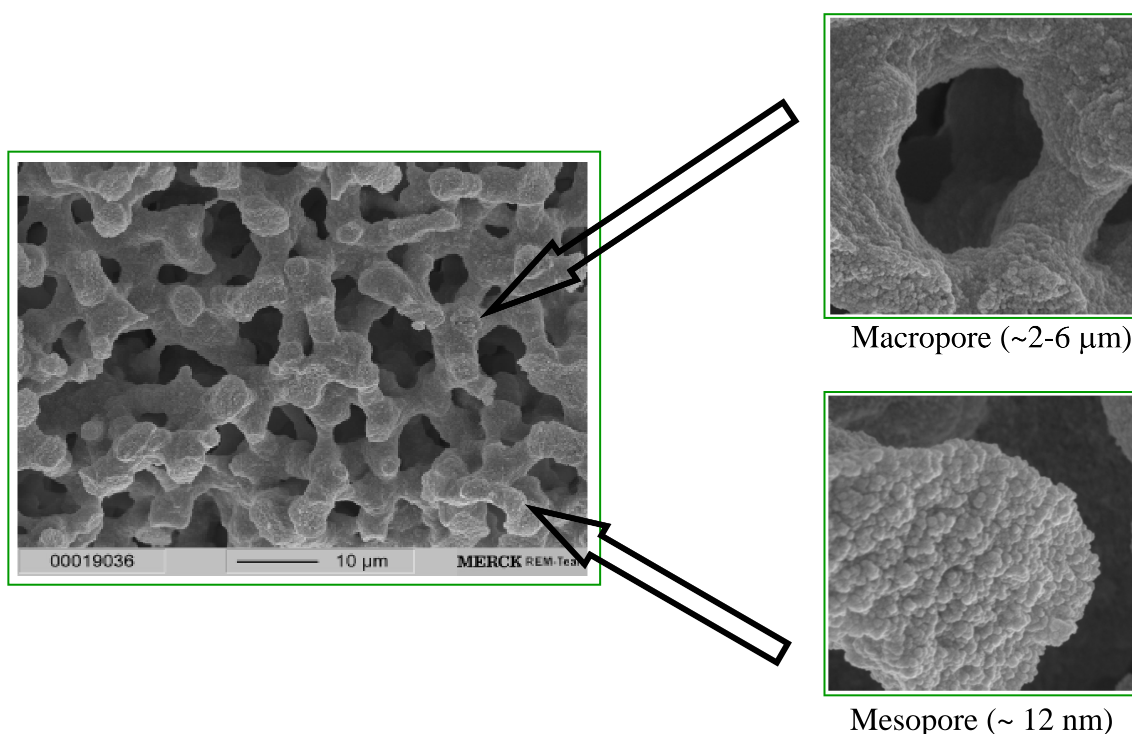


Figure 1.3. Scanning electron microscope picture of monolithic column [21].

1.1.2 Properties of Monolithic Material

Many research groups have investigated physical and chemical properties of monolithic material in terms of hydrodynamic porosity of the material, and

chromatographic properties such as selectivity and separation efficiency. These properties were commonly compared to that for conventional standard microparticulate column.

1.1.2.1 Physical properties

Several techniques have been used to physically characterize the morphology and structure of monolith such as scanning electron microscopy (SEM) [22-24], mercury porosimetry [25-26], size exclusion chromatography (SEC) [27-28] and N_2 adsorption [26].

1.1.2.1.1 Pore size, pore distribution and surface area of monolith

In order to investigate the morphology of monolithic material, SEM is usually used [22-24]. The technique provides data for skeleton and throughpores sizes. However homogeneity and pore distribution over the entire column can not be obtained from this technique unless longitudinal cut of the monolith are investigated.

Exact size and size distribution of the throughpores can be achieved by using mercury porosimetry technique [25-26]. Volume and size of both mesopores and throughpores can be determined. For determination of the throughpores, the monolith is wrapped with a mercury-tight membrane, and then the mercury pressure is increased to compress the monolith. The calculated reduction in the volume of the monolith from applying pressure is the volume of the throughpores. The mesopore volume can also be determined by linearly increasing the mercury pressure to the monolith without the membrane. Adsorption curve is used to determine the size and shape of the mesopores.

It is well known, that loading capacity, efficiency and selectivity are related to surface area of the chromatographic material. The surface area of the monoliths is determined by using N_2 adsorption technique based on

Brunauer-Emmett-Teller (BET) method [27]. N_2 gas is flowed into the monolithic column. By assuming that the adsorption is monolayer, the specific surface area of the monolith can therefore be calculated from the measured adsorbed volume of the N_2 gas and the size of N_2 molecule.

In addition to the N_2 adsorption technique, SEC can also use for determine mesoporosity of the monolith [27-28]. The method is based on size exclusion chromatography carried out by loading of a set of linear polystyrene standards with a molecular weight between 600 and 3.76106 Da (molecular radii of 2.7–453 nm, respectively). Size of the mesopores is achieved by comparing the result with standard material of known porous structure.

1.1.2.1.2 Hydrodynamic Property

Porosity of monolithic material is usually studied in terms of permeability (K) and column back pressure. The permeability (K) is calculated according to Knox equation [29] from the column back pressure observed from the experiment.

$$K = \mu\eta L/\Delta P \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where μ is the linear velocity of mobile phase, η is the mobile phase viscosity, L is the column length and ΔP is the back pressure.

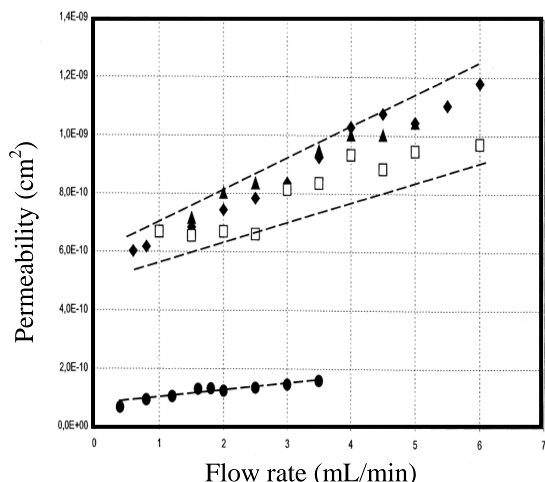


Figure 1.4. Permeability (K) as a function of the flow-rate F for three SilicaROD columns ((▲) Rod 178, (□) rod 216, (♦) rod 225), and the (•) Purospher RP 18e column [29].

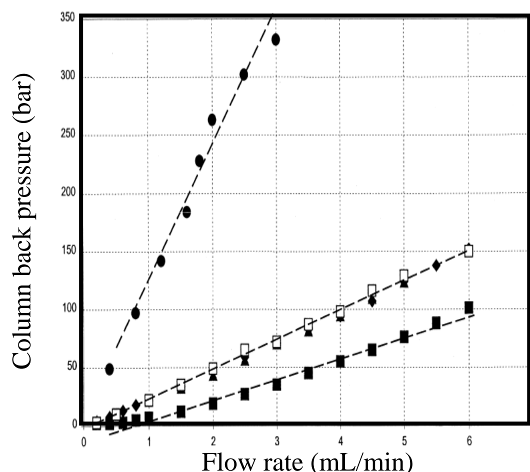


Figure 1.5. Column back pressure (D*p*) as a function of the flow-rate *F* on three monolithic and a microparticulate column. (■) Pressure drop due to equipment (without a column), ((▲) Rod 178, (□) rod 216, (♦) rod 225), and the (•) Purospher RP 18e column [29].

Figure 1.4 and Figure 1.5 are examples of hydrodynamic properties of monolithic and microparticulate columns [29]. Column back pressure and permeability obtained for the three representative monolithic columns (▲) Rod 178, (□) rod 216, (♦) rod 225), and (•) standard microparticulate columns (Purospher RP 18e column) were examined. It can be seen that at the studied flow rates, the monolithic columns provide significantly higher total column porosity (higher permeability or lower column back pressure) of about five times as compared to that for standard packed column is due to low flow resistance of the macropores in the monolithic structure.

1.1.2.2 Chromatographic properties of monolithic material

As shown in Figure 1.3 that monolithic column consists of mesopores and macropores structure. The macropores mainly control porosity of the column while the mesopores govern surface area, affecting the chromatographic performance of the column. Numerous works have shown that high porous monolithic materials have

equivalent chromatographic properties to that for standard microparticulate column in terms of selectivity, and column efficiency as shown examples below [7, 30, 32-34].

1.1.2.2.1 Selectivity

Cabrera *et al.* compared selectivity of a commercial silica monolith with 5 μm conventional HPLC packing column for separation of vitamin K under the same chromatographic condition [30]. The separation of the four vitamin K can be obtained with similar retention times as shown in Figure 1.6. The selectivity of the monolith was claimed to be similar to that for the common C18 reversed phase column [30].

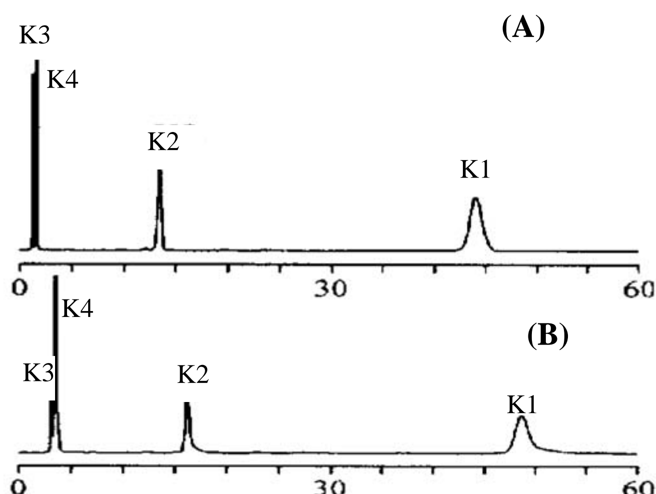


Figure 1.6 Chromatography of vitamin K (K1-4) on (A) LichroCART^{TM} LiChrosper^{TM} RP-18, 5 μm (125 x 4 mm) and (B) SilicaROD^{TM} RP-18 (83 x 7.2 mm). Chromatographic conditions : mobile phase, acetonitrile/water (95/5, v/v); flow rate 1 ml/min; detection, UV 280 nm; temperature, ambient [30].

1.1.2.3.2 Efficiency

Efficiency of monolithic columns in comparison with the efficiency of that for conventional particulate column have been intensively investigated using the van Deemter plot [38-40]. Figure 1.7 is an example of the comparison of the efficiency of two monolithic columns (C18 silica rods with 1.7 μm

through-pores) and three standard particulate columns (5 µm particles) using amylbenzene. The results demonstrate that the van Deemter plots of the monoliths were flatter with higher efficiency than that for the particulate columns. Similar results for efficiency studies of monolithic columns as compared to that for standard microparticulate columns have been also reported by other research groups [32-34]. It was claimed that the higher efficiency at high flow rate for the monolith was due to faster mass transfer of the compounds on the monolith [35-37]. This property allows the monolith to operate at a high flow rate to achieve high speed separations [7].

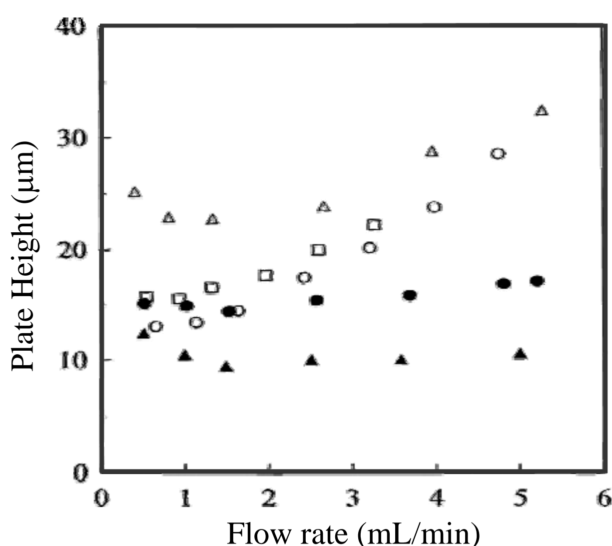


Figure 1.7 Van Deemter plots for silica C18 packed columns ((Δ-Capcellpak C18 SG (30 nm)), (□-Deltabond ODS (3o nm)), (○-Capcellpak C18 UG (12 nm)) and C18 silica monolithic rods ((•-SR-S-C18), (▲-SR-L-C18)) with amylbenzene as a solute. Mobile phase: 80% (v/v) methanol [7].

1.1.3 Preparation of Monolithic Column

Several parameters must be achieved for the monolithic chromatographic media such as material, surface chemistry, resistance to solvents, porosity, pore size distribution, and rigidity (mechanical property). These properties depend on the preparation method and polymer composition. Based on the preparation procedure and monomers used, monolithic material can be classified into inorganic and organic

polymers. The inorganic monolith is typically prepared from inorganic monomers by using sol-gel method while organic monolith is prepared from organic monomers by using *in situ* polymerization of monomers with cross-linkers and porogens [16].

In addition to differences in the preparation method of the organic and inorganic materials, the two types of materials have unique chromatographic features. The organic monolith have been found to be suitable for separation of larger molecules such as proteins, nucleic acids, and synthetic polymer while the inorganic monolithic material has been shown to give better separation efficiency for smaller molecules. Therefore, the two types of monolithic materials are complementary [16].

1.1.3.1 Organic monolith

Organic monolithic material is a polymer using organic monomers. It is sometime called 'polymer-based monolith' or 'organic polymer-based monolith'. These columns have been increasingly employed due to their wide working pH range, ease of preparation and modification with various available functional groups [16]. The preparation of organic monolith is achieved by *in situ* polymerisation of monomers and co-polymer and a suitable porogenic solvent. The synthesis of the polymer-based monoliths is usually carried out in only one step. Basically, the polymerisation solution consists of:

- Monomers
- Crosslinking monomer
- Porogenic solvent or porogen
- Initiator

1.1.3.1.1 Polymerization composition

Monomer

Surface chemistry and resistance to solvents of the monolith is mainly controlled by the chemistry of the monomers used in the preparation [38].

Many monomers have been reported for various surface chemistry or separation modes, such as reversed-phased [39-41], ion-exchange [42-43], affinity chromatography [44-45], molecular imprinting [46-48], and chiral separations [49-52].

Crosslinking monomer

The crosslinking monomer affects the chemistry, porosity and also the rigidity of the material. The main role of the crosslinker is to give rigid monolith and to decrease swelling of monolith under solvent used. At high percentage of the crosslinker, crosslinking between the polymer chains increases resulting in porous structures with larger surface areas and small pore size [38]. Figure 1.8 shows a commonly used crosslinker, ethylene dimethacrylate (EDMA).

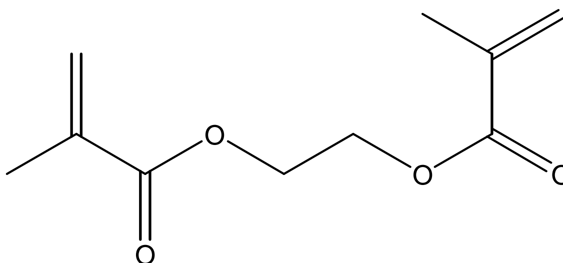


Figure 1.8 Chemical structure of ethylene dimethacrylate (EDMA)

Porogenic solvent

An important parameter controlling porosity of the monolith is the porogenic solvent (porogens). The porogens are not involved in the polymerisation reaction but controls the phase separation process of the polymerisation. The porogens must be able to dissolve the required amount of monomers and other component in the polymerisation solution to give a homogenous solution [38]. Binary or ternary porogens are sometime required to obtain a homogeous solution of the polymerisation solution. Fre´chet et al. studied the effect of important parameters on porous properties of a hydrophilic monolith, poly

(acrylamide-co-N, N'-methylenebisacrylamide) monolith rods including the effect of porogens [38]. The group reported that increasing in the percentage of porogens increased the porosity of monolith. They also found that solubility of the porogens affected the porosity of the monolith. Decrease of solubility of monomers in porogens resulted in promotion of phase separation (nucleation) at an earlier stage of the polymerisation. Large microglobules and large pores are formed under this condition. In contrast, when porogens with similar solubility with that of monomers were used, the monomers were not adsorbed into the nuclei or microglobules since the polarity of the separated nuclei is not very different from the surrounding medium. A large number of smaller microglobules with smaller pore size were therefore observed.

Initiator

Since the polymerisation of monomers occur via free radicals, a free radical initiator is necessary to start the polymerisation. The most common initiator for the organic monolith is azobisisobutyronitrile (AIBN), generating free radicals as shown in Figure 1.9. It has been shown by Fre´chet et al. that the concentration of the initiator affect the kinetics or rate of polymerisation [38]. The rate of polymerisation increased with increasing concentration of the initiator from 0.01% (w/w) to 1% (w/w). However increasing percentage of initiator also decreased porosity of the monolith. 1% (w/w) AIBN is commonly chosen, compromising between the rate of polymerisation and the porosity.

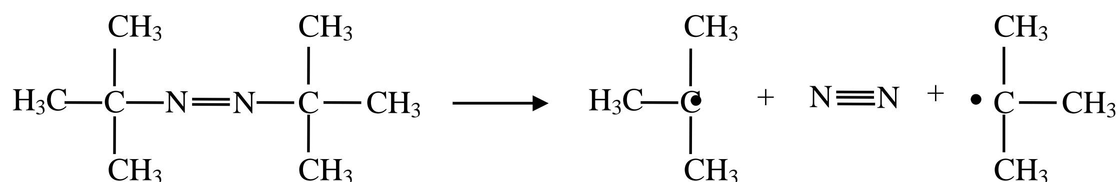


Figure 1.9 Generation of free radicals from AIBN

Polymerisation temperature

The rate of polymerisation increased with increasing the polymerisation temperature (50-90° C). However at higher temperature, smaller pores were observed [38]. The common temperature used for most monoliths is 60° C.

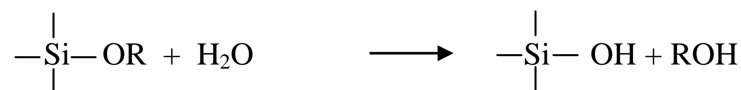
1.1.3.2 Inorganic monolith

Inorganic monolith is prepared from inorganic monomers. The most widely used is silica-based. Zirconia-based has also reported for this type of monolith [55].

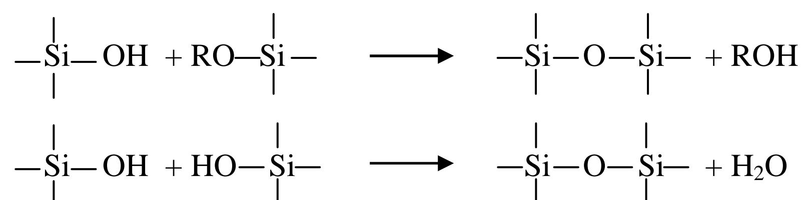
1.1.3.2.1 Silica-based monolith

The silica-based monolith is typically achieved from hydrolysis-polycondensation of alkoxysilane. The preparation is usually by sol-gel method. Tanaka *et al.* was the first group to report a sol-gel method for fabrication of porous silica rod monolith at low temperature and applied it to HPLC [7]. The monomers for synthesis of the silica based monolith are commonly alkoxysilanes such as tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) [17]. The sol-gel method involves gel formation (gelation) and phase separation. The gel-formation (formation of a monolithic network in a continuous liquid phase) is created from polycondensation of alkoxysilane in water containing water-soluble polymeric porogen, poly(ethylene oxide) (PEO) and a suitable catalyst, typically acetic acid. Polycondensation of the polymer chains create a silicate network. Chemical reaction for the sol-gel method can be described as follow. [17].

(i) Hydrolysis of alkoxysilane



(ii) Condensation



The gel formation step governs the skeleton size and domain size or structure of the final monolith. The skeleton size is the average thickness of the monolithic network and the domain size is the sum of the skeleton thickness and pore diameter [53]. The skeleton comparatively refers to particle size while the domain is equivalent to sum of interstitial void between particles and particle diameter. The macropores or through pores are formed during the gelation. The main parameter effecting the mesopore volume is the amount of the PEO.

After the polymerisation is complete, the column is washed with water and then acid neutralized with aqueous ammonium hydroxide (typically 0.01 M, for 9 hrs, at 40° C [54]) in order to generate the mesopores. The mesopores are formed by heat treatment and evaporative drying to decompose residual organic material and stabilize the surface of the hydrophilic silica gel by siloxane rings [38].

Properties of the formed sol-gel matrix including skeleton thickness, through-pore and mesopore sizes can be tailored by adjusting parameters affecting the gel formation step. The important parameters governing structure of the monolith are pH, temperature, reagent concentrations, reaction time, and the type of the catalyst used [17].

After the preparation, surface chemical modification of the monolith may be needed in order to achieve desired surface chemistry. Modifications of silica-based monolithic surface for reversed-phase, ion exchange and chiral separations have been reviewed [16].

Although the preparation of the silica-based monolith is relatively tedious and complicated compared to the polymer-based monolith, the silica based monolithic material offers higher mechanical stability, less swelling and shrinking when in contact with organic solvents [16]. However the drawback of the silica-based monolith is narrower working pH range. The material works well in the pH range of 2-10. It may not be suitable for application requiring at extremely low or high pH.

1.1.3.2.2 Zirconia-based monoliths

In order to over come the problem of poor stability of the silica-based monolith under extreme pH and high temperature, an alternative inorganic monolithic materials has been used, that is the zirconia-based monolith. This type of monolithic material has been reported to give good stability over a wide pH range and large electro-osmotic flow for CEC. Randon *et al.* reported two approaches for the preparation of the zirconia monolith, the zirconia coating of silica monolith and sol-gel method [55].

1.1.3.3 Preparation technology

Preparation technology for both inorganic and organic monolithic column can be typically performed in two formats. The first one is *in situ* preparation in a fused silica capillary. The second one is cladding of the monolith in a column support.

1.1.3.3.1 *In situ* preparation of monolith in a fused silica capillary

Monolithic material can be simply prepared *in situ* in a fused silica capillary. For organic monolith, the preparation can be divided in to four steps. These are, (i) pretreatment of inner capillary wall with bi-functional reagent, (ii) filling with polymerisation solution, (iii) polymerisation and (iv) washing steps. Before polymerisation, the inner surface of the capillary must be pre-treated with a bi-functional ligand to anchor the monolithic monomer onto the capillary surface. The common bi-functional ligand used is methacryloxypropyltrimethoxysilane or 3-(trimethoxysilyl) propyl methacrylate (γ-MAPS, with structure in Figure 1.10). The alkoxyl group of the γ-MAPS bonds with a silanol group of the capillary surface by condensation, while the second functional group is free to react with the monomer.

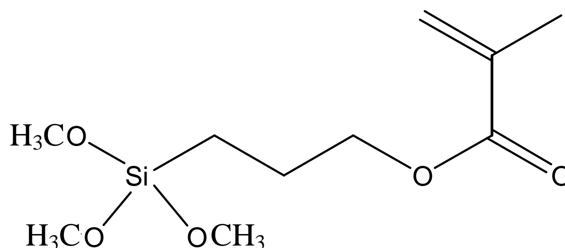


Figure 1.10 Chemical structure of γ-MAPS.

The pretreated capillary is then filled with the polymerisation solution and both ends of capillary sealed with septa. The polymerisation takes place either by thermal initiation or photochemical initiation. After the polymerisation is complete, the monolithic capillary column is washed with methanol or acetone by mean of a high pressure pump or electro-osmotic flow.

1.1.3.3.2 Preparation of monolith in a column support

In addition to *in-situ* preparation, monolithic material can be cladded into a support after the preparation. The method involves two main steps. Firstly the monolith is prepared in a mold with the desired dimension and then encase

in a column support such as polyether ether ketone (PEEK) or poly tetra fluoroethene (PTFE). The picture below is a diagram of the fabrication process of a monolithic silica column for HPLC. Briefly, the polymerisation solution is mixed and filled into a mold with the desired dimension. At optimal condition, polymerisation occurs which lead to gelation and phase separation. The macropores or through pores are formed in this step. After the polymerisation is complete, the mesopores is generated by aging with a basic solvent and heat treatment. After drying and surface modification, the monolithic rod is finally cladded into the column support.

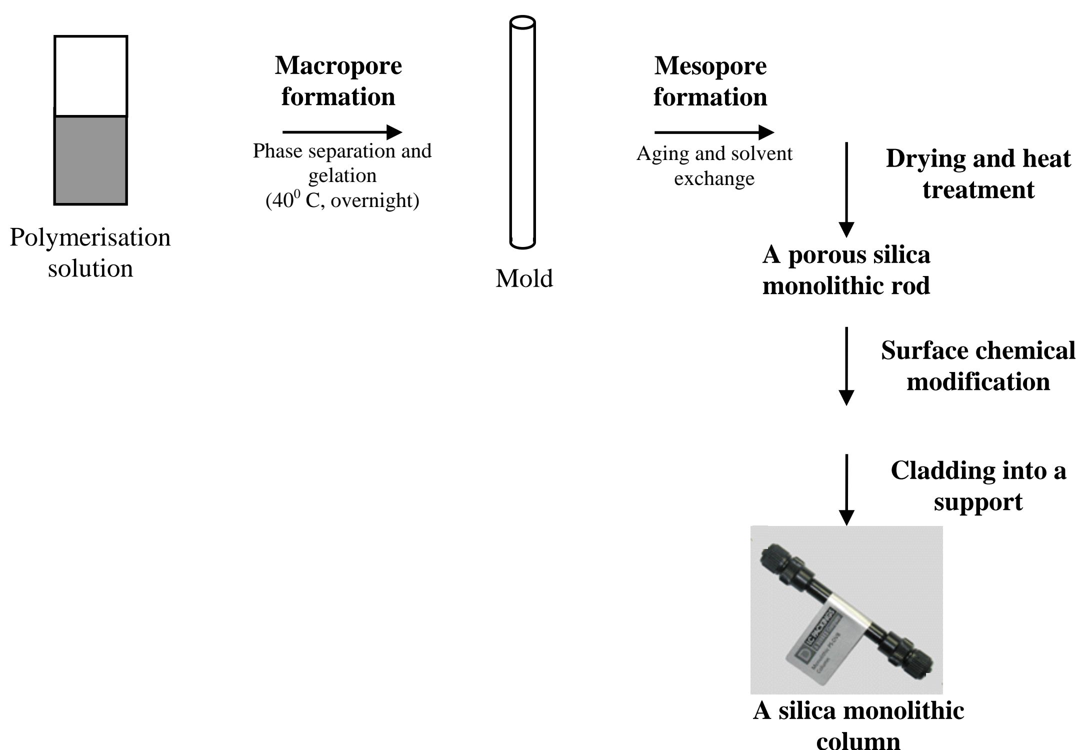


Figure 1.11 Example of the process for fabrication of a monolithic silica HPLC column.

1.2. Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) is one of the most powerful separation techniques. In contrast to chromatographic separation techniques such as liquid chromatography and gas chromatography, there is no stationary phase in CE. The separation is base

upon differences in mobility of charge compounds in a narrow fused silica capillary under an applied electric filed. Under an applied electric filed, there are two important driving forces governing the separation, that are electroosmotic and electrophoretic flows. The CE equipment mainly consists of: a high voltage power supply, solvent and sample vials, a fused silica capillary that simultaneously generates EOF and separates the analytes, and a detector that monitors the component peaks when they pass the detection position.

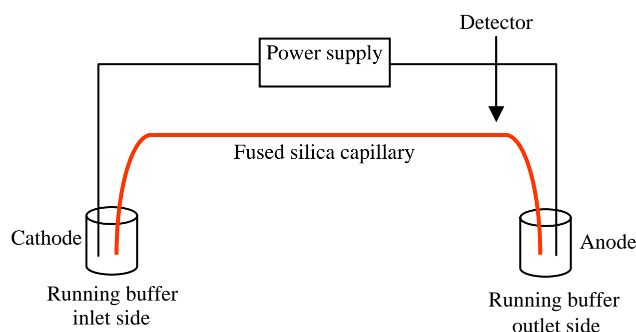


Figure 1.12 Simple diagram of capillary electrophoresis instrument

1.2.1. Electroosmotic flow

Electroosmotic flow or EOF is a bulk flow in CE which is generated from the charged surface of the fused silica material under an applied electric field. At pH higher than the pKa of the silanol group (pKa ~3, [56]), a negative surface is generated due to dissociation of the silanol groups (see Figure 1.13). Cations in the buffer contained in the capillary adsorb on the negative surface by electrostatic force giving a fixed and diffuse cation double layers. Under an electric field, the adsorbed solvated cations in the diffuse double layer move to anode side and drag solvated anions along with them, resulting in flow of bulk solution called electroosmotic flow or EOF.

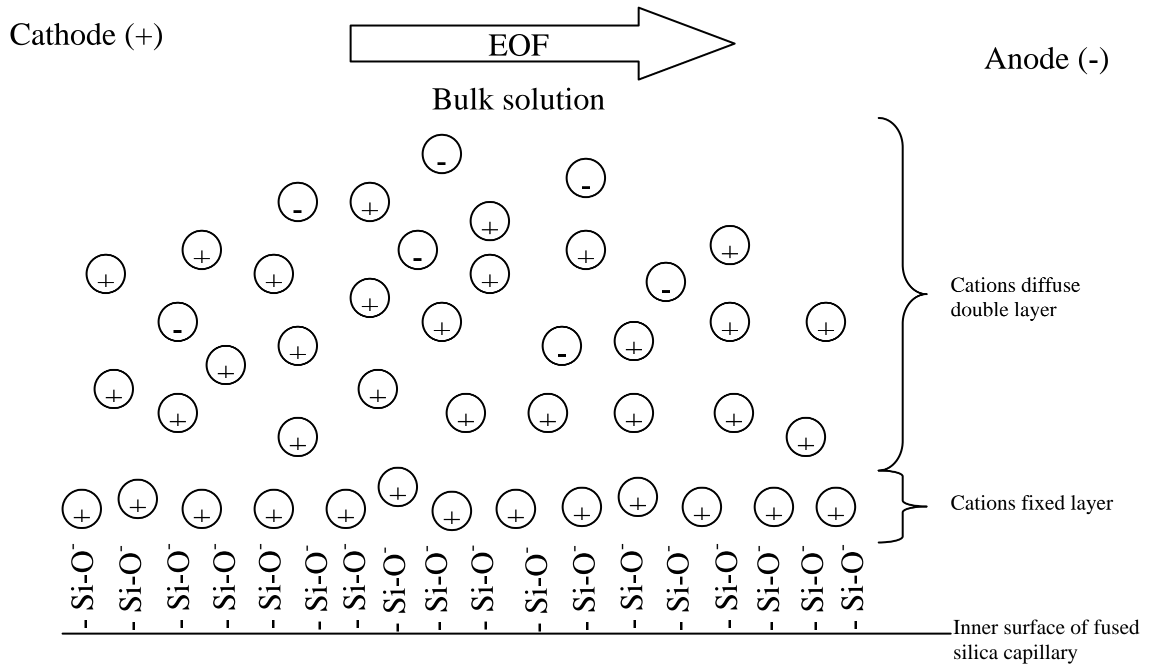


Figure 1.13 Schematic diagram of double layer and EOF formation in CE.

The magnitude of the EOF can be explained in terms of velocity or mobility as shown in eq.(2) and eq. (3) [57-58].

$$v_{eof} = \mu_{eof} E \quad \text{.............................(2)}$$

$$\mu_{eof} = \varepsilon_0 \varepsilon_r \zeta/\eta \quad \text{..........................(3)}$$

Where : v_{eof} = velocity

E = electric field

μ_{eof} = EOF mobility

ζ = zeta potential

ε_0 = permittivity of a vacuum

ε_r = dielectric constant of the electrolyte solution.

The zeta potential is dependent on the charges of the surface. Since dissociation of Si-OH on the surface depends on pH of the running buffer, the magnitude of EOF therefore significantly depends on pH of the running buffer. Figure 1.14 [56] shows the pH dependency of EOF for three capillary materials.

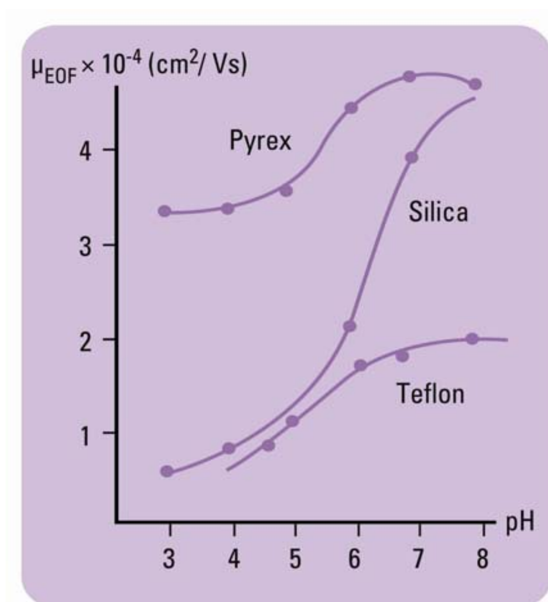


Figure 1.14 Effect of pH on electro-osmotic flow mobility in various capillary materials [56].

1.2.2. Electrophoretic flow

In addition to migration of ions by the EOF, the ions move under an electric field due to its charge. An anion moves to the positive electrode or cathode while a cation moves to a negative electrode or anode due to the electrostatic force.

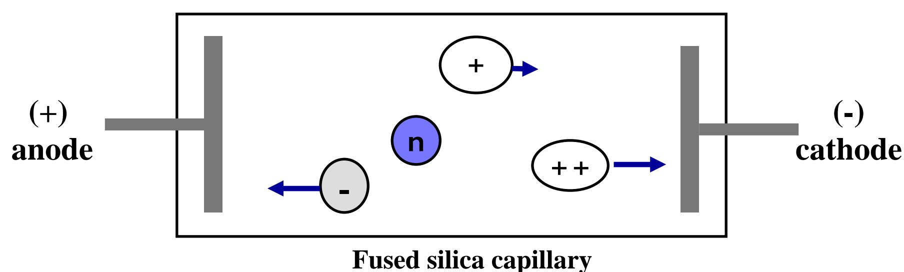


Figure 1.15 Direction of movement of ions due to electrophoretic mobility under an electric field

The movement of ions by its charge is called 'electrophoretic mobility' (μ_{ep}). The velocity and mobility of ion are be shown in eq (4) and (5).

$$v_{ep} = \mu_{ep} E \quad \text{.............................}(4)$$

$$\mu_{ep} = q / (6\pi\eta r) \quad \text{.......................}(5)$$

where : v_{ep} = ion velocity due to electrophoretic flow

μ_{ep} = electrophoretic mobility

E = electric field

q = ion charge

η = solution viscosity

r = ion radius

As shown in eq.(5) the electrophoretic mobility of ion depends on its chage and radius. Ion with high charge density has high mobility while ion with lower charge density migrate with the lower mobility (see Figure 1.15). In addition, ions move faster in low solvent viscosity.

1.2.3. Apparent mobility and migration time

Under an applied electric field, ion moves with apparent mobility due to both electro-osmotic flow, eq.(6) and (7). The apparent mobility of ion is a vector summation of mobility from both electro-osmotic and electrophoretic flows. Both the magnitude and direction of the two flows are considered.

$$\mu_{ap} = \mu_{ep} + \mu_{eof} \quad \text{.......................}(6)$$

$$v_{ap} = V_{ep} + V_{eof} \quad \text{......................}(7)$$

where : μ_{ap} = apparent mobility

μ_{ep} = electrophoretic mobility

μ_{eof} = electro-osmotic mobility

v_{ap} = apparent velocity

v_{ep} = ion velocity due to electrophoretic flow

v_{eof} = ion velocity due to electro-osmotic flow

Migration time (t_m) is time required for a solute to migrate from injection point to detection point and can be calculated by using the equation.

$$\mu_{ap} = l/ t_m E \quad = lL/ t_m V \ldots\ldots\ldots\ldots.(8)$$

where : $\mu_{ap} = \mu_{ep} + \mu_{eof}$

V = applied voltage

l = effective capillary length (from inlet to injector)

L = total capillary length

t_m = migration time

E = electric field

Schematic diagram of CE with cathodic EOF (EOF moving towards cathode side) is shown in Figure 1.16. If the magnitude of EOF is larger than μ_{ep} of anion, the direction of anion is to the cathode side.

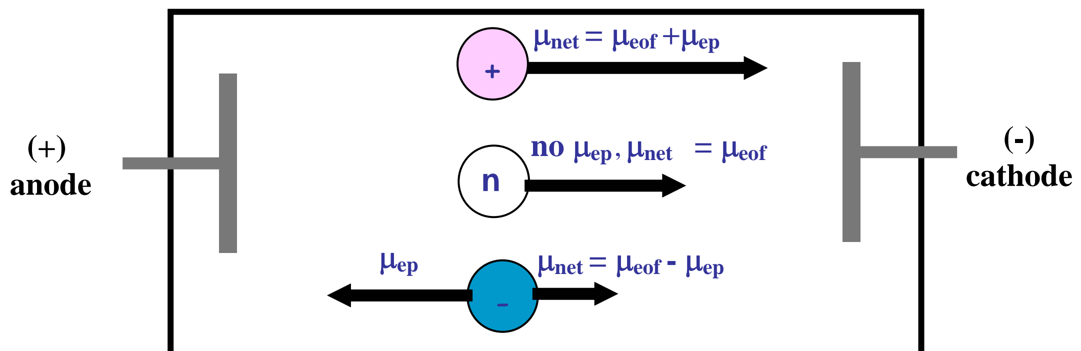


Figure 1.16. Net flow of ions under influences of EOF and its electrophoretic mobility.

1.2.4. Advantages of CE technique

One major advantage of CE is high separation efficiency due to the flat flow profile of the electroosmotic and electrophoretic flows as shown in Figure 1.17. Unlike the laminar flow due to applied pressure as in HPLC, ions in CE migrate with the same velocity resulting in flat flow pattern and therefore narrower peak with higher efficient separation are observed. In addition, CE technique has advantages in

terms of low reagent consumption, high speed of separation, ease in development and low cost as compared to HPLC.

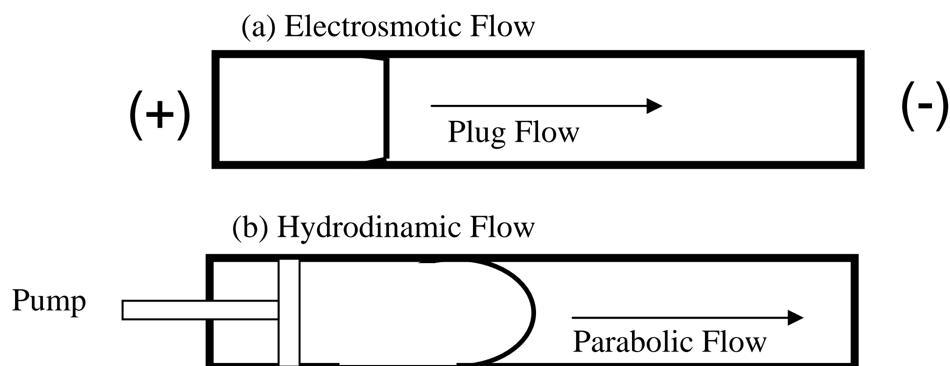


Figure 1.17 Flow profile in (a) electroosmotic flow profile in CE and (b) hydrodynamic flow profile in HPLC

1.2.5. Limitation of CE technique

Separation by CE has many advantages. However the small dimensions of the technique provides both advantages and disadvantages. The weakness of the technique is lack of sensitivity for UV detection which is the most common detector for CE. In order to improve the sensitivity, several approaches have been developed including modification of UV detection cell to increase the optical path length [60-62], using more sensitive detection methods such as laser-induced fluorescence detection [63-64], chemiluminescence [65-67], electrochemical detection [68-69] or ICP-MS [70-71]. On-capillary pre-concentration based on electrophoretic effects such as isotachophoresis [72-74], and field amplified sample injection [75-76] are also often used. However the methods are not suitable for samples with high matrix and have limitations with sample loading volume.

Another approach is sample pre-concentration on a solid sorbent called solid phase extraction (SPE). The SPE can be used for both the removal of potential interferences and enrichment of analytes. Significant sensitivity improvements in CE by coupling with SPE have been achieved with pre-concentration factor up to 2-4 orders of magnitude.

1.2.6. Coupling of SPE with CE for sensitivity enhancement

The early method beginning of coupling of SPE with CE used packed particle material [77-86]. Successful development of the coupling of SPE-CE were reported for off-line [77-78], at-line [79-70], on-line [81-83] or in-line [84-86] modes. Among these coupling methods, the on-line and in-line modes are preferable since they have short analysis times with less sample handling. However, the assembly of the packed SPE in CE is complicated and problems from high backpressure. An example of a packed particle SPE-CE connection reported by Staffan *et al.* [82] is shown in Figure 1.18.

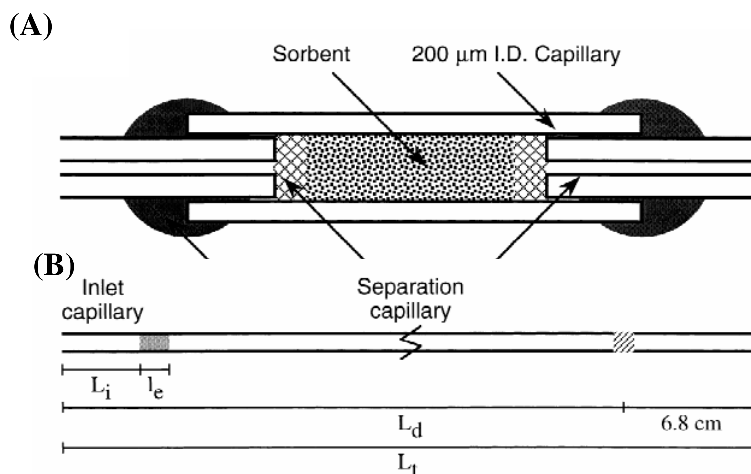


Figure 1.18 Cross-section of (A) the extractor and (B) the enrichment capillary where L_t (28–58 cm) is the enrichment capillary length, L_d (21.2–51.2 cm) is the length to the detector, L_i (5.4 cm) is the length of the inlet capillary and l_e (1–3 mm) is the extractor length [82].

To overcome these problems, some authors employed monolithic materials as SPE for the pre-concentration/extraction in CE.

Monolith is the newest technology in chromatographic stationary phase development. It has unique property of high porosity with high chromatographic efficiency [10-15]. Many types of monoliths have been applied as SPE for CE by preparing *in-situ* in a separation capillary. The coupling SPE-CE can be used with the

CE instrument without need of any connections or instrumental modification [87-91]. Monolith generally offers much higher porosity and permeability than the conventional microparticulate phase which allow the monolithic SPE-CE to be operated at a higher flow rate and a larger volume of sample loading with no problem of high back pressure.

The first in-line monolithic SPE-CE method was reported by Baryla and Toltl [87]. Methacrylic acid-ethylene dimethacrylate monolith (MAA-EDMA) was prepared *in situ* in a CE separation capillary using UV initiated polymerisation. The performance of the method was demonstrated with *S*-propranolol. Sensitivity improvement for LOD compared to that of conventional CE with pre-concentration factor of 100 fold, was observed. The coupling technique showed improvement in sensitivity without any significant difference in separation efficiency in terms of theoretical plates [87].

Lately, many groups reported further development of the in-line monolithic SPE-CE [88-91]. The coupling methods were applied for the extraction and pre-concentration of inorganic anions in seawater [88]. immunoglobulin G from human serum [89], organic anions [90], and antidepressants [91].

1.3 Capillary electrochromatography (CEC)

1.3.1 Basic principle

The concept of capillary electrochromatography (CEC) was first introduced in 1974 by Pretorius *et al.* [92]. The group reported use of high electric field for propelling solution through a chromatographic column in stead of using a high pressure pump. However the first CEC separation was reported by Jorgenson and Lukacs in 1981 [93].

The technique is a separation technique that combines separation mechanism and unique features from both liquid chromatography (LC) and capillary electrophoresis (CE). The separation takes place in a capillary containing chromatographic stationary phase under an electric field applied across both ends of the separation capillary. The separation is therefore based upon both electrophoretic and chromatographic processes which can be explained by terms of electromigration and distribution of analytes between mobile and stationary phases. Having a chromatographic stationary phase allow separation of both charged and uncharged compounds. The technique has therefore higher separation selectivity than CE but with high separation efficiency due to the flat flow profile of in CE. Another feature of the CEC is that since solution moves by electro-osmotic driven flow which is applied without limitation on the back pressure of the separation column usually found in HPLC [94], longer and smaller column can be used in order to increase efficiency. Comparison of efficiencies for HPLC and CEC are given in Table 1.1 [95]. Extremely high efficiencies of up to millions of plates per meter have been reported for partially ionized anionic-neutral compounds [96-97].

However the diameter of the particles controls the thickness of the electrical double layer in the flow channel [94]. In order to prevent overlapping of the electrical double layer, the diameter of the flow channel must be at least 20 times larger than the thickness of the electrical double layer. Wan [98] reported a relationship between the mean channel diameter and particle size (d_p) employing the interparticle porosity as shown in eq.(9).

$$d_p = (0.42\ d_p\varepsilon)/\ (1-\varepsilon) \quad \ldots\ldots\ldots\ldots\ldots.(9)$$

Where ε is the interparticle porosity, d_p is particle size. 0.42 is a random packing structure for a fairly well packed column.

Table 1.1 Comparison of efficiencies for HPLC and CEC for given column lengths and particle sizes [95].

	HPLC		CEC	
Particle size (μm)	Column length (cm)	Plates	Column length (cm)	Plates
5	50	45,000	50	90,000
3	30	50,000	50	150,000
1.5	15	33,000	50	210,000

Since the separation in CEC involves two mechanisms, chromatographic and electrophoretic, selectivity coefficient (capacity factor) for the CEC involves with both separation mechanisms are given in eq.(10) [99].

$$k_c = k + kk_e + k_e \quad \ldots\ldots\ldots\ldots\ldots \quad (10)$$

$$k = (t_r - t_0)/t_0 \quad \ldots\ldots\ldots\ldots\ldots\ldots (11)$$

$$k_e = (t_m - t_{eo})/t_{eo} \quad \ldots\ldots\ldots\ldots\ldots \ (12)$$

where k_c is selectivity factor for CEC, k is retention factor from chromatographic process ,

k_e is velocity factor from electrophoretic process,

t_r and t_m are elution and migration times respectively,

t_0 is the retention time of an unretained marker, and

t_{eo} is the retention time of a neutral solute moved only by the EOF.

The term kk_e is the result of simultaneous chromatographic and electrophoretic processes. For neutral compound which has no electrophoretic mobility or $k_e = 0$ then k_c equals k, and only chromatographic mechanism is involved the separation. In contrast, if the analyte is charged unretained compound, the term k = 0, then $k_c = k_e$ and only the electrophoretic process is the separation mechanism in this case. For charged retained compound, both the two mechanisms govern the separation as given by eq.(10) [99].

CEC can be operated with a CE instrument by using a separation capillary containing a suitable chromatographic stationary phase instead of a bare fused silica capillary. However slight modification may be needed in order to allow successful operation of the CEC, such as external pressure source and gradient system [100]. An external pressure source, with pressure up to 10 bars, is usually added to the CE system. This external pressure is commonly applied at both ends of the capillary during the separation to prevent problem from bubble formation which usually occur at the retaining frits of the packed particle capillary. Interruption of electrical current from the bubble formation leads to abortion of the separation process [100]. A very noisy baseline and spikes appear in the chromatogram are also observed. Application of pressure at capillary ends during the CEC separation is sometime called ‘pressurized CEC’ [101].

1.3.2 Column development in capillary electrochromatography

Stationary phase or column in CEC not provides only chemistry for the separation but also provides the driving force, EOF, to transport solvent and solute through the column. CEC column is typically divided in to three categories depends on the column fabrication: (i) open-tubular columns, (ii) packed columns and (iii) continuous-bed or monolithic columns [99].

1.3.2.1. Open-tubular column (OT-CEC)

Open-tubular column is simply prepared by attaching a stationary phase, which can be a coated polymer, bonded molecular monolayer, or a synthesized porous layer network, on to the inner surface of a fused silica capillary wall [94, 102]. The attachment can be either physical adsorption or chemical bonding of the coating on the silica surface. The stationary phase must provide functional group for separation and also charge functional group to generate the EOF.

Since the open-tubular column does not require any retaining frit, there is no problem from bubble formation which is commonly found in packed CEC. In addition, the stationary phase is more homogenous and the smaller internal diameter reduces joule heating with increase in efficiency. Column length can be also simply shortened. A higher voltage can be applied due to rapid heat dissipation from the narrow column is another advantage of the OT-CEC [102]. However the OT-CEC has drawback of low sample capacity due to the low surface area [94, 102]. Many groups have attempted to increase the phase ratio for the open-tubular column. Perek's group reported an approach by etching the surface area of the fused silica [103-105]. Horva'th and his group reported using porous-layer open-tubular columns [106]. In another approach reported by Sawada and Jinno, the phase ratio was increased by anchoring a polymeric stationary phase to the capillary wall via a bifunctional reagent [107]. However the sample capacity is still relatively low compared to that for packed and continuous-bed columns.

1.3.2.2. Packed column

Packed column is the most commonly used in CEC [91]. The column consists of two retaining frits, a packed section containing stationary phase and an open section (unpacked section) for making a detection window as shown in Figure 1.19.

A fused silica capillary is packed with particulates of the stationary phase. Two frits are formed by sintering the packing material to retain the packing particles. Coated polyimide is removed at a small section of the open section to generate a detection window.

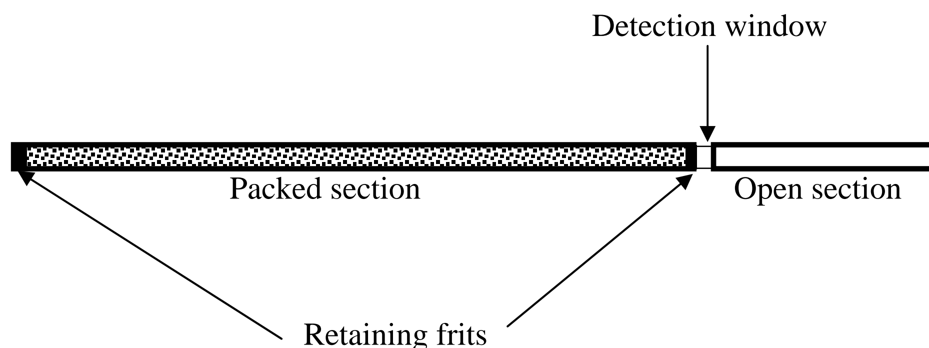


Figure 1.19 Diagram of a packed CEC column.

The EOF is generated by silanol groups on the surface of the fused silica capillary especially from the open section (bare capillary) and also by the packing particles surface[107]. In order to achieve adequate EOF, the packing particle must provide charged or ionizable groups. For silica-based material which is mostly used, the silanol groups are silanised to change the chemistry of the surface for the separation. However the residual silanols generates EOF when a mobile phase with pH higher than 3 is used. Non-endcapped packing material which contains higher residual silanol groups therefore generates higher EOF compared to that for the endcapped packing material [102]. Since dissociation of silanol groups is strongly pH dependent, variation of the EOF can be observed if the pH is not strictly controlled. In addition, the pH range should be 2-10 to prevent dissolution of the silica-based materials. The applications of packed column have been extensively reported [108-111].

Due to the larger surface area, the packed column provides higher sample capacity [107]. However the major disadvantages of the packed columns are bubble formation due to the retaining frits, difficulty in changing the column length and also the lower porosity of the frits can easily cause contamination or clogging when dirty samples are used. In addition, the column must be rigid and homogeneous to prevent a gap, generated due to movement of packing material when applying the voltage [102]. The preparation of the packed column is also relatively difficult

compared to open-tubular or monolithic columns. The process needs technical skill in order to obtain good quality packed column.

1.3.2.3. Continuous-bed or monolithic columns

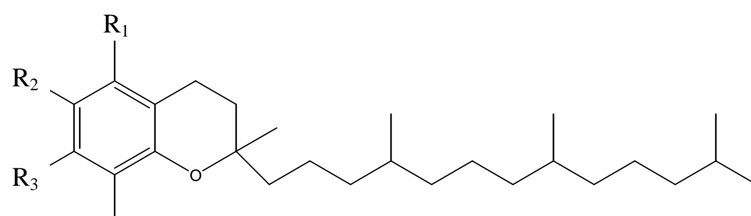
Continuous-bed or monolithic column is a relatively new column technology for CEC. Unlike the packed column, the monolith is a porous continuous skeleton with a rod-like shape. The column can be typically divided into silica-based and polymer-based depending on the monomers used and preparation procedure. Many monolithic stationary phases are available and have been applied for real samples [39-52]. Since the monolith can be *in situ* strongly bonded onto the glass surface of the fused silica, no retaining frits is needed. The problem from bubble formation is thus eliminated. In addition, this type of column can be readily prepared without need of complex apparatus. More details about monolithic column has been discussed in section 1.1.

1.4 Vitamin E

1.4.1 Introduction

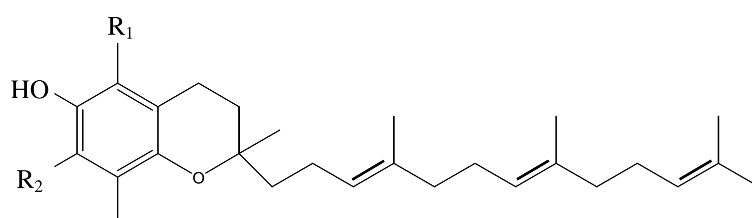
Vitamin E is a fat-soluble vitamin that exists in eight different forms, four tocopherols (TOHs) and four tocotrinols (T2). Both structures are similar but the tocotrinols contain of double bonds on the isoprenoid units (Figure 1.20). Since these vitamins are not synthesized in human, it is obtained from the normal diet or through vitamin supplements in order to prevent vitamin E deficiency. Vitamin E acetate or α-tocopherol acetate is the common form of the vitamin E in food or vitamin supplements. In addition to the anti-oxidant property, tocopherols play an important role in the prevention of certain chronic diseases such as, heart disease and cancer [112].

(a)



Compound	R_1	R_2	R_3
1. α-TOH	CH_3	OH	CH_3
2. β- TOH	CH_3	OH	H
3. γ- TOH	H	OH	CH_3
4. δ- TOH	H	OH	H
5. α- TAc	CH_3	$COOCH_3$	CH_3

(b)



Compound	R_1	R_2
1. α- T2	CH_3	CH_3
2. β- T2	CH_3	H
3. γ- T2	H	CH_3
4. δ- T2	H	H

Figure 1.20 Chemical structure of (a) four tocopherols and α-tocopherol acetate and (b) four tocotrinols.

Vitamin E acts as anti-oxidant which protects against lipid peroxidation (which could contribute to cell membrane weakness). The mechanism of vitamin E is to capture free radical as shown in Figure 1.21. Hydrogen from the hydroxyl group of the vitamin E is donated to the free radical, resulting in a vitamin E free radical. Since the unpaired electron can be delocalized in the aromatic ring of the vitamin E, the vitamin E free radical is therefore relatively stable [113].

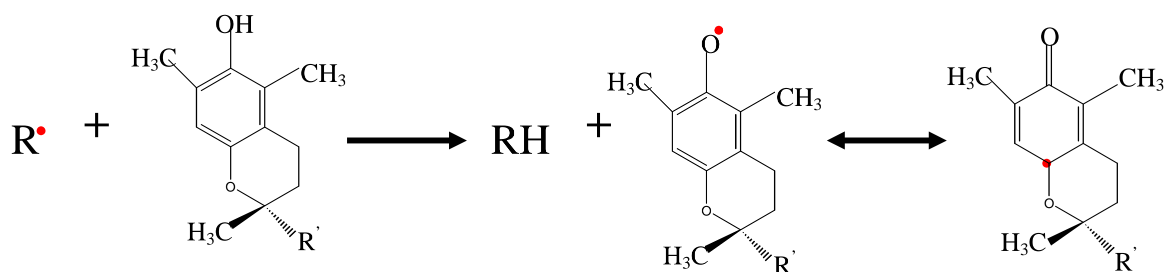


Figure 1.21 Free radical capture by vitamin E [113]

Each form of vitamin E has different biological activity as shown in Table 1.2 Among all the vitamin E forms, α-tocopherol has the highest biological activity.

Table 1.2 Biological activity of vitamin E [114].

Common name	Biological activity compared with α-tocopherol (%)
d-α-tocopherol	100
d-β-tocopherol	50
d-γ-tocopherol	10
d-δ-tocopherol	3
d-α-tocotrienol	30
d-β- tocotrienol	5
d-γ- tocotrienol	unknown
d-δ- tocotrienol	unknown

α-Tocopherol has the highest biological activity [115] and is the most abundant form in food [116. However there is evidence that other forms of tocopherols (β, γ, δ) also play important roles [117. An appropriate and reliable method for quantification of all the tocopherol isomers is therefore needed, for the investigation and comparison of the biological activity of the isomeric forms of tocopherol.

1.4.2 Analytical methods for determination of vitamin E

Many methods have been developed for speciation of vitamin E especially tocopherol in biological samples [118-120], pharmaceutical preparation [120-122] and food [123]. The analysis were carried out by high performance liquid chromatography (HPLC) [118-120, 124-135], microemulsion electrokinetic chromatography (MEEKC) [122, 135-137], and capillary electrochromatography (CEC) [124, 138-143].

Among these methods, the most common technique for separation of vitamin E is HPLC because of its high separation efficiency, robustness and reliability.

However due to the structural similarities of the β- and γ-isomers (positional isomers, see Figure 1.20 (a)), separation of these compounds is therefore challenging. Reversed-phase HPLC, with standard C8 or C18 stationary phases [128-131], have shown insufficient selectivity for separation of the problematic β-and γ-isomers. Normal-phase HPLC has been found to be able to separate all four tocopherol isomers [125-127]. Nevertheless normal-phase LC is less reproducible than reversed-phase separation. Normal-phase separation also has a major disadvantage of employing relatively toxic organic solvents.

Many works have attempted to develop a reversed-phase column for separation of the tocopherol isomers. In 1998, the group of Albert reported that a hydrophobic C30 stationary phase showed some selectivity for the two isomers. They found that partial separation of the β- and γ-tocopherols could be achieved by using a packed C30 stationary phase [134]. Application of the C30 stationary phase for these compounds was further extended to CEC technique with complete separation of all tocopherol isomers by Warner in 2001 [141]. Lately, Abidi *et al*. reported the use of a polar-reversed phase column called pentafluorophenylsilica (PEPS) for the separation of all forms of tocopherols and tocotrienols [152]. The separation of all four isomers of tocopherols and tocotrinols could be achieved in 30 minutes. Recently the group of Rodríguez-Gonzalo reported the use of a polar embedded C18 stationary phase, called ULTIMA C18, for the complete separation of tocopherol isomers and compared the results to that for the conventional C18 and C30 stationary phases [143].

From the literature, it has been shown that in order to achieve success in separation of all tocopherol isomers, an extremely hydrophobic stationary phase or polar reversed-phase stationary phase is required. However all reported works have been carried out on microparticulate column. There also has been no reported use of monolithic stationary phase for the separation of the vitamin E.

Pentaerythritol diacrylate monostearate (PEDAS, neutral monolith (see structure in Figure 1.22)), was first introduced and evaluated in CEC by M. Bedair and Z. E. Rassi in 2002 [144]. It contains both hydrophilic hydroxyl and hydrophobic C17

groups as functional ligands which is similar to the concept of the polar embedded C18. It is therefore expected to show good selectivity for separation of the β- and γ-tocopherols which are normally not well separated by standard C8 or C18 stationary phases, as observed from the polar embedded C18.

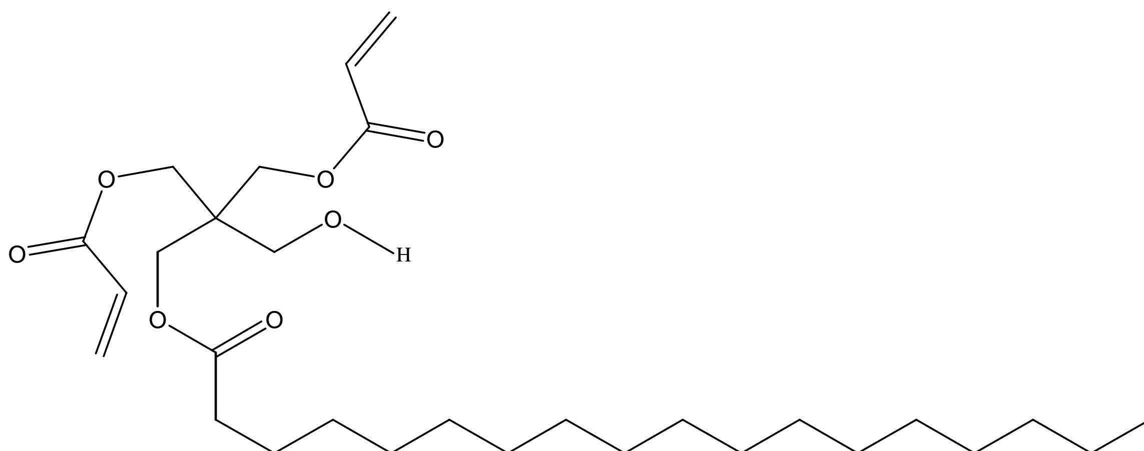


Figure 1.22 Pentaerythritol diacrylate monostearate (PEDAS)

CHAPTER II
OBJECTIVES

Due to the unique advantages of monolithic material over conventional packed based material, monolith was used in this work with two objectives, for pre-concentration and for separation purposes.

The first was investigation of in-line coupling of monolithic solid phase extraction to capillary electrophoresis for pre-concentration and extraction. In this part, performance of the monolith was also compared to conventional packed particle phase. The second application was use of monolithic column as stationary phase for separation of vitamin E in the form of tocopherols and tocopherol acetate by capillary electrochromatography. The objective of this work is divided into two parts as given below.

2.1 Part I : Investigation of polymer based monolithic and silica based packed particle phases for pre-concentration/extraction purposes for capillary electrophoresis by in-line coupling

2.1.1 To develop an in-line coupling of solid phase extraction to capillary electrophoresis for sensitivity enhancement

2.1.2 To develop a simple and high sensitive SPE-CE method for determination of terbutaline and 4-hydroxy-3-methoxy-methamphetamine.

2.1.3 To compare performances of polymer-based monolithic SPE-CE to silica based packed particle phases.

2.2 Part II : Development of a monolithic capillary electrochromatographic method for separation and determination of tocopherols and tocopherol acetate

2.2.1 To examine and develop a monolithic capillary electrochromatographic method for separation of α-, β-, δ-, γ-tocopherols and α-tocopherol acetate on a pentaerythritol diacrylate monostearate -ethylene dimethacrylate monolithic column.

2.2.2 To apply the develop method for determination of tocopherols and α-tocopherol acetate in vitamin E supplement.

CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals and reagents

Detail for all chemicals and reagents used in this thesis are listed in the below Table.

Table 3.1 List of chemicals and reagents

Chemicals	Supplier	City/Country
1. Acetonitrile	Sigma-Aldrich	Poole, UK
2. Azobisisobutylronitrile	BDH	Lutterworth,
3. 1,4- Butanediol	Sigma-Aldrich	Poole, UK
4. Benzyl alcohol	Sigma-Aldrich	Poole, UK
5. Butylated hydroxytoluene	Sigma-Aldrich	Poole, UK
6. Butylmethacrylate	Sigma-Aldrich	Poole, UK
7. Decanol	Sigma-Aldrich	Poole, UK
8. Ethylene dimethyacrylate	Sigma-Aldrich	Poole, UK
9. Ethylene glycol	Sigma-Aldrich	Poole, UK
10. 4-Hydroxy-3-methoxy-methamphetamine	from Dr. Andrew Kicman, Drug Control Center, King's College	London, UK
11. Methacrylic acid	Sigma-Aldrich	Poole, UK
12. Methanol	Sigma-Aldrich	Poole, UK
13. Natural vitamin E-400	Holland&Barrett Naturally	Warwickshire,
14. Natural vitamin E-oil	Holland&Barrett Naturally	Warwickshire,
15. Pentaerythritol diacrylate	Sigma-Aldrich	Poole, UK
16. Pentanol	Sigma-Aldrich	Poole, UK

Table 3.2 List of chemicals and reagents (continued)

Chemicals	Supplier	City/Country
17. Phosphoric acid	BDH	Lutterworth, UK
18. Sodium dihydrogen phosphate	BDH	Lutterworth, UK
19. Sodium phosphate	BDH	Lutterworth, UK
20. di-Sodium hydrogen orthophosphate anhydrous	BDH	Lutterworth, UK
21. Terbutaline	Sigma-Aldrich	Poole, UK
22. 3-(Trimethoxysilyl)propyl	Sigma-Aldrich	Poole, UK
23. Tris(hydroxylmethyl)	Fisher Chemicals	Loughborough,
24. Thiourea	Sigma-Aldrich	Poole, UK
25. α-Tocopherols	Sigma-Aldrich	Poole, UK
26. β-Tocopherols	Sigma-Aldrich	Poole, UK
27. γ-Tocopherols	Sigma-Aldrich	Poole, UK
28. δ-Tocopherols	Sigma-Aldrich	Poole, UK
29. α-Tocopherol acetate	Sigma-Aldrich	Poole, UK
30. Toluene	Sigma-Aldrich	Poole, UK

3.2 Instrumentations

All used instruments and apparatus are shown in the below Table.

Table 3.3 Instruments and apparatus

Instruments/	Model	Supplier	City/Country
1. Capillary electrophoresis system	HP^{3D}	Hewlett Packard	Waldbron/Germany
2. pH-Meter	Mettler Delta 320 pH meter		Switzerland
3. Fused-silica capillaries	i.d. 75 and 100 µm with 360 µm o.d	Composite Metal Service Ltd.	Worcestershire/UK
4. Spherisorb ODS I	5 µm	Waters	Milford/USA
5. Spherisorb ODS I	3 µm	Waters	Milford/USA
6. High pressure pump		Shandon Southern Products Ltd.	Runcorn/USA
7. Oven	63505	Kendo	Germany
8. Sonicator	208H	Ultrasonik™	Germany
9. Frit maker		Innovatech	Herts/UK
12. Balance	CP225D	Sartorius	Germany
14. ELGA Maxim Water system	MAM 142782	ELGA Maxim	Buckinghamshire, UK
15. Microscope	CX21	Olympus	Hamburg/Germany
16. Drill bit	0.4 mm	Supelco	USA

3.3 Preparation of used solutions

In this section, procedures for preparation of solutions used in this work are described.

3.3.1 Preparation of solutions for Part I : Investigation of polymer based monolithic and silica based packed particle phases for pre-concentration/extraction purposes for capillary electrophoresis by in-line coupling

3.3.1.1 Preparation of phosphate buffer

One hundred milimolar phosphate buffer was prepared by dissolving 0.78 g of di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) in 100 mL DI water. The solution was then diluted with DI water to achieved desire concentration. The solution was adjusted pH by adding same concentration of sodium phosphate (Na_3PO_4) for making basidic solution (pH 11) or adding sodium dihydrogen phosphate (NaH_2PO_4) for pH 7.

For acidic phosphate buffer (pH 3), sodium dihydrogen phosphate solution was adjusted pH by adding phosphoric acid.

3.3.1.2 Standard solutions of BZ, TER and HMMA

Stock 1000 ppm standard benzyl alcohol (Bz), terbutaline (TER) and 4-hydroxy-3-methoxy-methamphetamine (HMMA) were prepared in methanol by dissolving individual 0.0100 g of each standard in 10.00 mL methanol. The stock solutions were then diluted with water or 10% (v/v) ACN : water for preparing working solutions.

For in-line packed particle SPE-CE, the mixed standard solution of the three compounds was prepared in 10 mM phosphate buffer (pH 7).

3.3.1.3 Fifty milimolar Tris in methanol (pH 8.2)

Fifty milimolar of Tris in methanol was prepared by dissolving 0.600 g of tris(hydroxymethyl) methylamine (Tris) in 100 mL methanol. The solution was then adjusted pH by adding small amount of concentrated hydrochloric acid to get the observed pH of 8.2.

3.3.2. Preparation method of solutions used for Part II : Development of a monolithic capillary electrochromatographic method for separation and determination of tocopherols and tocopherol acetate

3.3.2.2. Ten, fifty, one hundred and two hundred milimolar Tris buffer (pH 9.3)

Ten, fifty, one hundred and two hundred milimolar of Tris buffer (pH 9.3) were prepared by dissolving tris(hydroxymethyl) methylamine of 0.12, 0.6, 1.2 and 2.4 g, respectively in 100 mL DI water. Five molar of hydrochloric acid was added into the solutions to obtain buffer solution pH of 9.3.

3.3.2.3. Standard solutions of TOHs and TAc for construction of calibration curves

Stock 1,000 ppm solutions of all standard tocopherols and tocopherol acetate were prepared in methanol and kept in dark vials at 4° C. Working standard solutions for construction of calibration curves, were daily prepared by diluting the stock solutions in 2%(v/v) water in methanol containing 50 ppm butylated hydroxytoluene (BHT, added for preventing auto-oxidation of the TOHs and TAc).

3.3.2.4. Vitamin E supplement samples : Natural Mixed tocopherols capsule and Natural vitamin E-oil

Three capsules of Natural Mixed tocopherols were individually prepared for analysis by CEC by dissolving each capsule of the sample in 10.00 mL methanol. The stock sample solution was serially diluted two fold or one hundred fold with 2%(v/v) water in methanol containing 50 ppm of BHT for the analysis of β-, γ-, δ-TOHs or α-TOH, respectively. The average values from the three capsules for each tocopherol were reported as representative data.

For natural vitamin E oil, 0.03xx g of the sample was dissolved in 10.00 mL methanol containing 50 ppm BHT. One hundred microliter of the sample solution was diluted with 900 μL of 2%(v/v) methanol:water before injected into the CEC.

Percent recoveries for TOHs and TAc in the samples were carried out by spiking known concentration of each standard TOH or TAc into the sample. The measured amount of spiked standard TOH and TAc was compared to that for the standard solutions.

3.3.2.5. Mobile phase for CEC experiment for separation of the TOHs and TAc

Mobile phase for separation of the TOHs and TAc was prepared by mixing 100 mM Tris buffer (pH 9.3) with acetonitrile or methanol in various ratios. The mobile phase was filtered through 0.45 μm membrane before use.

3.4 Experiment

3.4.1 Capillary pretreatment

Before preparing a monolith inside a fused silica capillary, the capillary has to be treated with a bifunctional ligand (3-(trimethoxysilyl)propyl methacrylate), γ-MAPS for providing anchor site for binding the monolith. The pre-treatment was carried out by flushing and filling the capillary with 1 M NaOH then sealing both ends of the capillary with rubber septa (Figure 3.1) and then kept at 100^0 C for 2 hr. The capillary was then washed with water and methanol for 30 min each. After that, the capillary was dried under a N_2 stream. The capillary was filled with silanisation solution containing 50% (v/v) γ-MAPS in methanol, sealed with rubber septa and kept at 60^0 C overnight. After silanisation, the capillary was rinsing with methanol and dried by purging with nitrogen gas. The capillary was then ready for preparation of monolith.

Figure 3.1 Capillary containing polymerisation solution sealed off with rubber septa.

3.4.2 On-line coupling of MAA-EDMA monolithic solid phase extraction with capillary electrophoresis

The polymerisation composition for synthesis of the MAA-EDMA for on-line coupling with capillary electrophoresis was carried out, according to the method described by Y. Fan *et al.* [145], with slight modification of the composition:

Monomers (33%(w/w) compared with porogen)

: 0.4270 g EDMA (90%)

: 0.0481 g MAA (10%)

Porogen (67% (w/w) compared with monomer)

: 0.1175 g toluene (12%)

: 0.8628 g decanol (88%)

Initiator : 0.0045 g AIBN (1% (w/w) compare with monomer)

Note: EDMA = ethylene dimethyacrylate, MAA = methacrylic acid, AIBN = azobisisobutylronitrile

The solution was mixed ultrasonically until a homogenous solution was formed. Subsequently, the solution was purged with nitrogen gas for 3 min to remove oxygen before aspirating into the capillary. Both ends of the capillary were sealed with rubber septa and then the capillary was kept at 60^0 C overnight for polymerisation. The capillary was then washed with methanol for 1 hr. A five centimeters length of monolithic capillary was cut and connected to the separation capillary via a short piece of PTFE tubing. A short PTFE piece (~0.3 cm length, 254 µm ID x 1.56 mm OD) was drilled with 400 µm OD drill bit. A short piece of monolith and fused silica CE capillary were then inserted into the PTFE tubing for on-line coupling as shown in Figure 3.2.

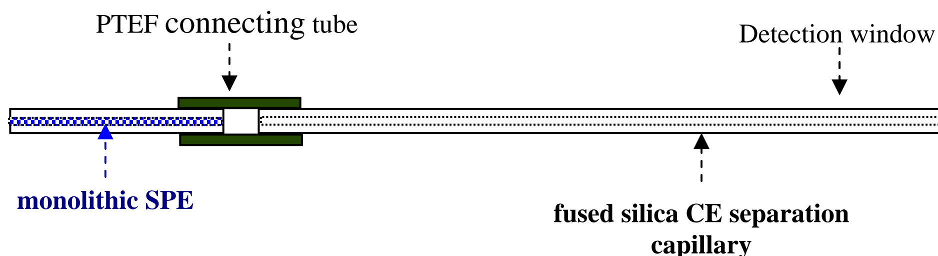


Figure 3.2 Schematic diagram of on-line monolithic SPE-CE system.

3.4.3 In-line coupling of MAA-EDMA and BMA-EDMA monolithic solid phase extraction with capillary electrophoresis

A 75 μm ID fused silica capillary was first pre-treated with the bi-functional reagent, γ-MAPS as described in section 3.4.1.

The preparation of the MAA-EDMA monolithic SPE was then carried out, according to the composition previously described in section 3.4.2. The polymerisation mixture was filled into one end of the γ-MAPS pre-treated capillary by means of capillary action, to a depth of approximately 5 cm (i.e., a length of 5 cm from the filling end). Both ends of the capillary were sealed with rubber septa, and the capillary then placed in an oven at 60° C overnight for polymerisation. After the polymerisation, the capillary was washed with MeOH for 1 h. A small section of the monolith-containing end of the capillary was cut off to give a 1-cm long monolithic SPE phase in a 33-cm total length capillary.

A BMA-EDMA monolith was prepared using the same method as for the MAA-EDMA monolith, the polymerisation composition consisting of 0.1414 g EDMA, 0.1799 g BMA, 0.0032 g AMPS, 0.0721 g water, 0.4460 g 1-propanol, 0.2055 g 1,4- butanediol and 0.0050 g AIBN. Simple diagram of the in-line monolithic SPE-CE is shown below.

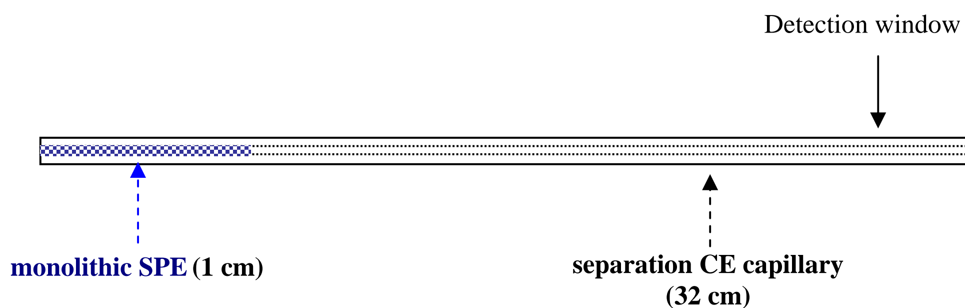


Figure 3.3 Schematic diagram of in-line monolithic SPE-CE system.

3.4.4 In-line coupling of packed particle silica based solid phase extraction with capillary electrophoresis

A 100 µm ID fused silica capillary was packed with 5 µm Spherisob ODSII as shown in Figure 25. One end of the capillary was immersed into a silica slurry and the resulting wedge of silica heated in order to make a retaining frit. The stationary phase packing suspension in acetone was then pumped into the capillary at high pressure (about 8,000 psi) using an ultrasonic probe as described by Boughtflower et al [146] (step I, Figure 3.4). The packed capillary was subsequently washed with water (step II). Two frits were created at another position (near the end connected to the pump) in order to make a 0.5 cm packed SPE (step III). The unwanted packing was then removed by eliminating the first frit at the outlet side (step IV) then cut to obtain a 33 cm long capillary. A detection window was then made at 8.5 cm from the outlet end (step V).

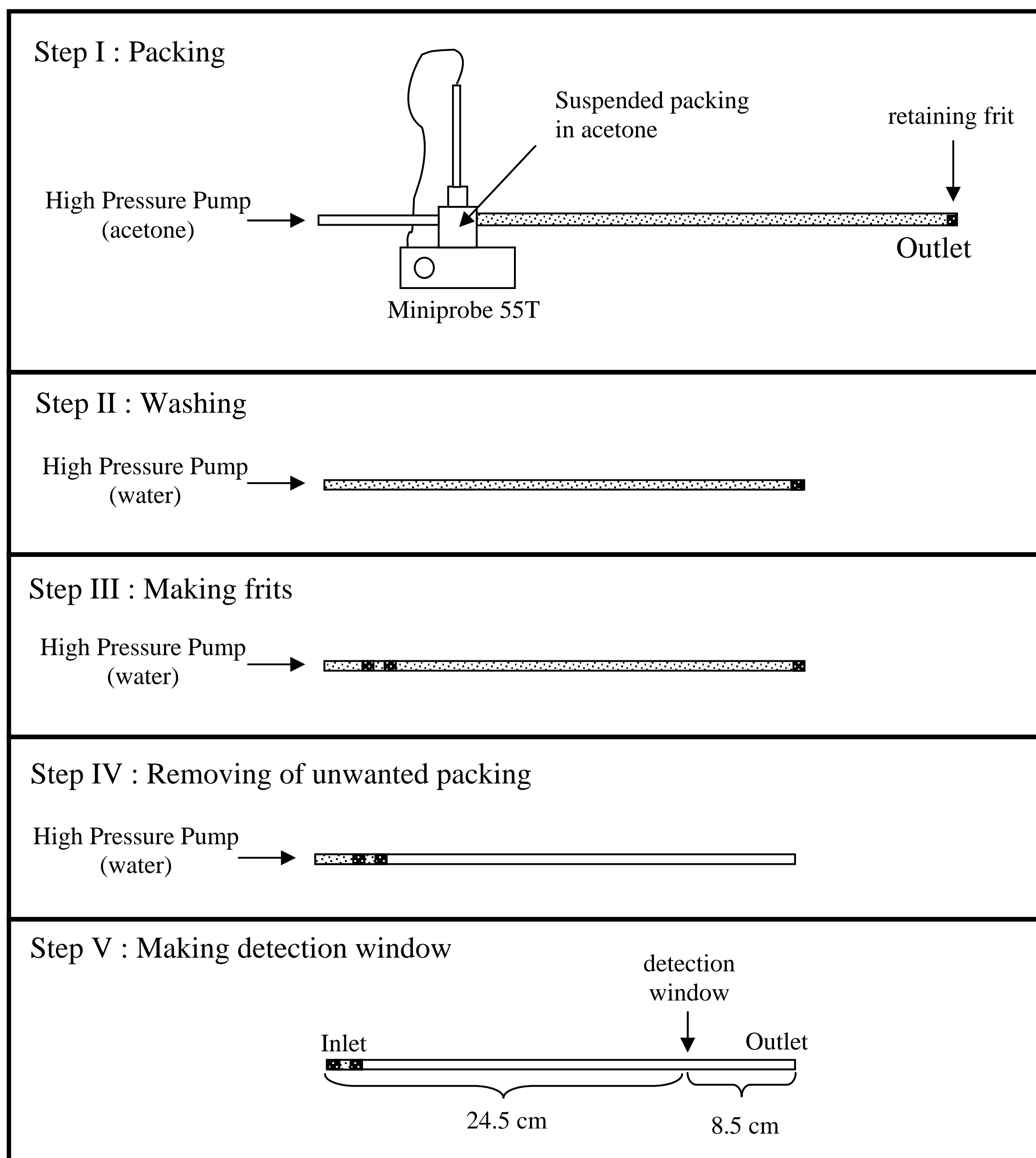


Figure 3.4 Procedure for making a packed particle SPE capillary.

3.4.5 Synthesis of PEDAS-EDMA monolith

Preparation of the PEDAS-EDMA monolith was carried out, according to the method previously reported by Bedair and Rassi [144], with slight modification. The polymerisation composition is shown below.

Monomers (30%(w/w) compared with porogen)

: 0.3325 g pentaerythritol diacrylate (PEDAS) (95%)

: 0.0175 g ethylene dimethacrylate (EDMA) (5%)

Porogen (70% (w/w) compared with monomer)

: 0.5148 g pentanol (79.2%)

: 0.1118 g ethylene glycol (17.2%)

: 0.0234 g water (3.6%)

Initiator : 0.0035 g AIBN (1% (w/w) compare with monomer)

The solution was homogenised by sonication. The solution was then purged with nitrogen gas for ~ 5 min and subsequently filled into a 100 µm id γ-MAPS pre-treated capillary. Both ends of the capillary were then sealed with rubber septa, and kept overnight at 60° C for polymerisation. After polymerisation, the capillary was washed with methanol for 1 hr. A detection window was made by pyrolysing the monolith at the outlet end to give a capillary 32 cm long with a 23.5 cm effective separation length.

3.4.6 Column packing procedure for C18 packed particle (ODS I) capillary column

Commercial 3 µm C18-silica particles (ODS I) were packed into a 100 µm id capillary by following the procedure of Boughtflower *et al.* [146]. One end of the capillary was immersed into a silica slurry and the resulting slug of silica was heated in order to make a retaining frit. 3 µm ODS I particles were suspended in acetone and then pumped into the capillary under high pressure (about 8,000 psi) with the aid of an

ultrasonic probe. The second frit was made by heating part of the packed capillary near the outlet end. The capillary was subsequently washed with water to remove extra particles outside the two frits. The detection window was made near the outlet frit to give a capillary 32 cm total with 23.5 cm effective separation length. Schematic of the procedure for packing ODS II column is shown below.

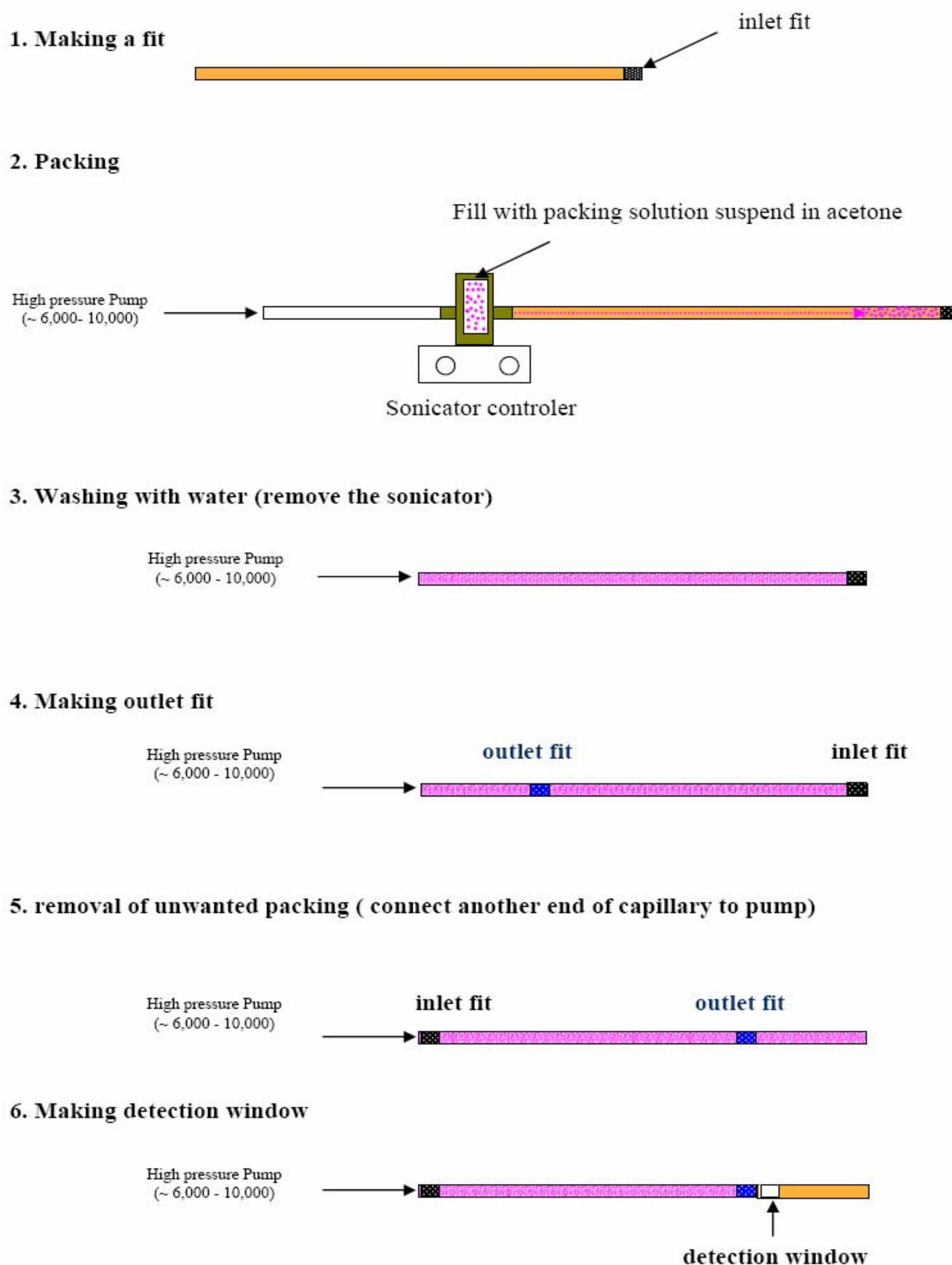


Figure 3.5 Procedure for preparation of packed column.

3.4.7 Making of detection window

Before installing a capillary into the CE or CEC instrument with UV-Vis detection, a clear detection window is required. The window was generally made near the outlet end. For HP^{3D} capillary electrophoresis used in this work (Hewlett Packard, Germany), the detection window must be made 8.5 cm from the outlet end. In this work, a detection window was achieved by heating the capillary with a burner as, shown below in Figure 3.6.

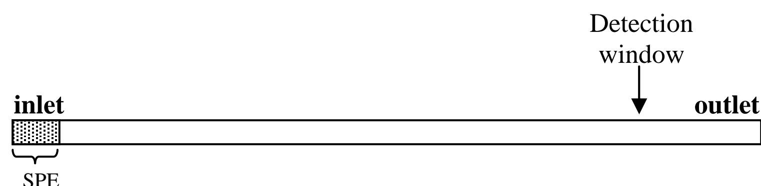
Figure 3.6 Procedure for making detection window.

3.4.8 Operational steps for investigation of SPE

The monolithic and particle packed SPEs were examined by using the CE equipment in a non-CE mode (no applied voltage). Experiments comprised of a conditioning, washing, loading and elution step. Nitrogen gas was used for propelling solutions through the SPE capillaries. Since all operational steps were performed by means of applying pressure (8 bars), the applied pressure was also monitored during the experiment in order to confirm the applied pressure was constant from run to run. All experiments were carried out at 25^{0} C and the monitored UV absorbance at the wavelength of 200 nm. Studies of the SPEs were carried out for repeatability and eluting solvents (section 3.4.8.1 and 3.4.8.2).

3.4.8.1 Repeatability test

Repeatability of the SPE for pre-concentration and extraction were examined by extracting the compounds and then comparing the observed signal for each extraction. The repeatability was studied for two systems with operational steps as shown in Figure 3.7. The two systems differ at only in the solvent used for sample loading and washing. In the first system, water was used as the solvent while 10% (v/v) acetonitrile : water was employed for the second system.



Operational steps ; Conditioning : 3 min with methanol

Washing : 2 min with water (or 10% (v/v) ACN : water for the second system)

Loading : 1 min loading of 100 ppm standard solution of TER, HMMA or BZ in water (or in 10% (v/v) acetonitrile : water for the second system)

Elution : elution with 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3)

Figure 3.7 Operational steps of the SPE experiment.

3.4.8.2 Eluting solvent study

In this part, eluting solvent for extraction of the three compounds, Bz, HMMA and TER was examined. The experiment was similar to that given in section 3.4.8.1 in which 10% (v/v) acetonitrile : water was used as solvent for washing and loading of the standard solution (Figure 3.7).

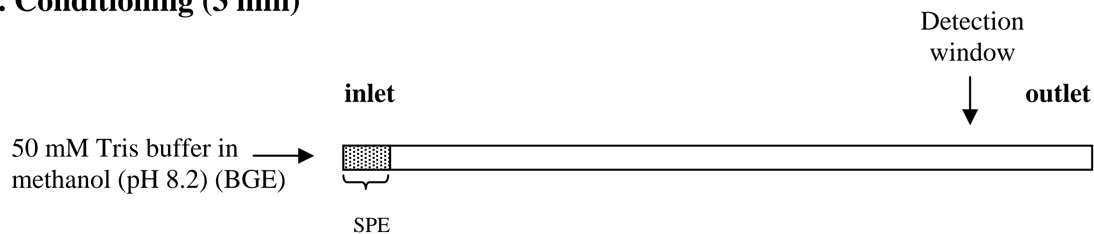
Methanol, acetonitrile, 50 mM Tris in methanol (pH 8.2), 70% (v/v) acetonitrile: water and 70% (v/v) acetonitrile: 50 mM phosphate buffer (pH3) were studied for eluting the trapped compounds from the SPEs.

3.5 Operational steps for in-line polymer based monolithic solid phase extraction-non-aqueous capillary electrophoresis

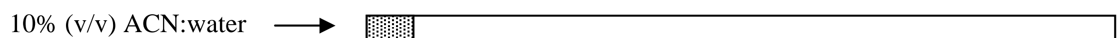
The MAA-EDMA monolithic SPE was in-line coupled with CE as described in section 3.4.3. Operational steps for this experiment are shown in Figure 3.8.

Before extraction, the SPE was conditioned with methanol and then washed with water. Standard mixture of 3 ppm TER,HMMA and 1 ppm Bz in 10%(v/v) acetonitrile:water was loaded into the SPE. The CE employed non-aqueous 50 mM Tris in methanol (pH 8.2) as running buffer or background electrolyte (BGE). Since the BGE can elute the trapped compounds (Bz, HMMA and TER) from the SPE, the BGE was filled into the capillary from the outlet end after the loading step. The BGE was then filled in to the inlet end in order to elute the compounds. An applied high voltage of +25 kV was used for the CE separation.

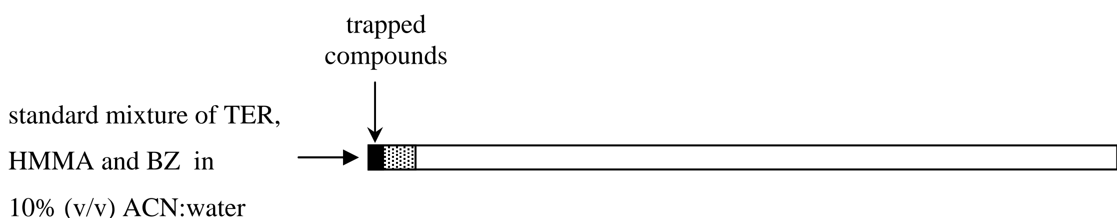
I. Conditioning (3 min)



II. Washing (1 min)



III. Loading of the compounds



IV. Filling in the running buffer

V. Elution by the running buffer (BGE)

VI. Applying the separation voltage

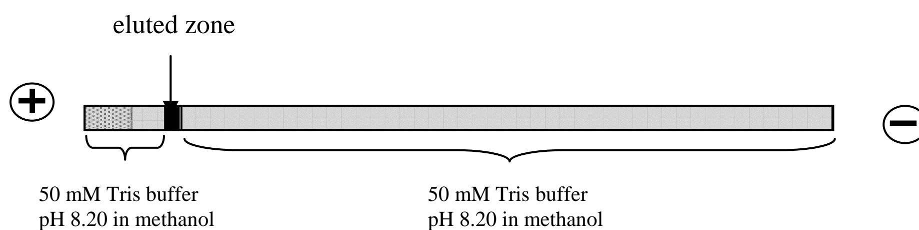


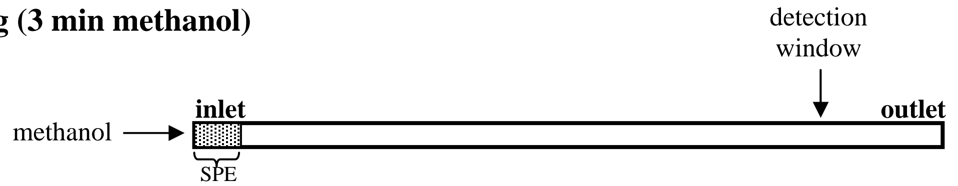
Figure 3.8 Operational steps for use of the in-line monolithic SPE-CE.

■ sample zone □ running buffer ▒ SPE part

3.6 Operational steps for in-line packed particle solid phase extraction -aqueous capillary electrophoresis

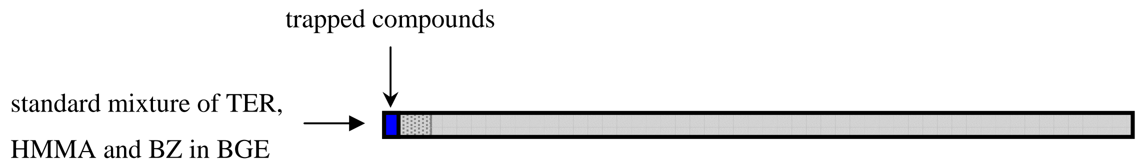
For in-line coupling of packed particle SPE with CE, silica based packing was *in situ* packed inside a fused silica capillary as described in Section 3.4.6. The operational step consisted of 5 steps (Figure 3.9). The first two steps were conditioning with methanol and washing with water. In step III, the standard mixture of 3 ppm TER, 0.5 ppm HMMA and 1 ppm BZ in BGE (10 mM phophaste buffer (pH 7)) was loaded into the SPE. The next step was elution of the trapped compounds by filling the inlet end of the capillary with 70% (v/v) ACN : 50 mM phosphate buffer (pH 3). High voltage of +10 kV was finally applied in order to carry out the CE separation.

I. Conditioning (3 min methanol)



II. Washing (1 min)

III. Loading of the compounds



IV. Elution by the running buffer (BGE)

V. Applying the separation voltage

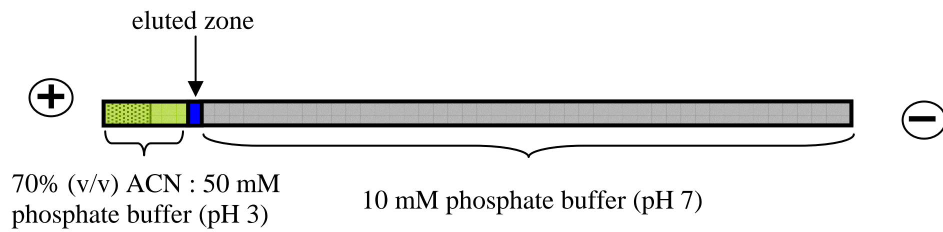


Figure 3.9 Operational step for the in-line packed particle SPE-CE.

sample zone running buffer SPE part Eluting solvent

3.7 CE experiment for separation of TER, HMMA and Bz

In order to compare chromatography of TER, HMMA and Bz between normal CE without SPE enrichment and the coupling SPE-CE, CE experiment were performed on a 100 μm i.d. with 33 cm long fused silica capillary in both aqueous and non-aqueous CE modes.

The new capillary was conditioned with, 1M NaOH for 40 min, then 0.1 M NaOH and finally DI water for 10 min each.

3.7.1 Aqueous CE

Before each injection the capillary was pre-conditioned with 0.1 M NaOH, DI water and running buffer or background electrolyte (10 mM phosphate buffer pH 7) for 2 min each. Injection was by hydrodynamic injection at 5 mbar for 5 second. The separation was carried out at of +10 kV, at 25^0 C.

3.7.2 Non-aqueous CE

For non-aqueous CE, the capillary was conditioned with 0.1 M NaOH, DI water and running buffer or background electrolyte for 2 min each before each injection. In this experiment, 50 mM Tris in methanol (pH 8.2, apparent pH in methanol medium) was used as background electrolyte. Hydrodynamic injection was performed at 10 mbar for 5 second. The separation was carried out at +25 kV and 25^0 C.

3.8 CEC experiment for separation of TOHs and TAc

Both monolithic and packed columns were conditioned with mobile phase prior to use by applying a voltage of 5 kV for 5 min, then 30 kV for 30 min or until a stable current and UV baseline were observed. For separation of the tocopherols and tocopherol acetate, 30 kV was employed. A pressure of 8 bars was applied to both ends of the capillary during separation for the packed ODS I column in order to

prevent bubble formation. Sample introduction was performed by electrokinetic injection at 10 kV for 30 seconds. All experiments were performed at 30^0C. The signal was monitored at 200 nm.

CHAPTER IV
Results and Discussion

Due to the unique advantages of monolithic materials over conventional packed based materials, monolithic stationary phases have been increasingly used in chromatography for both sample preparation (pre-concentration/extraction or sample clean up) and separation purposes.

In this thesis, two investigations of monolithic materials were examined. The first was the investigation of in-line coupling of monolithic solid phase extraction (SPE) to capillary electrophoresis (CE) for pre-concentration and extraction. The work also employed conventional packed silica SPE. The second was using a monolithic column as stationary phase for the separation of vitamin E in the forms of tocopherols and tocopherol acetate by capillary electrochromatography (CEC). The method was applied to analysis of commercial vitamin E supplements.

4.1 Part I: The investigation of polymer based monolithic and silica based packed particle phases for pre-concentration/extraction purposes by in-line coupling with capillary electrophoresis

Since monolithic materials have been shown to have high porosity with high chromatographic efficiency and can be prepared *in situ* in a fused silica capillary, it is therefore attractive for use as an in-line SPE for sensitivity enhancement in CE. Due to the high porosity of the monolith and also ease of *in situ* preparation, the monolithic SPE can be coupled with CE without the need of any instrumental modification, eliminating leakages which is usaully found with packed SPE-CE systems [82].

4.1.1 Connection for coupling a monolithic SPE to a CE capillary

The first study was to find an effective method for coupling a monolithic SPE to a CE separation capillary.

Two types of approaches, on-line and in-line couplings were investigated. Both could be carried out without any need of instrumental adaptation or modification. The coupled SPE-CE capillaries were easily installed into the commercial CE instrument.

4.1.1.1 On-line coupling

For on-line coupling of monolithic SPE-CE, a short piece of fritless monolithic SPE was simply connected to a CE separation capillary via a PTFE tube as shown in Figure 31 (described in section 3.4.2). The connecting PTFE sleeve (0.25 mm ID) was drilled out with a drill bit (0.40 mm) in order to perfectly connect the monolithic SPE and CE capillaries.

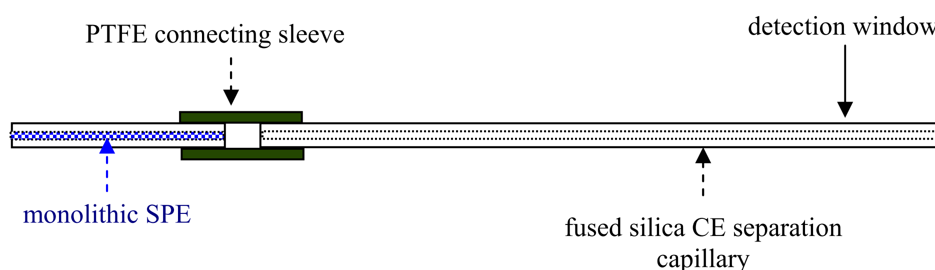


Figure 4.1 Schematic diagram of on-line monolithic SPE-CE system.

With this approach, the system was flexible for replacing the monolithic SPE or CE separation capillary. The coupled capillary was then tested for electrical connection, leakage and bubble formation when applying a high voltage.

The experiment was set up by connecting a methacrylic acid-ethylene dimethacrylate (MAA-EDMA) monolith to a CE separation capillary via a short PTFE

connecting sleeve. After installing the coupled capillaries into the CE instrument, the capillary was filled with a buffer (phosphate buffer (pH 11)) by applying nitrogen gas at the inlet end immersed in the buffer vial. After filling, both ends of the capillary were immersed in the running buffer vials. A high voltage of +20 kV was then applied across the ends of the capillary. The current, which indicates stability and electrical cennection of the system, was monitored during application of the voltage.

It was found that loss of electrical connection was observed when using phosphate buffer at a concentration higher than 10 mM (see Figure 4.2). Due to the high ionic strength, the high joule heating lead to bubble formation. Thus separation of the test compounds could not be achieved with a buffer concentration higher than 10 mM.

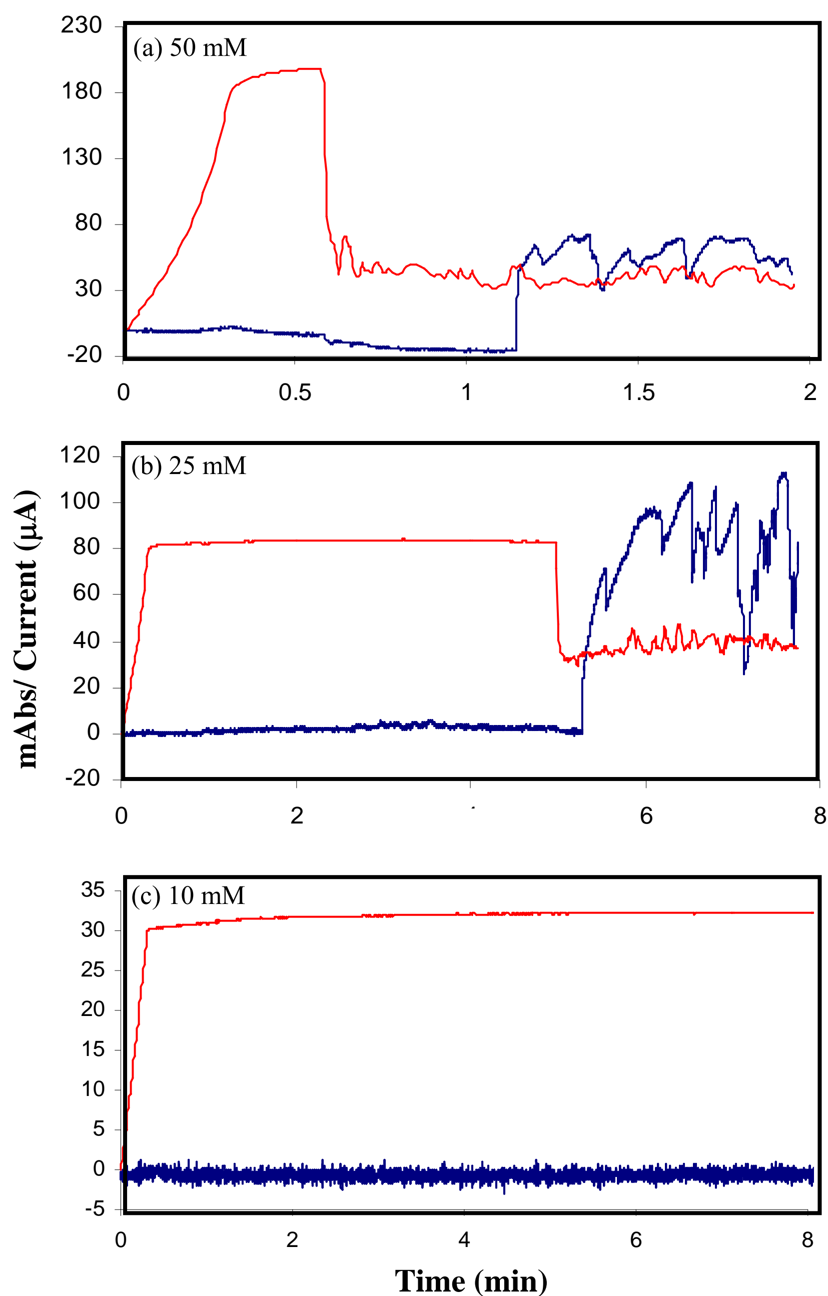


Figure 4.2 Current and UV signal profiles obtained from (a) 50 mM, (b) 25 mM and (c) 10 mM phosphate buffer (pH 11) using a 34 cm x 50 µm i.d. capillary, at applied voltage +20 kV, and 25 °C. (——— absorbance at 200 nm, ——— Current).

The on-line coupling method was prone to problems from bubble formation or leakage leading to loss of electrical connection. Therefore an in-line coupling of monolithic SPE-CE was investigated.

4.1.1.2 In-line coupling of monolithic SPE to CE

In order to overcome the problem found with the on-line coupling system, an in-line coupling method was studied. The in-line coupling was carried out by *in situ* preparing of the MAA-EDMA monolith inside a fused silica capillary after treating the capillary with bi-functional ligands in order to provide sites for attachment of the monolith to the capillary surface, as described in section 3.4.1. Since the monolith is strongly bound on the capillary surface, the monolith can be prepared inside the CE separation capillary with no connection required. Problems from bubble formation or leakage should thus be eliminated. Moreover the method was simpler. A simple schematic diagram of the monolithic SPE-CE is shown in Figure 4.3.

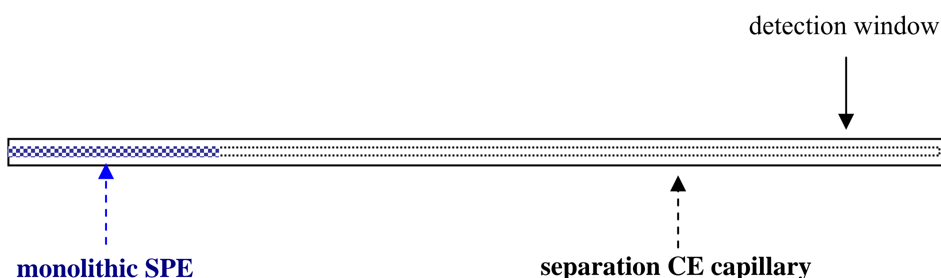


Figure 4.3 Schematic diagram of in-line monolithic SPE-CE system.

For this system, a stable baseline and current were observed even at high concentrations of running buffer without break of electrical connection as shown in Figure 4.4. This coupling approach was therefore chosen for further studies.

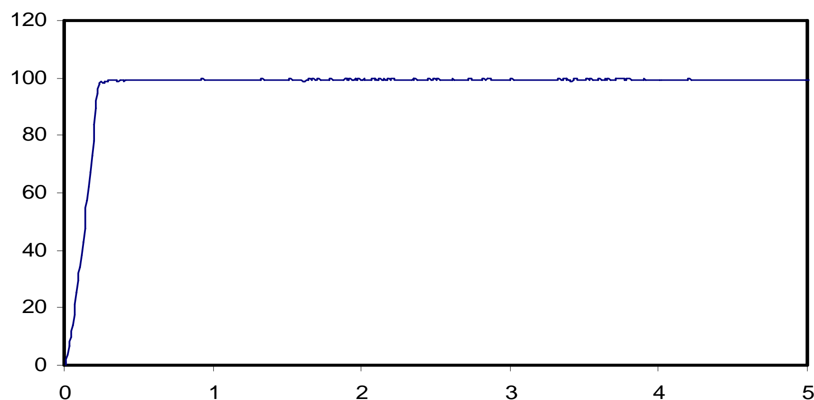
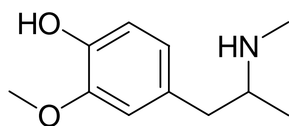
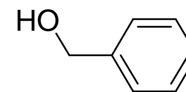


Figure 4.4 Current signal obtained with 25 mM phosphate buffer (pH 11) using a 34 cm x 50 µm i.d. capillary, applied voltage +20 kV, 25 ^{o} C.

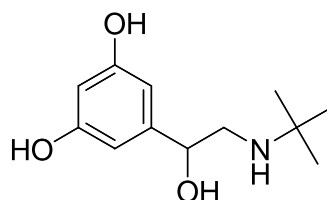
4.1.2 Monolithic solid phase extraction (monolithic SPE)

In this part of the study, methacrylate based monoliths, methacrylic acid-ethylene dimethacrylate (MAA-EDMA) and butyl methacrylate-ethylene dimethacrylate (BMA-EDMA), were investigated for use as a pre-concentration column or SPE of three model compounds, benzyl alcohol (Bz) terbutaline (TER) and 4-hydroxy-3-methoxy-methamphetamine (HMMA) (see Figure 4.5 for chemical structures). The MAA-EDMA and BMA-EDMA monolithic SPE capillaries were prepared *in situ* in a fused silica capillary as described in section 3.4.3. The chemical formulae of the monomers used for the two monolithis are shown in Figure 4.6.



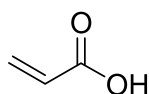


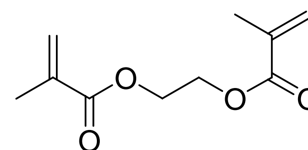
4-hydroxy-3-methoxy-methamphetamine (HMMA) Benzyl alcohol (Bz)



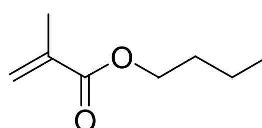
Terbutaline (TER)

Figure 4.5 Chemical structures of the three model compounds, BZ, HMMA, and TER.





(a) methacrylic acid (MAA) (b) ethylene dimethacrylate (EDMA)



(c) butyl methacrylate (BMA)

Figure 4. 6 Chemical structures of monomers used for MAA-EDMA and BMA-EDMA monoliths. (a) MAA, (b) EDMA, (c) BMA.

Note : MAA-EDMA monolith provides EOF but there was no EOF from the neutral co-polymeric monolith, BMA-EDMA

4.1.2.1 Performance study of the monolithic SPE

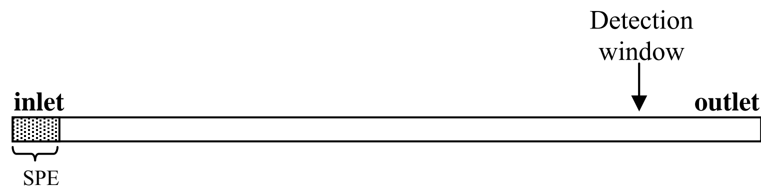
The aim of this experiment is to examine the stability and performance of the MAA-EDMA and BMA-EDMA monolithic SPEs for extraction of Bz, HMMA, and TER. Microscopic picture of the synthetic MAA-EDMA monolithic SPE is shown below.

Figure 4.7 Microscopic picture of the in-line monolithic SPE-CE capillary.

Before carrying out the stability and performance tests, the mechanical strength of the monoliths was first tested by connecting the monolithic SPE capillaries to a high-pressure pump and pumping with methanol. A pressure of up to 200 bars was applied to test the bonding strength of the monolith to the capillary. No movement of the monoliths from the capillary wall was observed from monitoring with a microscope, indicating strong bonding of the monoliths to the inner surface of the capillary.

The two monoliths were then examined for their potential to extract the test compounds. The experiment is described in section 3.4.8.1. Briefly the SPE was first conditioned with methanol then washed with water. After the washing step, aqueous standard solutions of the compounds were individually loaded onto the SPE for extraction/pre-concentration purposes. The trapped compounds were then eluted with the eluting solvent. All the solutions were filled into the capillary by means of

applied pressure (nitrogen gas) at 8 bars. UV absorbances of the compounds were monitored at 200 nm.



Operational steps ;

Conditioning	:	3 min methanol
Washing	:	2 min water
Loading	:	1 min 100 ppm aqueous standard solution of TER, HMMA or Bz
Elution	:	elution with 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3)

Figure 4.8 Operational steps and simple diagram of the SPE experiment.

For both the MAA-EDMA and BMA-EDMA monoliths, it was observed that large peaks were observed for each compound due to trapping of the compounds on the monoliths, resulting in pre-concentration and a greater signal. However non-reproducible results for elution time and peak area of the three compounds for both monoliths were observed (Figure 4.9A and 4.9B). Shifting of elution times and smaller peaks were observed. The experiments were repeated three times, but similar results were still observed. When ploting the elution time with the number of injection, the elution time was shown to increase in a linear manner as shown in Figure 4.10.

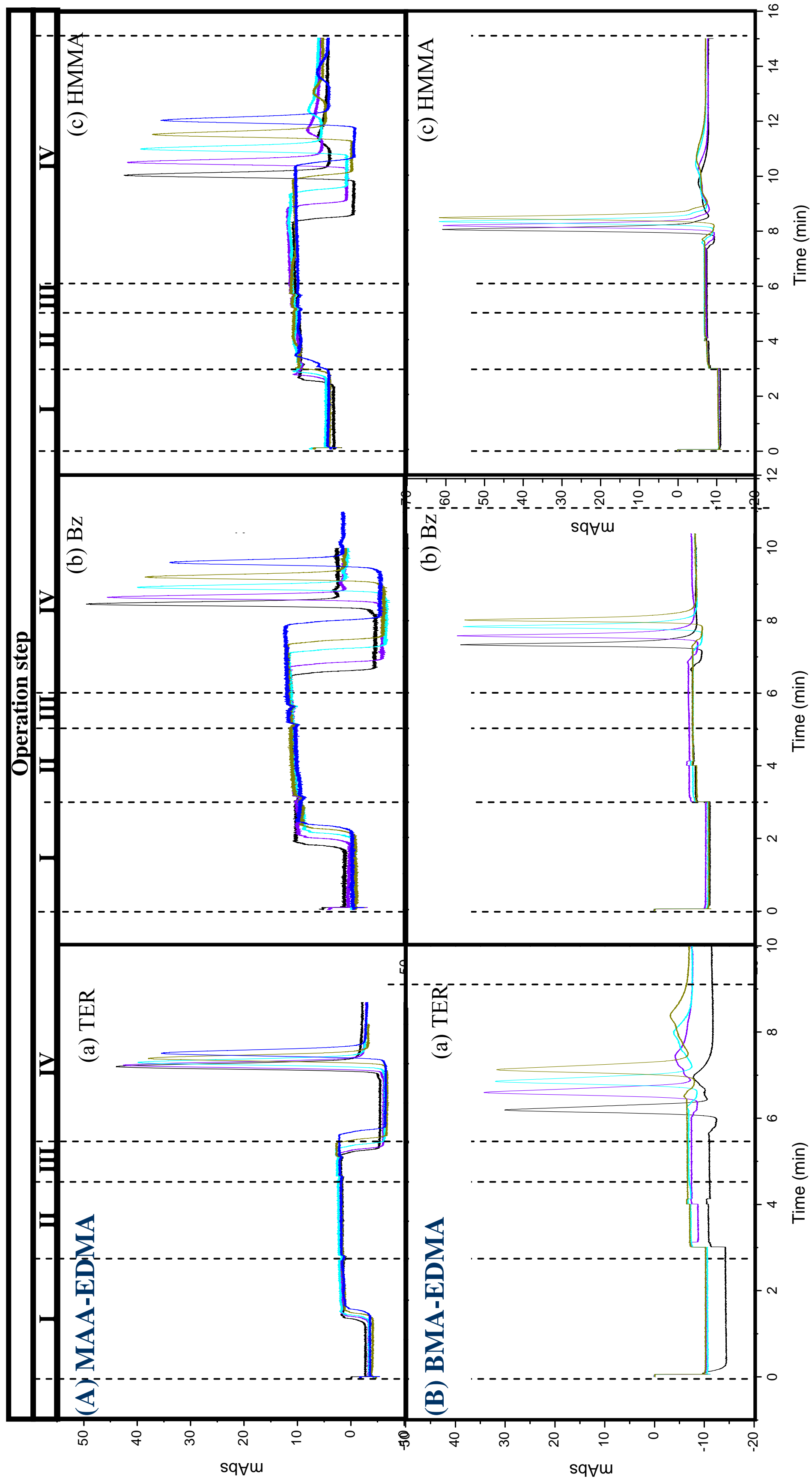


Figure 4.9 Replicate injections of 100 ppm TER, Bz and HMMA in water for (A) MAA-EDMA and (B) BMA-EDMA monolithic SPEs. Other conditions are given in Figure 4.8 — 1st, 3rd, 5th, 7th, 9th injection.

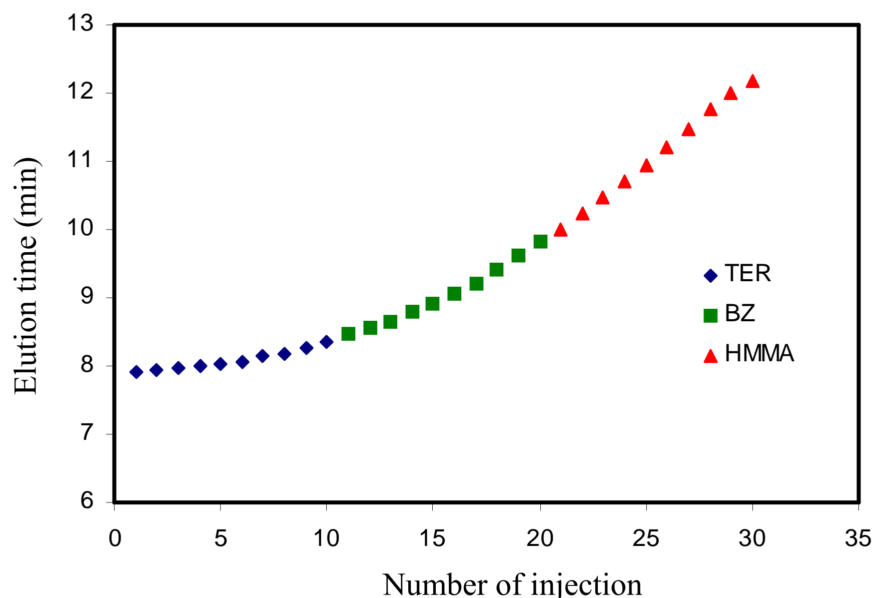


Figure 4.10 Elution time for TER (1st-10th injection), Bz (11th-20th injection) and HMMA (21st-30th injection) from the MAA-EDMA monolith at each replicate injection. Conditions are shown in Figure 4.9.

However it was found that the repeatability of the monoliths could be improved by using an aqueous medium containing at least 10% (v/v) of organic solvent such as methanol or acetonitrile. The experiments were carried out similar to the previous experiment but the three compounds were individually dissolved in 10% (v/v) acetonitrile: water for loading to the monolithic SPEs. Better repeatability for both MAA-EDMA and BMA-EDMA monoliths were observed as shown in Figure 4.11A and 4.11B. RSD% for elution time and peak area using these conditions for the three compounds were less than 0.6% and 5.4% for the MAA-EDMA and 0.5 and 3.5% for the BMA-EDMA respectively.

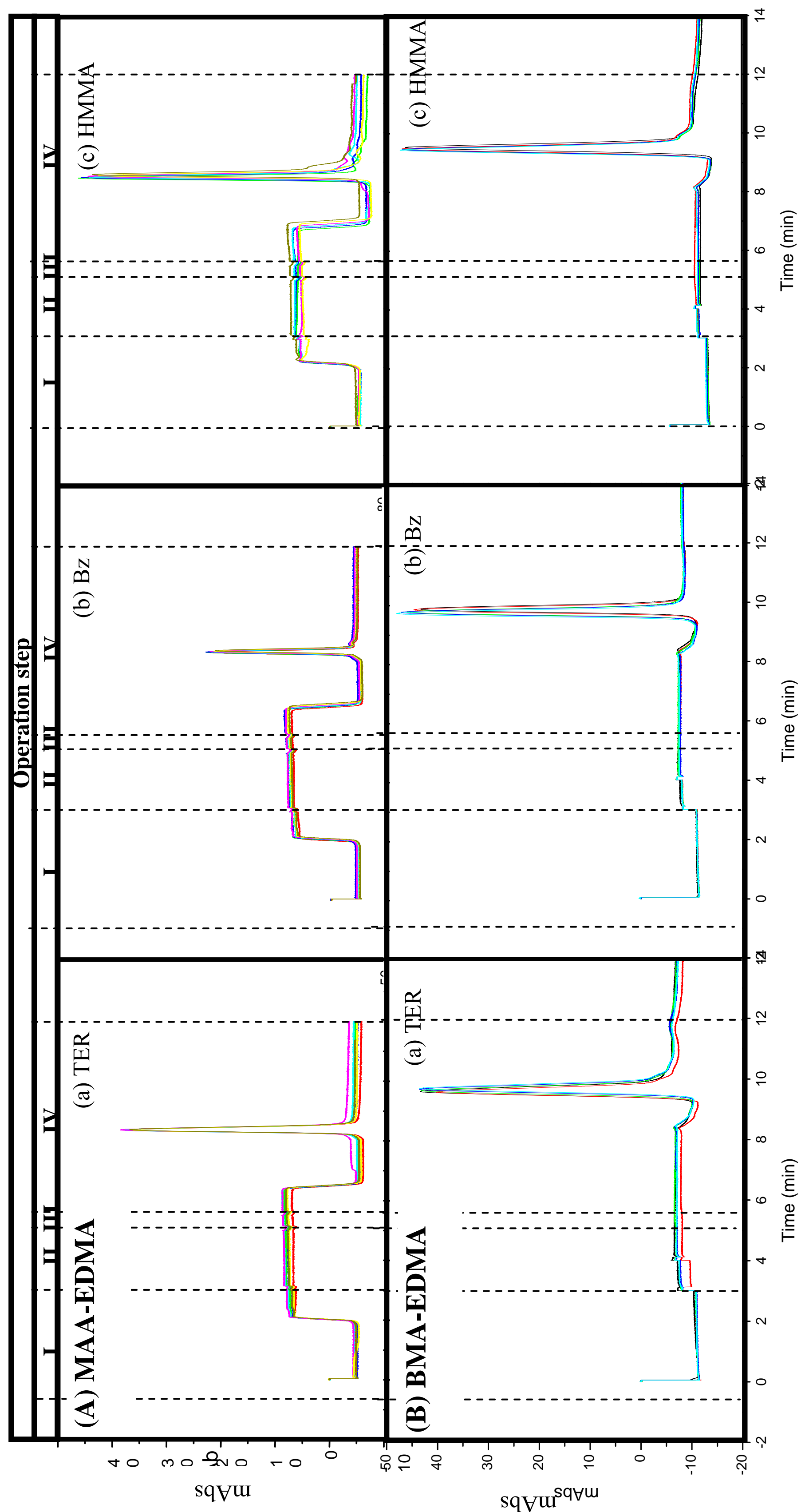


Figure 4.11 Seven replicate injections of 100 ppm TER, Bz and HMMA (A) MAA-EDMA and (B) BMA-EDMA monolithicSPEs.

Condition ; same as in Figure 39 but 10%(v/v) acetonitrile:water was used as washing and loading solvents.

4.1.2.2 Eluting solvent studies

As shown in Section 4.1.2.1 the two monoliths were not compatible with pure aqueous medium. The next experiment to find a suitable eluting solvent was therefore carried out by keeping the amount of organic solvent in all the solutions at least 10% (v/v). After loading of the compounds, various solvents were examined for eluting the trapped compounds, including acetonitrile, methanol, Tris-methanol solution and mixture of acetonitrile and phosphate buffer (pH 3).

For MAA-EDMA monolith (Figure 4.12), it was found that Bz could be eluted by all the eluting solvents (Figure 4.12(a)). In contrast, acetonitrile, methanol and mixtures of acetonitrile and water did not give a good elution peak or complete elution of TER and HMMA from the SPE (Figure 4.12 (b and c)). It was observed that complete elution of compounds could be achieved by adding salt to the pure methanol (Tris-methanol solution) or using a solution of acetonitrile and 50 mM phosphate buffer (pH 3). These results indicated that under the employed condition, interaction of the Bz on the monoliths was mainly hydrophobic while both hydrophobic and coulombic interactions are involved in the retentions of TER and HMMA on the MAA-EDMA monolith. The coulombic interaction between the two compounds and the monolith could be due to the polar carboxylic/ester groups of the monolith (see Figure 4.6a and b).

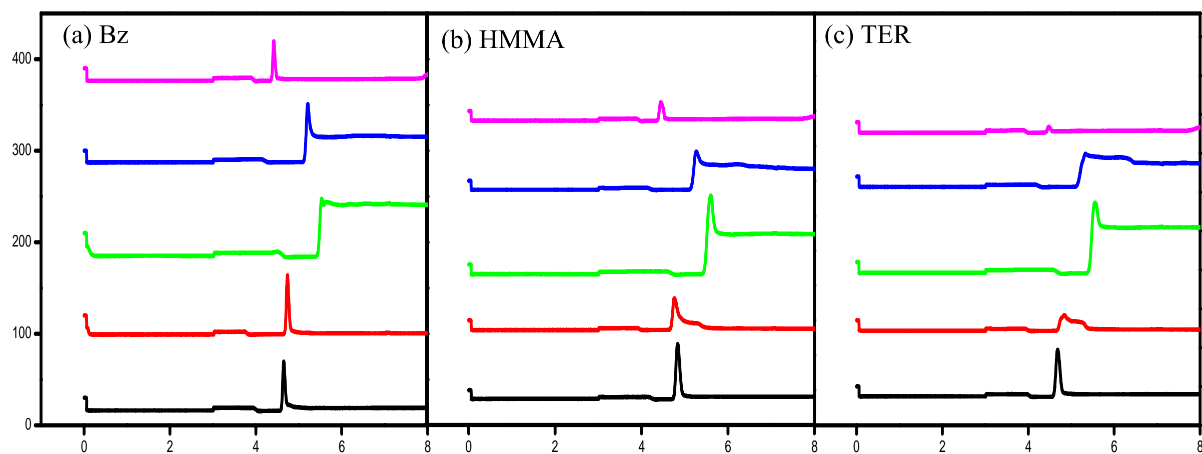


Figure 4.12. MAA-EDMA monolith. Signal profiles of 100 ppm (a) Bz, (b) TER and (c) HMMA eluted by eluting solvents. Condition; 3 min conditioning with acetonitrile, step II : 0.5 min loading of 100 ppm TER in 10% (v/v) acetonitrile:water, step III : eluted with investigated eluting solvents, step V : eluted with 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3).

70% (v/v) acetonitrile : 50 mM PPB pH 3

70% (v/v) acetonitrile : water

50 mM Tris buffer pH 8.2 in MeOH

MeOH

ACN

4.1.3 In-line coupling of monolithic pre-concentrator-CE

In this section, in-line coupling of the MAA-EDMA monolith as a pre-concentrator column for the CE system is described. As shown in the previous section both the BMA-EDMA and MAA-EDMA were not compatible with an aqueous medium, the MAA-EDMA was thus coupled to a non-aqueous CE in order to maintain the integrity of the monolithic SPE. The monolithic SPE was coupled to a CE system according to the method described in section 3.5 and shown below.

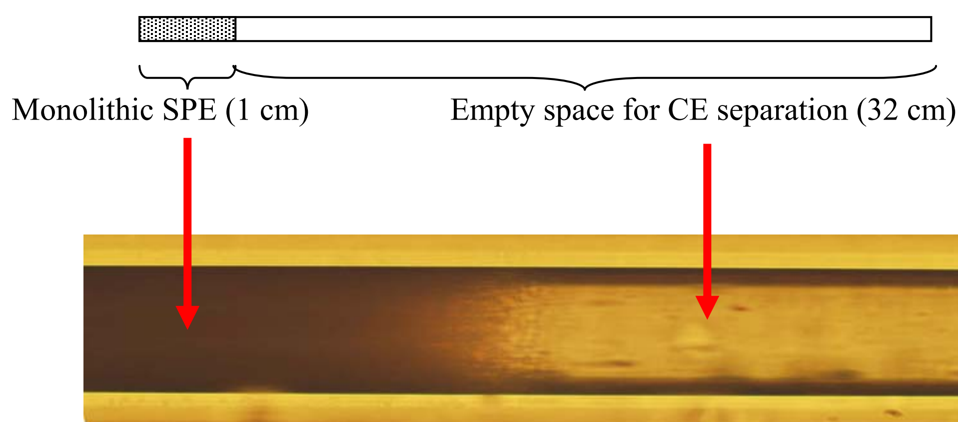
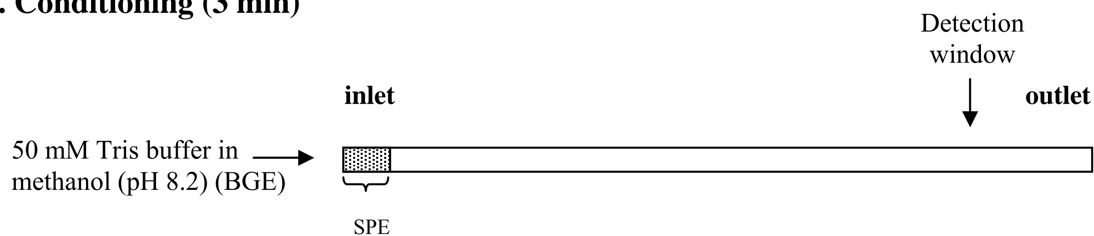


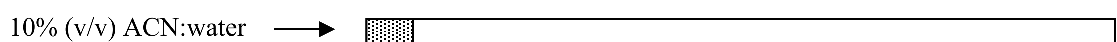
Figure 4.13 Microscopic picture of the in-line monolithic SPE-CE capillary (not to scale).

In order to simplify the coupling method, an eluting solvent with good separating properties is preferred so that filling of another separating solvent after elution can be eliminated. Although both 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3) and 50 mM Tris in methanol (pH 8.2) have been shown to be good eluting solvents for the three compounds, poor separation was obtained when using the 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3) as separating solvent. Better separation was observed from the non-aqueous solvent, 50 mM Tris buffer in methanol (pH 8.2). Therefore this solvent was selected as background electrolyte (BGE) for the CE separation of the coupling technique. The operational steps for the coupling technique consisted of 6 steps as shown in Figure 4.14.

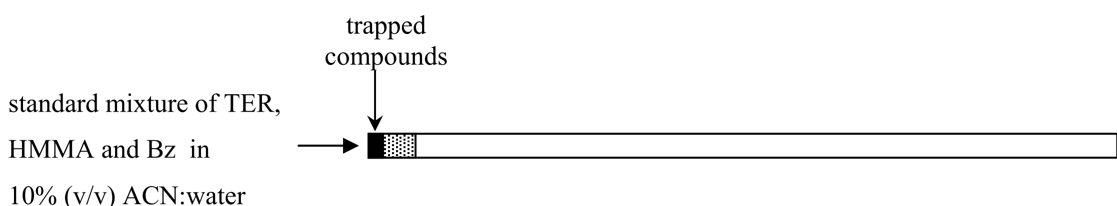
I. Conditioning (3 min)



II. Washing (1 min)



III. Loading of the compounds



IV. Filling of the running buffer

V. Elution by the running buffer (BGE)

VI. Application of the separation voltage

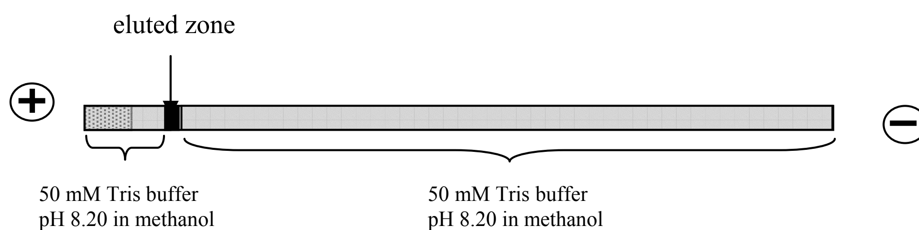


Figure 4.14 Operational steps for use of the in-line monolithic SPE-CE.

■ sample zone □ running buffer ▩ SPE part

In steps I to V (Figure 4.14), liquids were forced into the capillary by applying nitrogen gas at pressure of 8 bars. The first and second steps were conditioning and washing. The standard mixture of the three compounds in 10% (v/v) acetonitrile : water was then loaded into the SPE. After the loading step, the capillary had to be refilled in step IV with BGE (50 mM Tris buffer in methanol (pH 8.2)), prior to elution of the compounds from the SPE and subsequent CE separation. Since BGE acts as the separating and as eluting solvent, filling of BGE must be carried out from the outlet end. This is to prevent loss of the retained compounds from the SPE before the CE separation. In step V, the retained compounds were eluted from the SPE by flowing BGE into the inlet end. Step VI was the CE separation step, in which the dc voltage of +25 kV was applied and the nitrogen pressure no longer applied. For this set up, the filling time of BGE in step IV was examined.

4.1.3.1 Effect of filling time (in step IV, Figure 4.14)

In step IV, the BGE had to be filled from the outlet end of the capillary up to the SPE in order to prevent elution of the retained compounds from the SPE. However insufficient filling of the BGE will result in residual aqueous solution from the loading step. Figure 4.15 shows a schematic diagram of the SPE-CE in step IV and V corresponding to various filling times in step IV. The filling time in this step was investigated and the results are displayed in Figure 4.16.

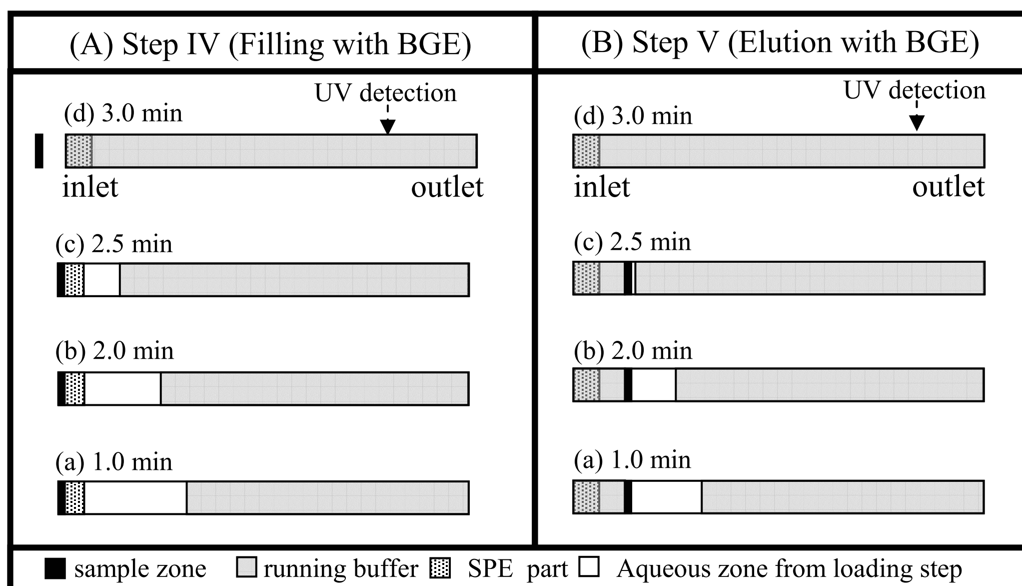


Figure 4.15 Schematic diagram of the SPE-CE in step IV and V (elution) in Figure 4.14.

Results in Figure 46 show that the separation improved with increase in the filling time (from 1.0 to 3.0 min). However filling at 3.0 min led to overfilling and the compounds are eluted out of the SPE monolith. No peaks are observed (Figure 4.16d and Figure 4.15A(d)). According to the electropherograms in Figure 46, a filling time of 2.5 min gave the best results for this experiment (Figure 4.16(c)). Under this condition, large signals for both TER and HMMA at concentration for which no signal for normal CE-UV (without the enrichment SPE) were obtained.

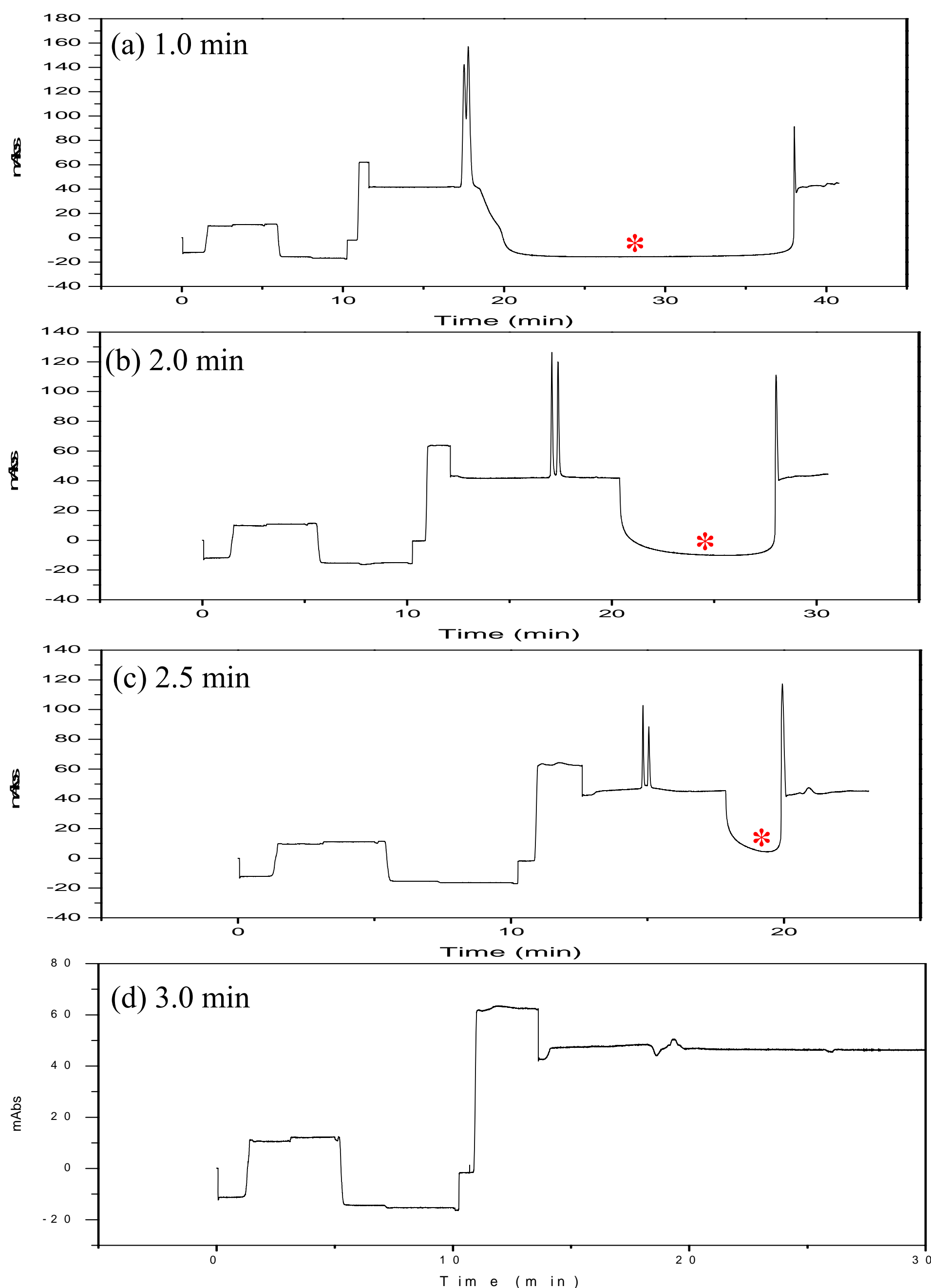


Figure 4.16. Electropherograms (a) showing the effect of BGE filling time of the SPE-NACE system. The results were obtained from injections of a mixture of 1 ppm of TER, HMMA, and Bz, carried out at 4 periods of filling times in step IV (1 to 3 min). Operating condition: step I, II, III and VI are the same as that shown in Figure 4.6; step IV: varied as depicted; step V: 0.3 min; applied voltage: +25 kV, 25 ^{o}C. (* represent the residue aqueous zone left from sample loading step).

4.1.3.3. Repeatability of the in-line monolithic SPE-CE for separation of Bz, HMMA and TER

As shown in the previous section the coupling technique gave a significant sensitivity enhancement for Bz, TER and HMMA. The repeatability of the method was then examined by repeated injecting of a standard mixture under the selected condition. Results for 8 replicate injections of the standard mixture are displayed below.

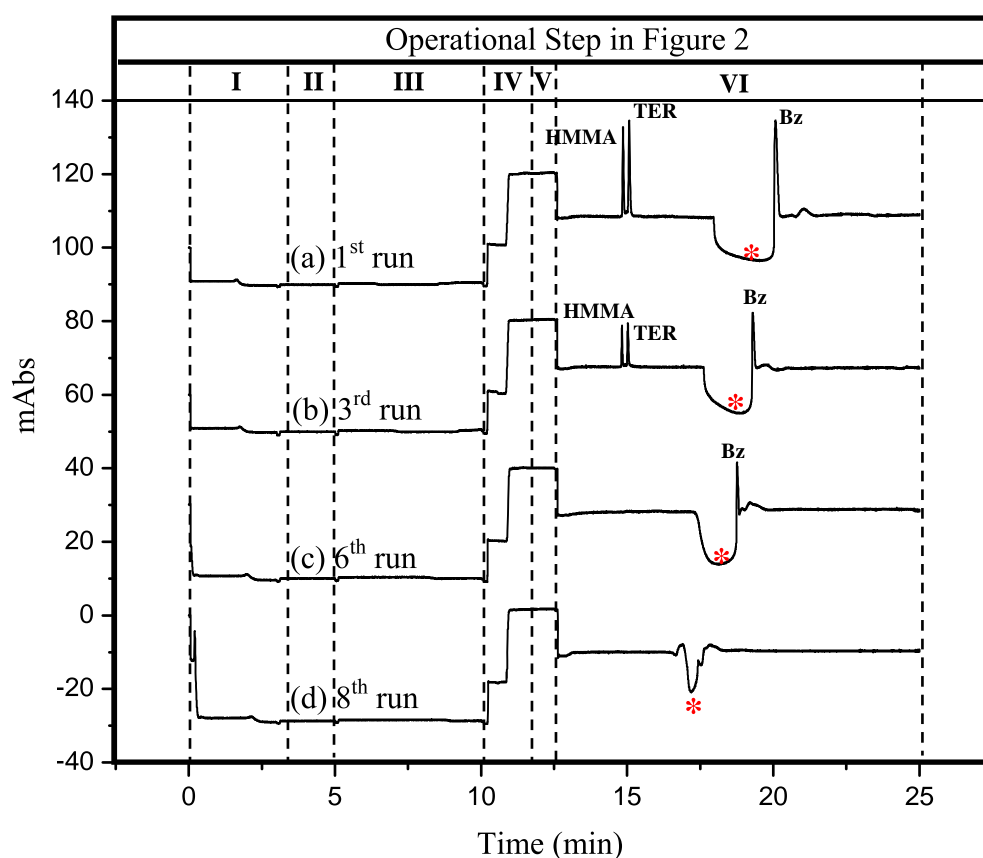


Figure 4.17. Electropherograms obtained from the (a) 1st, (b) 3rd, (c) 6th, and (d) 8th run from the in-line monolithic MAA-EDMA–CE. Condition; step I : 3 min conditioning with MeOH, step II : 2 min washing with 10% (v/v) ACN:water, step III : 5 min loading mixture of 3 ppm TER, HMMA and 1 ppm Bz in 10% (v/v) ACN:water, step IV : 2.5 min filling of BGE (50 mM Tris buffer (pH 8.2) in MeOH) from outlet, step V : 0.3 min filling in of BGE from the inlet, applied pressure: 8 bars (step I–V), applied voltage :+25 kV (step VI). (* represent the residue aqueous zone left from sample loading step).

Although under the selected conditions, a big improvement in sensitivity was observed, repeatability between runs was poor as shown in the electropherograms in Figure 4.17. It was found that peak areas for all three compounds quickly decreased and no peaks were observed from the 8th injection onward. We also studied a combination of voltage and pressure for the filling of the BGE from the outlet as recommended by two reports [87,91]. However, acceptable reproducibility could still not be obtained. This could have resulted from non-reproducible filling of the BGE before the elution. The BGE must be filled from the outlet end up to the SPE in order to prevent loss of the trapped compounds. This filling step must therefore be very precise in order to obtain reproducible results. It can be seen in Figure 4.17 that in addition to decreasing of peak areas for the compounds, a negative peak (marked by *) due to the residual solvent from the loading step (10% (v/v) acetonitrile:water), was also smaller indicating that the filled BGE increased from run to run. This could be due to imprecise applied pressure in the filling step or increasing in porosity of the polymeric-based monolith. Changing in properties of the monolith may occur due to passing different solvents from conditioning, loading, eluting and separation steps.

4.1.4 Packed particle solid phase extraction

The polymer based monolith has been shown to be unsuitable for our application. A silica-based monolith could possibly work better for this case. However since the preparation of silica-based monoliths is complicated and time consuming, in order to test the idea, the silica based packed particle was employed in this part.

4.1.4.1 Performance study of the particle packed solid phase extraction

Unlike previous work reported on coupling particle packed SPE-CE [84-86], in this work, the C18 (ODS II) particles was packed *in situ* inside a separation capillary which is simpler. A microscopic picture of the packed particle SPE capillary is shown in Figure 4.18. Two frits were made in order to retain the packing inside the capillary as decribed in section 3.4.4. By using this method, a connecting tubing was not required and the problem of leakage was eliminated.

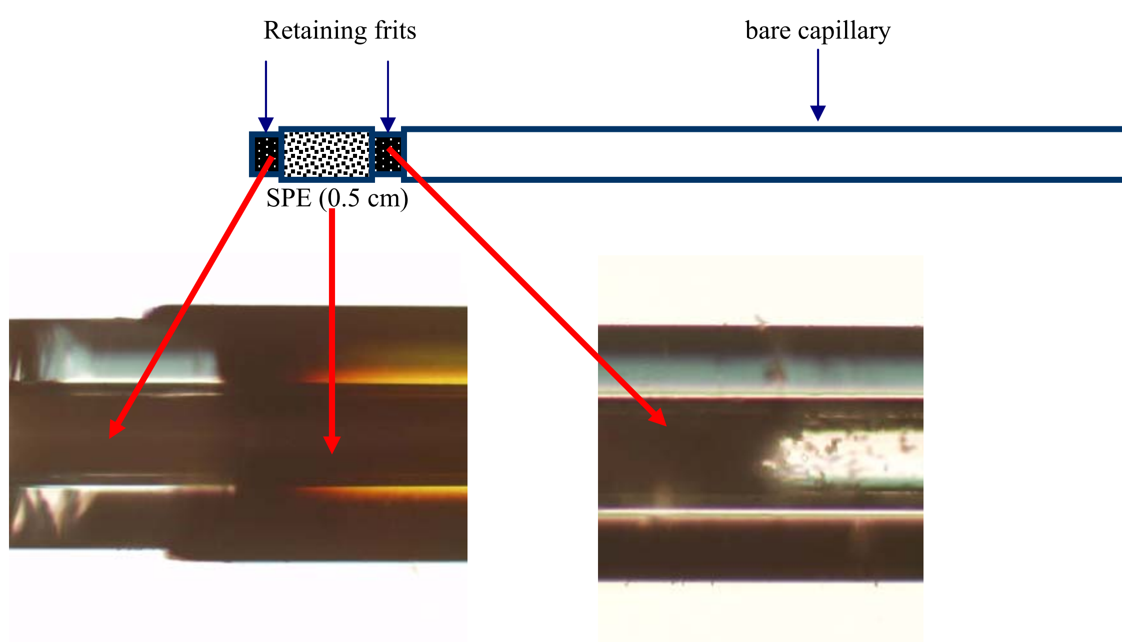


Figure 4.18 Microscopic picture of the packed particle SPE capillary (not to scale).

Performance tests of the packed SPE showed excellent repeatability for both peak areas and elution times for the three compounds with RSD% for ten replicate injections of less than 1.35 and 0.39 respectively. The signal profiles are shown in Figure 4.19.

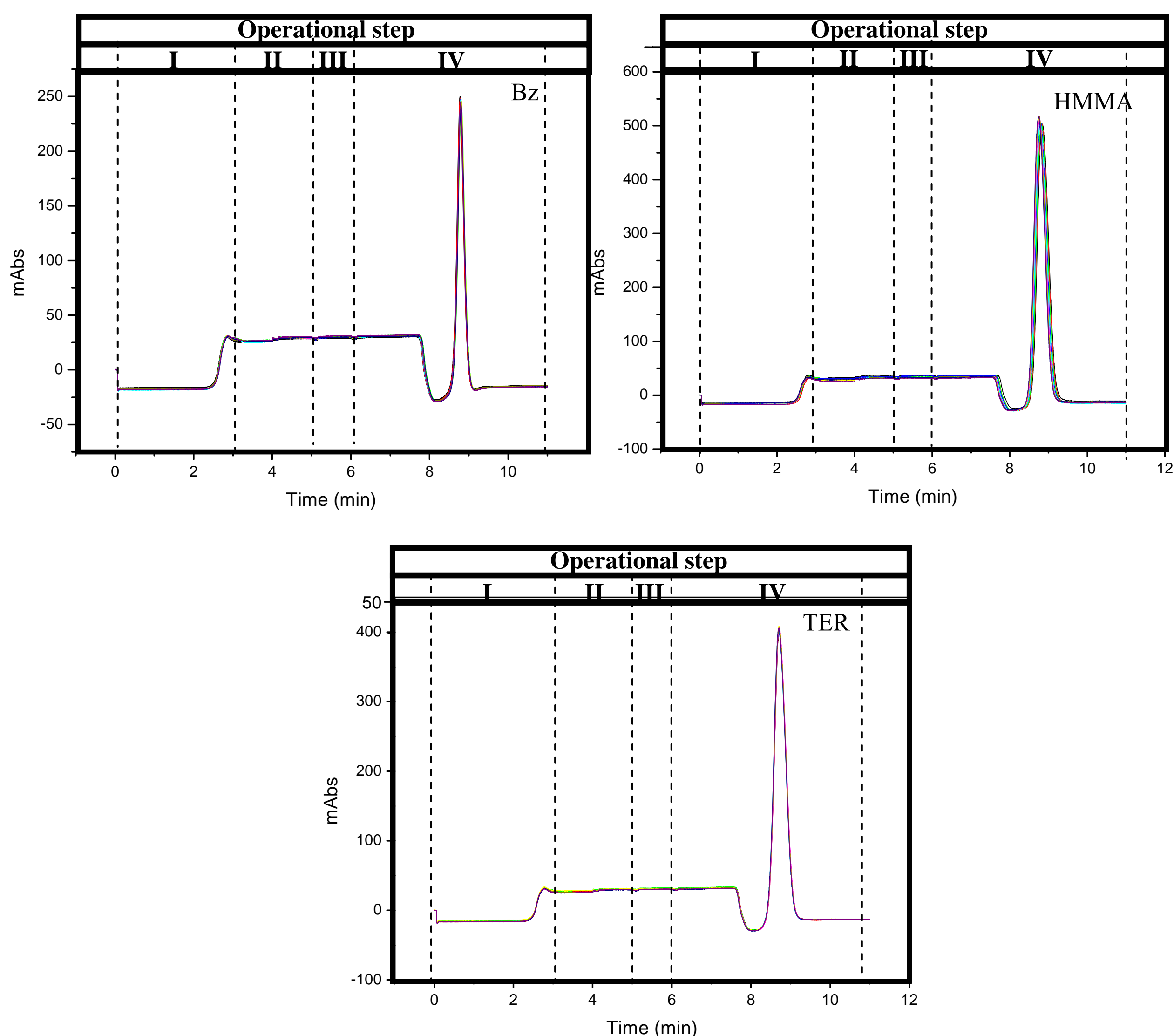


Figure 4.19 Ten replicate injections of 100 ppm TER, Bz and HMMA in water for packed particle SPE. Condition; step I : 3 min conditioning with methanol, step II : 2 min washing with water, step III : 1 min loading, step IV : elution with 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3). Absorbance was monitored at 200 nm.

Eluting solvent was also studied for this type of SPE with the same experimental set up as for the monoliths (described in section 4.1.2.2). Similar results to that for the MAA-EDMA and BMA-EDMA monolithic materials were observed (Figure 4.20) indicating similar retaining interaction of the compounds on the surface of the monoliths and the packed particle SPEs. Although the ODS II is a reversed-phase C18 phase which has mostly bonded C18 on the surface, residual silanol groups on the particles could result in coulombic interaction with the TER and HMMA as for the monoliths. From this study, 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3) was selected as eluting solvent for further investigation.

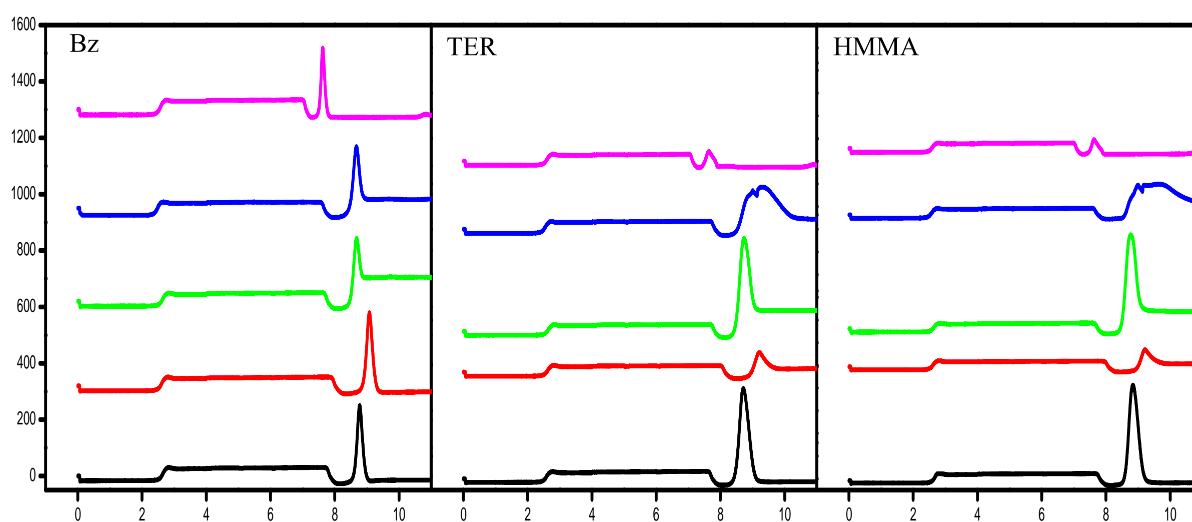


Figure 4.20 Signal profiles of 100 ppm Bz, TER and HMMA eluted by investigated eluting solvents. Condition; 3 min conditioning with acetonitrile, step II : 0.5 min loading of 100 ppm TER in 10% (v/v) acetonitrile:water, step III : eluted with investigated eluting solvents, step V: eluted with 70% (v/v) acetonitrile : 50 mM phosphate buffer (pH 3).

70% (v/v) acetonitrile : 50 mM PPB pH 3

70% (v/v) acetonitrile : water

50 mM Tris buffer pH 8.2 in MeOH

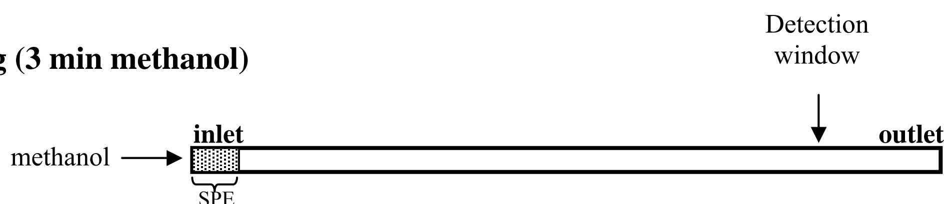
MeOH

ACN

4.1.5 In-line packed particle solid phase extraction-capillary electrophoresis

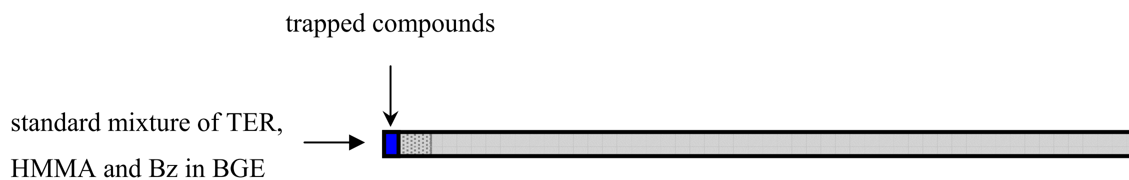
Since the SPE showed no problem with both aqueous or non-aqueous solvents, CE separation of the test compounds was performed by using aqueous background electrolyte (BGE). 10 mM phosphate buffer (pH 7) was selected as BGE due to satisfactory separation for the three compounds. The packed particle SPE was therefore coupled with aqueous CE as shown in Figure 4.18. Operational steps for this experiment are shown in Figure 4.21. The method was simplified by dissolving the three test compounds in the BGE (10 mM phosphate buffer (pH 7)).

I. Conditioning (3 min methanol)



II. Washing (1 min)

III. Loading of the compounds



IV. Elution by the running buffer (BGE)

V. Applying the separation voltage

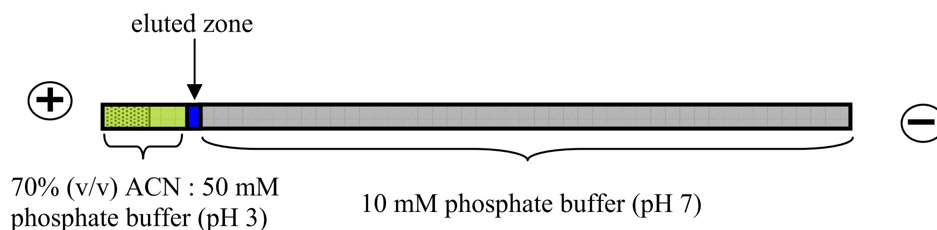


Figure 4.21 Operational step for the in-line packed particle SPE-CE.

sample zone running buffer SPE part Eluting solvent

In order to confirm that there was no loss of the compounds in the BGE medium during loading, the signals were compared with that for loading in water. The signals from both media were not significantly different as shown in Table 4.1.

Table 4.1 Peak area of eluted peak loaded in water and BGE (average ± sd)

Loading solvent	Peak area (mAbs*sec)		
	Bz	HMMA	TER
water	207 ± 3.2	1370 ± 44	757 ± 68
10 mM phosphate buffer (pH 7)	214 ± 1.3	1344 ± 46	834 ± 24

This way, the CE separation could be performed without the need to fill the capillary with the BGE after the loading step. Therefore only the elution step (step IV, Figure 4.21) needs to be optimised before performing the CE separation. Basically, the elution period or elution time should be kept to a minimum. This is to avoid shortening of the length of capillary for the CE separation. Three elution periods were investigated as shown in Figure 4.22. It was observed that as the elution time was increased (Figure 4.22B-a), the resolution slightly decreased (Figure 4.22A-a). This can be explained by the effect that the elution time has on the position of the eluted zone within the capillary. With longer elution times, the eluted zone was displaced towards the outlet end (detection end), resulting in a shorter CE separation length. However a short elution time may not be sufficient for complete elution of the retained compounds. Under selected conditions, no peaks were observed. (Figure 4.22A-c). From these experiments, an elution time of 0.2 min was selected as optimum (Figure 4.22A-b).

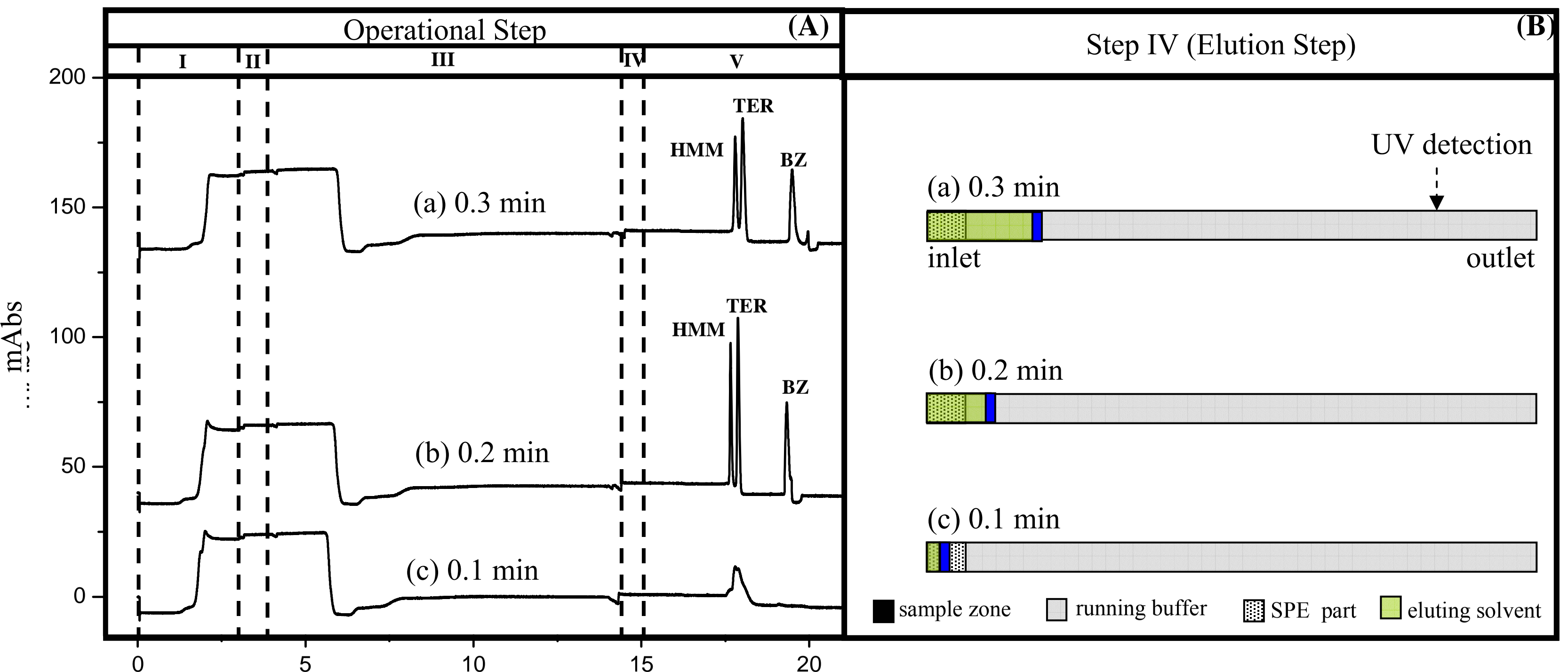


Figure 4.22. Electropherograms arious length of time from 0.1 to 0.3 min for elution time from the in-line particl packed SPE-CE. The schematic diagram (B) illustrates the decrease in the separation distance, with increasing elution time. Conditioı step I : 3 min conditioning with methanol, step II : 1 min washing with water, step III : 10 min loading mixture of 3 ppm TER, 0.5 ppı HMMA and 1 ppm Bz in BGE (10 mM phosphate buffer pH 7.0), step IV : elution with 70% (v/v) acetonitrile : 50 mM phosphate buffe (pH 3) at 0.1 min, 0.2 min and 0.3 min, applied pressure: 8 bars (I-IV), applied voltage +8 kV (step V).

The applied voltage was also studied in order to obtain an adequate separation time (Figure 4.23). An applied voltage of +8 kV was found to be a suitable potential. Separation at this applied voltage gave resolution for the HMMA and TER of 1.68 with separation time of 20 min (Figure 4.23 (b)).

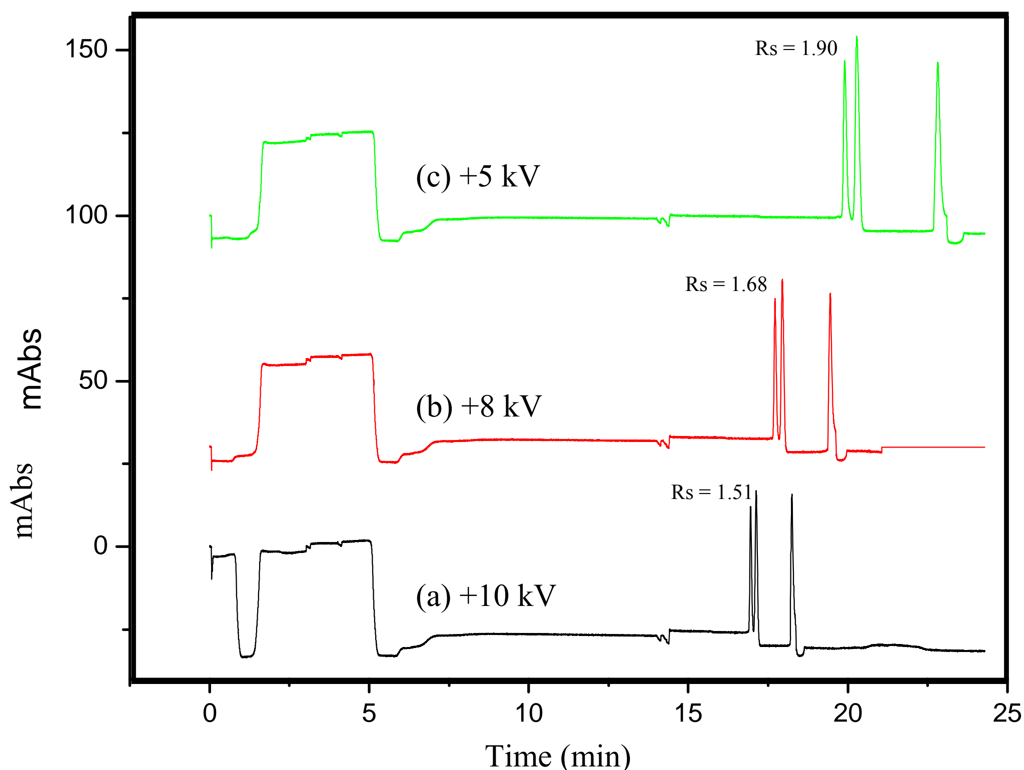


Figure 4.23. Varying applied voltage for CE separation. Condition; 3 min methanol, 1 min washing with water, loading 10 min 0.5 ppm HMMA, 3 ppm TER and 1 ppm Bz in 10 mM phosphate buffer pH 7.2, BGE : 10 mM phosphate buffer (pH 7), 0.2 min eluting time, +25° C temperature, absorbance of 200 nm.

4.1.5.1 Analytical performance of the method

At these optimum conditions, the method showed a significant improvement in the sensitivity in the CE technique. Figure 4.24 shows the comparison of the electropherograms between normal CE's (Figure 4.24a) and the in-line packed SPE-CE's (Figure 4.24b). Figure 4.24 demonstrates that the signals of the three compounds following in-line SPE, were greater than the signals of the compounds at lower concentrations by factor of about 50 and 150 for TER and HMMA, respectively (calculated by using peak heights).

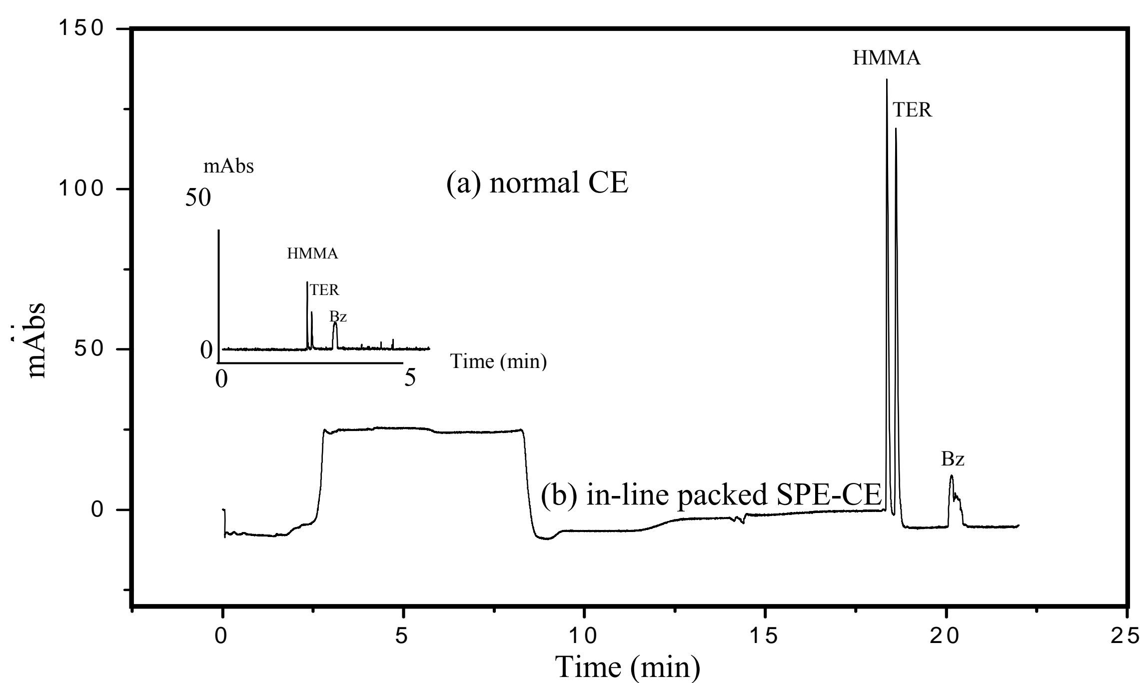


Figure 4.24. Electropherograms of (a) mixture of 10 ppm TER, HMMA and 50 ppm Bz from normal CE (without the enrichment SPE), condition; capillary : 100 µm ID x 33 cm, electrokinetic injection: 5 mbar x 5 sec, BGE: 10 mM phosphate buffer (pH 7), applied voltage : +10 kV, temperature: 25 ^{o}C, detection wavelength : 200 nm. (b) mixture of 3 ppm TER, 0.5 ppm HMMA and 1 ppm Bz from the in-line particle packed SPE-CE. Same conditions as in Figure 4.22A-b.

The method showed excellent repeatability for both peak area and migration times for all the compounds as shown in Table 4.2.

Table 4.2 Peak area (PA) and retention time (t_m) of HMMA, TER and Bz from repeatability study (n = 20)

	HMMA		TER		Bz (EOF marker)
	PA	t_m	PA	t_m	t_m
average	517	18.36	527	18.62	20.15
Sd	31	0.06	17	0.07	0.11
%RSD	4	0.2	2.3	0.2	0.3

With the in-line packed particle SPE and 10 min sample loading, the pre-concentration factors for HMMA and TER were found to be 1000 and 333 respectively (calculated from the LOD's). However the sensitivity could be readily improved by increasing the loading time.

In terms of analysis time, it should be noticed that with a pre-concentration step, the analysis time was extended. The analysis time of the in-line SPE method was approximately 20 min. This includes the time for the pre-concentration step and for the CE separation. Figure 4.24a shows that the time for the separation in normal CE (without pre-concentration) was approximately 9 min (3 min for separation and 6 min for conditioning step before each injection). However, the total analysis time would have been much longer using an off-line approach, and with a more tedious procedure and sample handling. Besides, this developed CE method requires minimal amount of sample (approximately 300 µL for 10 min loading).

Linearity for TER and HMMA were studied in the range of 25-1000 ppb and 150-6000 ppb, respectively (see Figure 4.25a for electropherograms). Calibration curves in Figure 4.25a and 4.25b shows good linearity with correlation coefficient higher than 0.996 for the two compounds. The analytical performance of the method is summarised in Table 4.3.

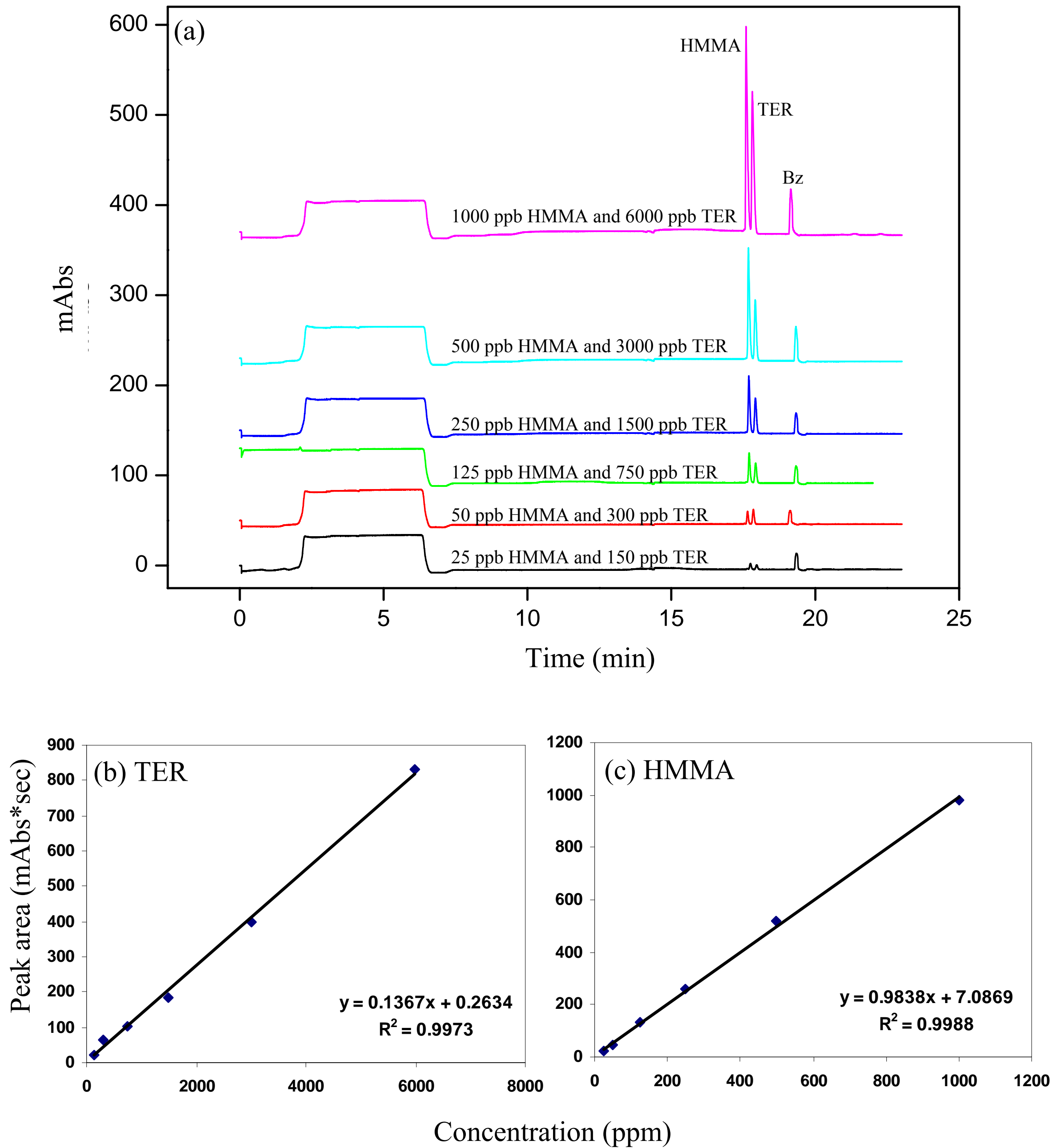


Figure 4.25 Electropherograms for standard calibration curve solution (a) and calibration curves for TER (b) and HMMA (c). Condition as explained in Figure 4.22A-b.

Table 4.3 Analytical performances of the in-line particle packed SPE-CE

	HMMA	TER
LOD (S/N = 3)	2.5 ppb	15 ppb
Preconcentration factor[a]	1000	333
Studied linearity range	25-1000 ppb	150-6000 ppb
Calibration equation[b]	Y = 0.98X + 7.08	Y = 0.13X + 0.26
Linear regression (R^2)	r^2 = 0.999	r^2 = 0.997
Precision (RSD% for PA and t_m)[c]	4.5%, 0.2%	2.3 %, 0.2%

[a]calculated from the LOD from CE with and without the enrichment packed particle SPE

[b] Y is peak area (mAbs*sec) and X is concentration (ppb)

[c](n = 20), PA = peak area, t_m = migration time

4.1.6 Comparison between in-line monolithic solid phase extraction and packed particle phase solid phase extraction - capillary electrophoresis

Comparison between monolithic and silica based packed phases, showed that the silica based packed SPE was more compatible to coupling with a CE system as pre-concentrator than the polymer based MAA-EDMA and BMA-EDMA monoliths in term of repeatability. The two monoliths were found not to be compatible with a pure aqueous medium. Nevertheless, eliminating the use of an aqueous medium did not produce acceptable repeatability. The in-line packed SPE-CE was successfully developed with good reproducibility and showed a big improvement in sensitivity for HMMA and TER compared to CE using a commercial UV detector without the enrichment SPE.

However, the preparation of the monolithic SPE was much easier than the packed SPE even though the preparation needed a longer time, but the method is simple and does not require special technical skill. In addition, the monolithic phase

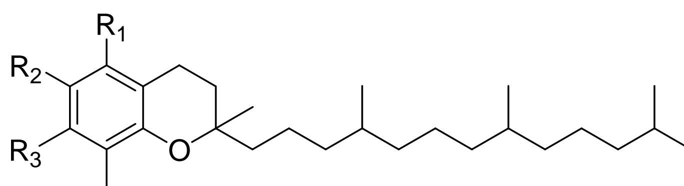
offers more choices for the stationary phase. The low back pressure is another advantage of a monolithic SPE providing the possibility of higher sample loading. In order to obtain low column back pressure, the packed particle SPE needs to be short resulting in lower capacity. In addition, the retaining frits for the packed particle SPE are fragile and have the potential to cause bubble formation during the CE separation step. Silica based monoliths could offer an answer to the problem. Summary for the comparison is shown in the below Table.

Table 4.4 Comparison data for the in-line MAA-EDMA and BMA-EDMA monoliths-CE and silica based packed SPE-CE.

Comparison	MAA-EDMA and BMA-EDMA monoliths	Packed Particle
Pre-concentration	Good	Good
Solvent Compatibility	Poor	Good
Simple Preparation method	Yes	No
Preparation time	Long	Short
Option for stationary phase	Wide	Narrow
Backpressure	Low (Good)	High (Poor)
Capacity for sample loading	High	Low
Robustness	Good	Fair

4.2 Part II : Capillary electrochromatography (CEC) for the separation of vitamin E isomers

Vitamin E is commonly found as tocopherol (TOH) which exists naturally as four isomers that are α-, β-, γ-, and δ-TOH. Due to the structural similarity of the β- and γ-isomers (Figure 4.26), it has been reported that common reversed-phase packed columns such as C4, C8 and C18 [118-120, 124-135] have no selectivity for the two isomers.



Compound	R_1	R_2	R_3
1. α-TOH	CH_3	OH	CH_3
2. β- TOH	CH_3	OH	H
3. γ- TOH	H	OH	CH_3
4. δ- TOH	H	OH	H
5. α- TAc	CH_3	$COOCH_3$	CH_3

Figure 4.26 Chemical structures of TOHs and α-TAc.

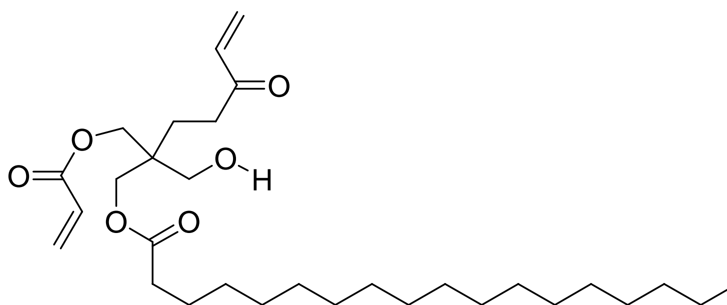
In this part of the thesis, an amphiphilic monolithic column, a polar hydroxyl C17 material namely pentaerythritol diacrylate monostearate was examined for the separation of the four TOHs and α-tocopherol acetate (a synthetic vitamin E, α-TAc).

4.2.1 PEDAS-EDMA

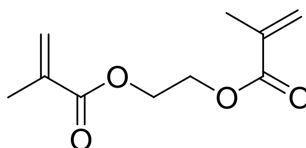
It has been shown in the literature that either an extremely hydrophobic or polar reversed-phase stationary phase is required for the reversed-phase separation of all tocopherol isomers. An amphiphilic monolithic CEC column with pentaerythritol

diacrylate monostearate as monomer (PEDAS, see structure in Figure 4.27) was investigated for the separation of the TOHs and α-TAc.

The PEDAS monolith contains both hydrophilic hydroxyl/ester and hydrophobic C17 groups as functional ligands (polar reversed-phase stationary phases). The PEDAS-EDMA was investigated for separation of the four TOHs, α-TAc, butylated hydroxytoluene (BHT, an antioxidant added for preventing oxidation loss of the tocopherols) and thiourea (EOF marker). It was observed that the separation efficiency was improved with the addition of EDMA as co-polymer in the synthesis of the monolithic stationary phase. Therefore, a co-polymeric monolith of PEDAS-EDMA (Figure 4.27) was employed in this work.



(a) pentaerythritol diacrylate monostearate



(b) ethylene dimethacrylate (EDMA)

Figure 4.27 Chemical structure of (a) PEDAS and (b) EDMA.

4.2.2 EOF for the neutral PEDAS-EDMA monolith

It is well known that an important factor in capillary electrophoresis (CE) and capillary electrochromatography (CEC) is the electroosmotic flow which is commonly generated from the charged surface of the fused silica material or stationary phase. Migration of the adsorbed ions in the diffuse double layer on the surface under an electric field results in movement of bulk solution called electroosmotic flow or EOF. However Rassi *et al*. have reported that even the neutral PEDAS-EDMA monolith provided sufficient EOF using an aqueous phosphate buffer mixed with acetonitrile as mobile phase [144]. They proposed that the observed EOF was due to adsorption of phosphate ions in the mobile phase onto the PEDAS surface resulting in a negative charged surface, leading to a cathodic EOF (direction of the EOF is from anode to cathode) [144].

In this work, a non-aqueous mobile phase was employed (the aqueous Tris buffer is only 3% of the mobile phase) and a cathodic EOF was also observed. In addition to the explanation provided by Rassi *et al*. of adsorbed ions on the surface of the monolith, an additional source for the EOF is the dissociation of the –OH groups on the PEDAS monolith generating a negatively charged surface. The zeta potential between this negative surface and cations from the Tris buffer can lead to the observed EOF.

4.2.3 Retention study of tocopherols and tocopherol acetate on the PEDAS-EDMA

Retention of the compounds on the PEDAS-EDMA was carried out in three mobile phase systems namely methanol, acetonitrile and mixtures of methanol and acetonitrile. Plots of capacity factor (k') and retention time for the compounds against mobile phase composition are shown in Figure 4.28.

Retention depends on the polarity of compounds. Since the four TOHs contain a hydroxyl group on the molecule (Figure 4.27), they are more polar than the

α-TAc. The TOHs therefore interact with both the nonpolar C17 and the polar hydroxyl/ester groups on the PEDAS. However the elution order of the TOHs according to their polarities indicated that the reversed-phase mechanism was predominant. δ-TOH is the most polar and therefore has the lowest k' amongst the four TOHs (Figure 4.28). β- and γ-TOHs are less polar and α-TOH will be the least retained due to the presence of the two methyl groups.

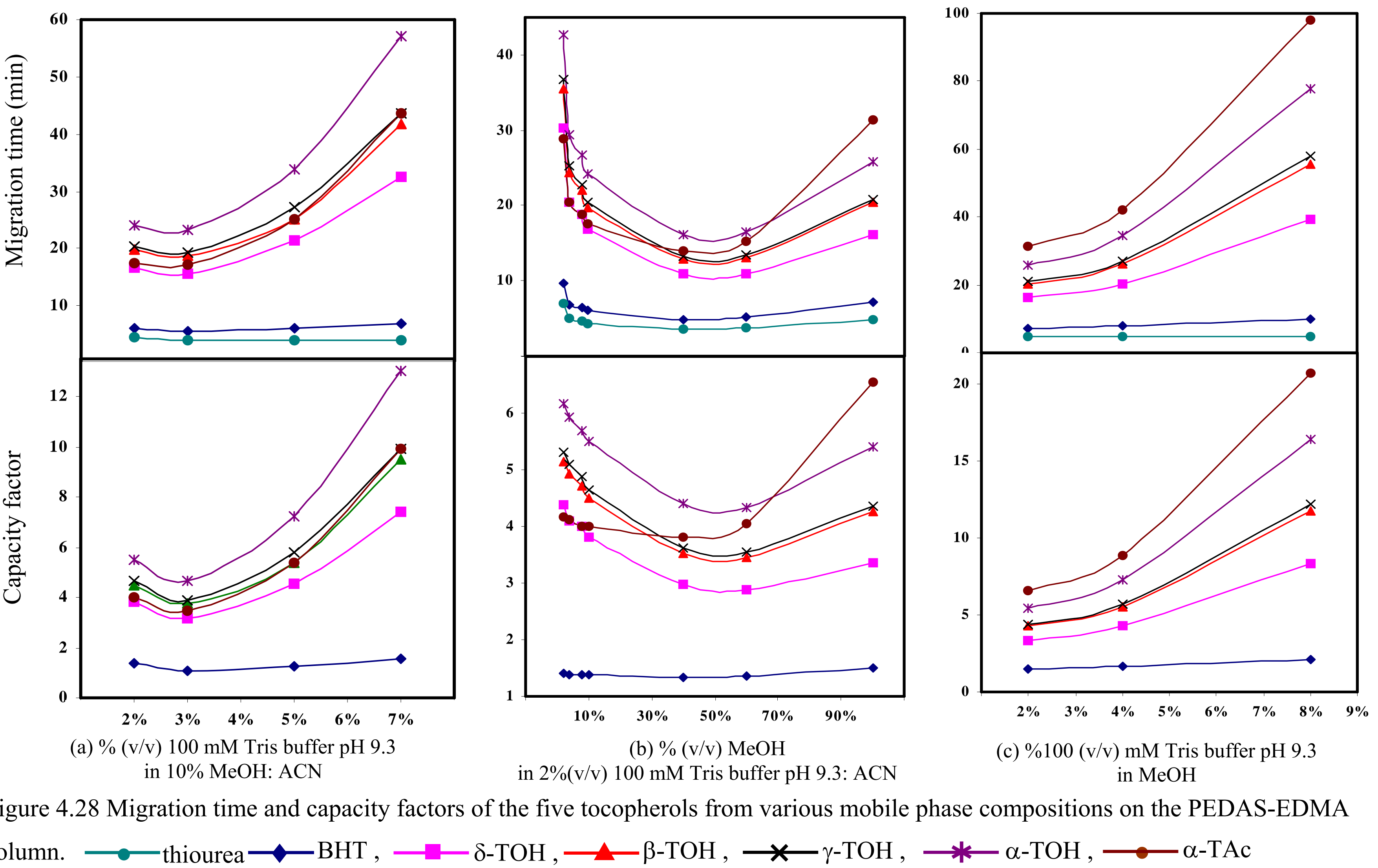


Figure 4.28 Migration time and capacity factors of the five tocopherols from various mobile phase compositions on the PEDAS-EDMA column. thiourea , BHT , δ-TOH , β-TOH , γ-TOH , α-TOH , α-TAc

For ACN/MeOH mobile phase medium containing 2% (v/v) 100 mM Tris buffer (pH 9.3), the compounds interact with both the non-polar C17 and the polar hydroxyl/ester groups on the PEDAS. The TOHs which contain polar –OH group therefore were retained on the column longer than the TAc (Figure 4.29a, Table 4.5). When the aqueous Tris buffer in the mobile phase was changed from 2 to 3% (v/v), the compounds were eluted faster due to the increased EOF with slightly lower k' (Figure 4.28a). A significant increase of k' was observed when the amount of the aqueous Tris buffer increased from 3% to 7%(Figure 4.28a). This indicated that the reversed-phase mechanism was predominant for these conditions. Noticeably, a change in elution order of α-TAc was found when increasing the aqueous buffer in the mobile phase (Figure 4.28a). This could be explained by the fact that α-TAc retains on the PEDAS only by hydrophobic interaction, while the TOHs interact with both the polar –OH/ester and non-polar C17 groups. The separation of the TOHs therefore combined normal and reversed-phase mechanisms with only a reversed-phase mechanism present for the α-TAc separation. Increasing the water content of the mobile phase, therefore greatly effects the retention of α-TAc.

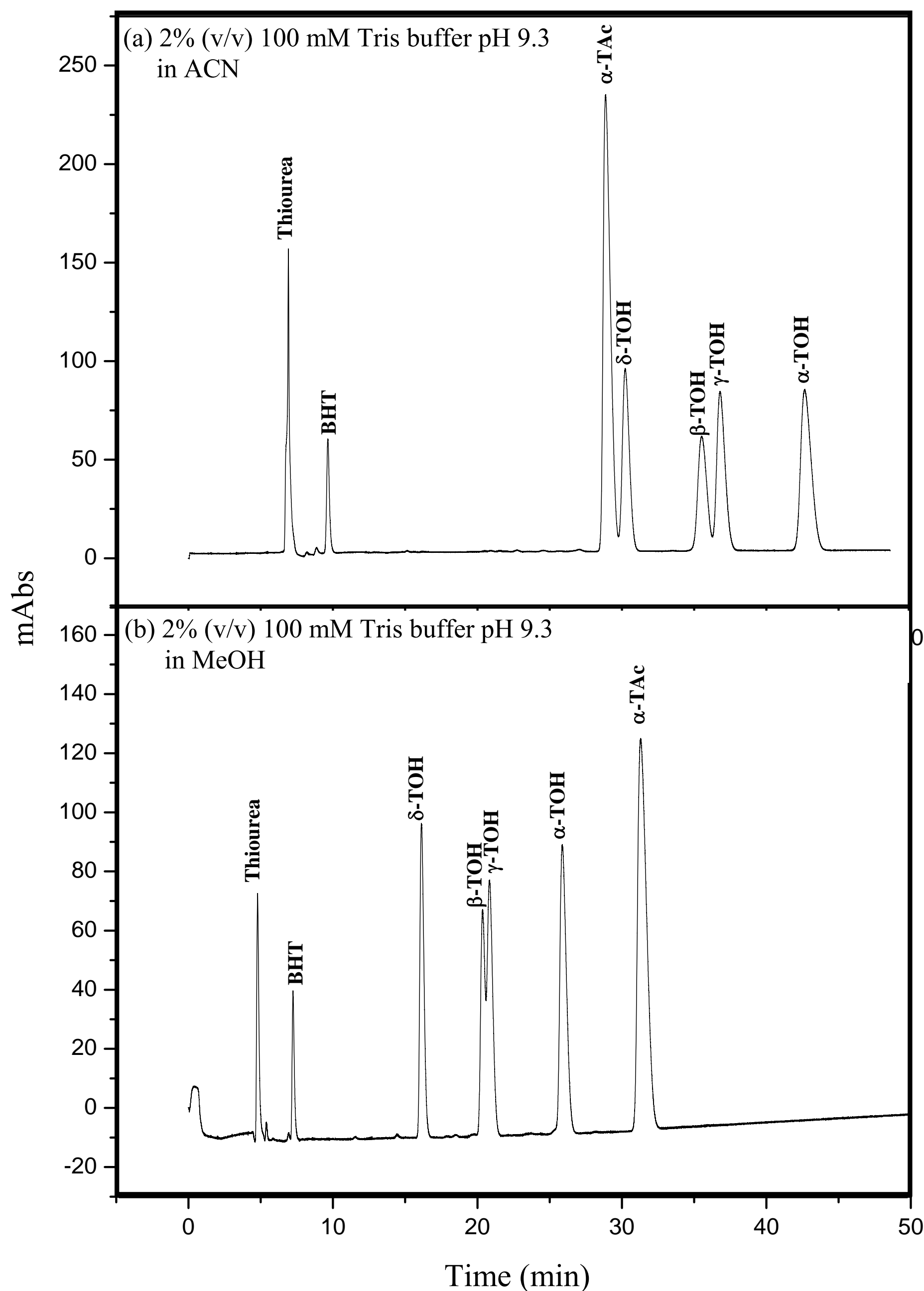


Figure 4.29 Separation of BHT and the five tocopherol homologues on PEDAS-EDMA monolithic column. Capillary column, 23.5 cm effective length, 32 cm total length × 100 μm id; applied voltage, +30 kV; column temperature, 35°C; electrokinetic injection at +10 kV for 30 second; detection wavelength, 200 nm.

Table 4.5 Retention times and capacity factors (k') using 2%(v/v) 100 mM Tris buffer (pH 9.3) in methanol or acetonitrile as mobile phase. Other conditions as in Figure 4.29.

Compounds	Retention times (min)		Capacity Factors[c] (k')	
	MeOH mobile phase[a]	ACN mobile phase[b]	MeOH mobile phase	ACN mobile phase
1. Thiourea (EOF maker)	4.788	6.916	-	-
2. BHT	7.239	9.649	0.51	0.40
3. δ-TOH	16.127	30.23	2.37	3.37
4. β-TOH	20.373	35.528	3.26	4.14
5. γ-TOH	20.836	36.792	3.35	4.32
6. α-TOH	25.877	42.647	4.40	5.17
7. α-TAc	31.294	28.872	5.54	3.17

[a]2% (v/v) 100 mM Tris buffer pH 9.3 in methanol as mobile phase

[b]2% (v/v) 100 mM Tris buffer pH 9.3 in acetonitrile as mobile phase

[c]k = Capacity Factors = $(t_m - t_{EOF})/t_{EOF}$

In contrast to an ACN medium, when methanol with 2% (v/v) 100 mM Tris buffer (pH 9.3) was used as mobile phase, α-TAc eluted last and no change in elution order was observed. This could be due to methanol molecules forming H-bonding with the hydroxyl group of PEDAS, as shown in Figure 4.30.

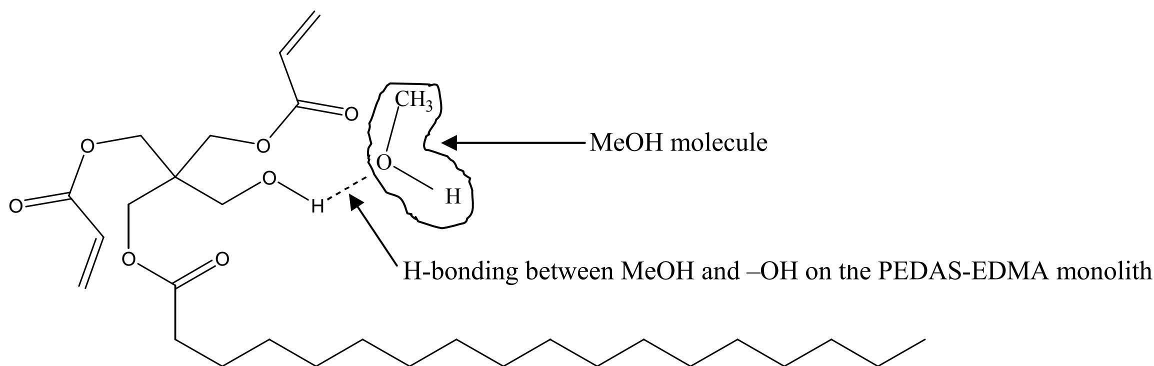


Figure 4.30. Schematic illustration of H-bonding of methanol (MeOH) and the PEDAS.

When there is a block of the –OH group, the compounds interact less with the hydroxyl on the monolith, leading to decrease retention for the four TOHs (α-, β-, γ-, and δ-tocopherol) but greater retention for the α-TAc. There is a change in the elution order compared to the acetonitrile medium, as shown in the chromatogram and retention data in Figure 4.29a and 4.29b and Table 4.5.

It was found that the retention times and capacity factors of all the compounds increased with increasing ratio of aqueous buffer or with decreasing strength of the mobile phase (Figure 4.28b), as expected in a reversed-phase separation. No change in elution order of the α-TAc was observed for this type of mobile phase.

Increasing of k' for the compounds with increasing amount of the aqueous Tris buffer, indicated that with both ACN and MeOH media, reversed-phased mechanism was predominant for all the tocopherols on the PEDAS-EDMA column.

In order to confirm the assumption that when ACN was used there is less H-bonding leading to greater contribution of the –OH group, the methanol composition was varied from 2% (v/v) to 100% (Figure 4.28b). Increasing methanol composition in the acetonitrile mobile phase from 2 to 60% resulted in decreased retention of the TOH's as a result of methanol competing with the TOH's for the polar ester/hydroxyl groups on the PEDAS-EDMA, leading to less retention of the TOH's. However the capacity factor, k' increased when the methanol composition was higher than 60% (Figure 4.28b). Increased H-bonding could make the hydrophobicity of the monolith surface more dominant as most of the –OH group is bound to methanol. It should be noted that the elution order for all TOH's still remain unchanged with increasing methanol concentration. α-TAc contains no polar hydroxyl group on the molecule. Its capacity factor is however very dependent on the composition of the mobile phase (Figure 4.28a and 4.28b). For a methanol concentration between 2-40%, retention of α-TAc remained constant whereas there is decreased retention of the TOH's with increasing methanol concentration. For methanol content greater than 40%, retention of α-TAc increased significantly. This corresponds to the increased hydrophobicity of the monolith surface and α-TAc is the last to elute in 100%

methanol. The functionality of –OH group on the monolith can be adjusted by varying the methanol composition.

The results show that the PEDAS-EDMA has better selectivity for the positional TOH isomers, β- and γ-TOHs compared to a C18 reversed-phase column. The column has been shown to separate the problematic TOH isomers, (β- and γ-pair) (Figure 4.29). This can be credited to its amphiphilic nature, having both polar hydroxyl/ester and non-polar C17 groups. The extent of the polar hydroxyl/ester group interaction depends upon the major component of the mobile phase.

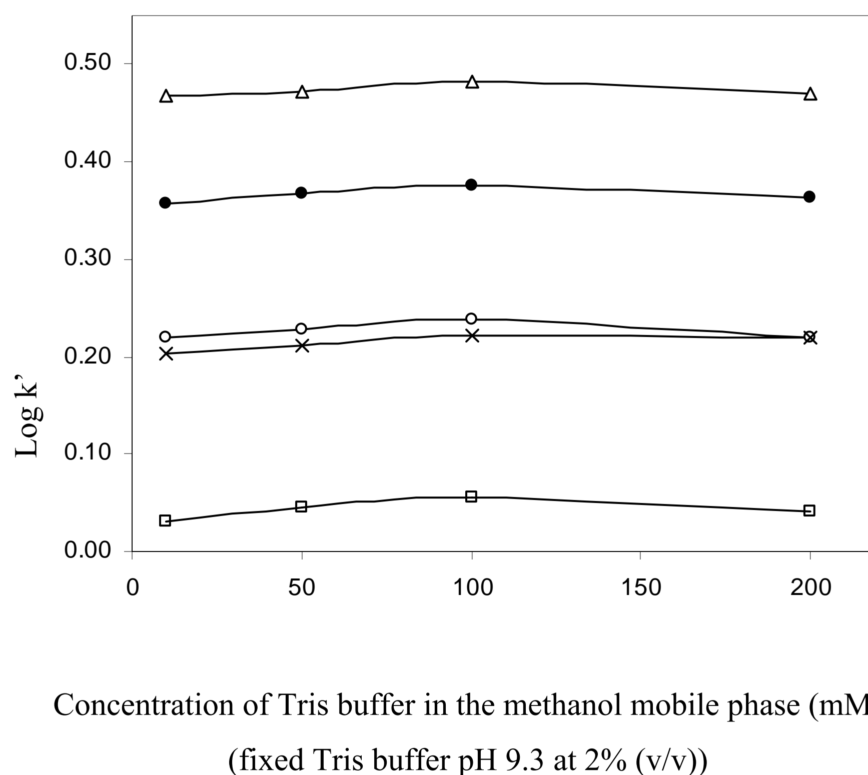
4.2.4 Selection of the optimal mobile phase composition for the separation of the TOHs and α-TAc

Since the compounds are extremely non-polar, separation therefore requires high organic content of the mobile phase in order to elute from a reversed-phase column in a reasonable time. Tris buffer was selected for controlling the pH of the mobile phase, due to its ability to dissolve in organic solvents. In order to achieve an optimal condition for separation of the compounds, important parameters were optimized including concentration and pH of the buffer and the amount of organic solvent in the mobile phase.

The effect of Tris concentration was studied in methanol, from 10 to 200 mM at 2% (v/v) Tris buffer in methanol. Increasing the buffer concentration resulted in a higher EOF velocity, leading to a reduced analysis time (Table 4.6). In addition Tris concentration from 10 to 100 mM slightly increased capacity factors (k') for all the compounds as shown in plot of log k' versus the Tris concentrations (Figure 4.31). However the capacity factor decreased and resolution was insufficient for the β, γ-pair when Tris buffer was greater than 100 mM. In order to obtain a short separation time with acceptable resolution, a Tris buffer concentration of 100 mM was selected.

Table 4.6. Retention time of the compounds at various Tris concentrations in the methanol mobile phase containing 2%(v/v) of the Tris buffer.

Tris concentration (mM) in methanol	Retention time (min)					
	Thiourea (EOF marker)	δ-TOH	β-TOH	γ-TOH	α-TOH	α-TAc
10	10.3	21.3	26.7	27.4	33.8	40.6
50	6.9	14.6	18.3	18.7	23.1	27.6
100	6.3	13.4	16.8	17.2	21.3	25.5
200	5.9	12.4	15.7	15.7	19.6	23.3



Concentration of Tris buffer in the methanol mobile phase (mM)
(fixed Tris buffer pH 9.3 at 2% (v/v))

Figure 4.31. Plot of log k' for TOH's and TAc versus concentration of Tris buffer in the mobile phase. Other conditions are in Figure 29 .

—□— δ-TOH, —×— β-TOH, —○— γ-TOH, —●— α-TOH, —△— α-TAc

The pH of the Tris buffer was also studied from pH 7 to 10. Although the Tris buffer in the mobile phase was only 2%, the pH dramatically affected the EOF velocity. It was found that the EOF decreased very rapidly when the pH was less than 9. In fact no EOF was observed for pH 8 and 7. This could be due to the decreased dissociation of the –OH group on the PEDAS at the lower pH. Therefore, subsequent work was conducted at pH about 9.

Although the analysis time in acetonitrile (Figure 4.29a) was slightly longer than in methanol (Figure 4.29b), acetonitrile was selected as solvent for the mobile phase, since the resolution between β- and γ-TOHs was greater in acetonitrile compared to methanol. However, methanol was added to the acetonitrile-Tris solution in order to achieve separation within 25 min.

For this work, 100 mM Tris buffer (pH 9.3): methanol: acetonitrile 3:10:87 (v/v/v) was selected as a suitable composition of mobile phase for the CEC separation on the PEDAS-EDMA monolith. Under these conditions, acceptable resolution (R_s = 1.2) of the β- and γ-tocopherols was achieved. The resulting chromatogram is shown in Figure 4.32.

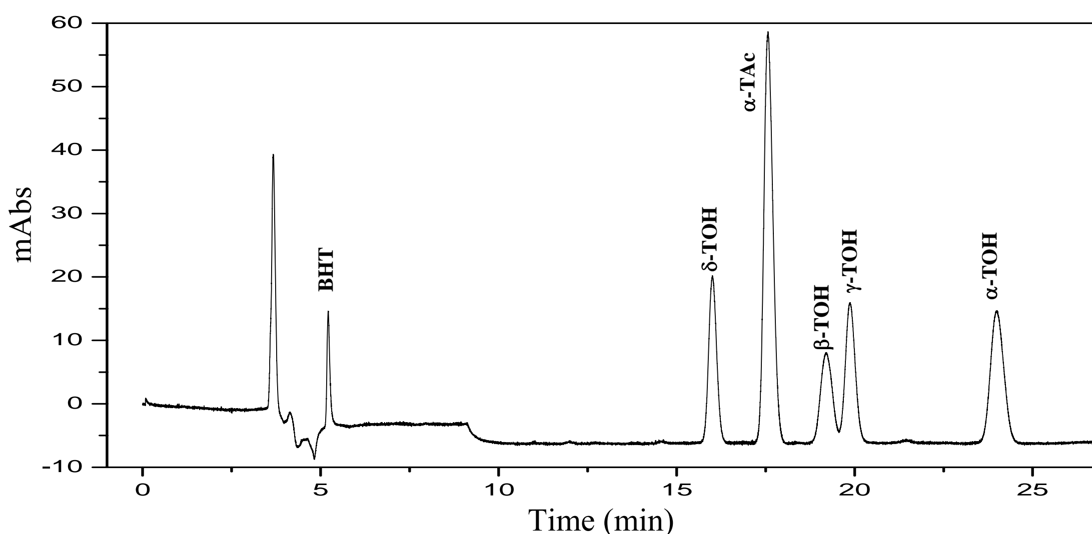


Figure 4.32 Separation of thiourea, BHT, TOHs and TAc on the PEDAS-EDMA monolithic column; mobile phase, 3:10:87% (v/v/v) 100 mM Tris buffer pH 9.3 : MeOH : ACN Other conditions are as given in Figure 4.29.

4.2.5 Selectivity of a PEDAS-EDMA monolith compared to commercial packing materials

It has been shown that common C8 and C18 reversed-phase materials have no selectivity for the separation of the β- and γ-TOHs [118-120, 124-135]. We also carried out studies using a commercial packed-particle C18 phase for the separation of these compounds. As has been previously reported [118-120, 124-135], purely hydrophobic reversed-phase interactions on a C18 or C8 material could not separate these two analogues. Increasing the ratio of the aqueous buffer in the mobile phase in order to decrease the strength of the mobile phase, did not lead to improvement in the separation of the β- and γ-isomers. The two isomers still co-elute even when the separation time was as long as 40 min (Figure 4.33).

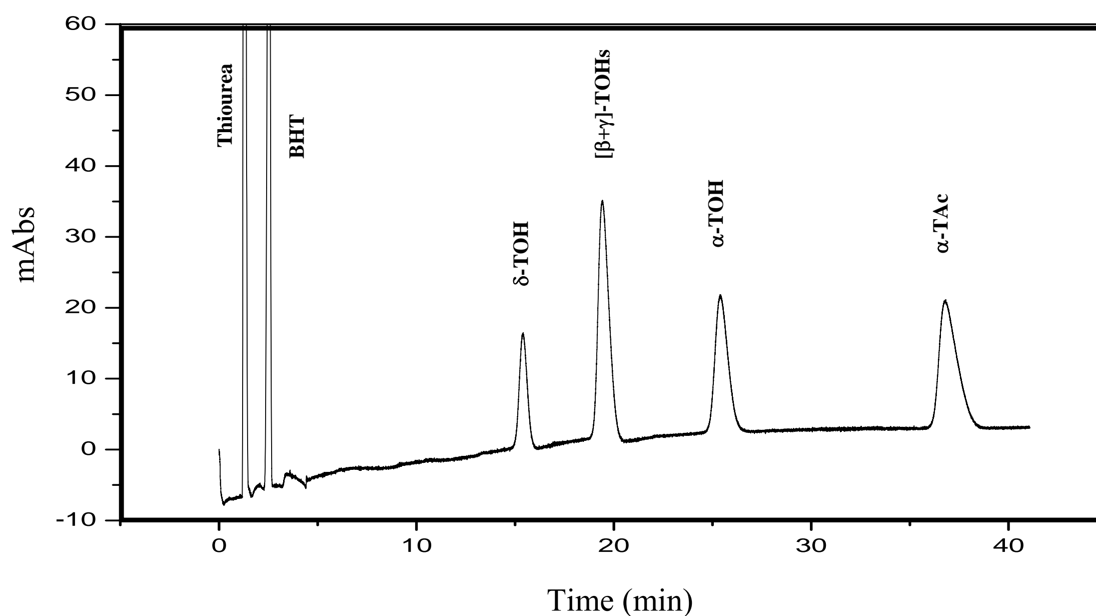


Figure 4.33 Separation of thiourea, BHT, TOHs and α-TAc on C18 packed column; mobile phase, 8:10:82% (v/v/v) 12 mM Tris buffer pH 9.3 : MeOH : ACN, Other conditions are as given in Figure 4.29.

Results in Figures 4.33 clearly demonstrate that C18 does not have sufficient selectivity for β- and γ-TOHs as previously reported. Retention for all the

compounds were increased with increasing water content in the mobile phase or by reducing the mobile phase strength (Figure 4.34) as commonly found in reversed phase chromatography. No change in elution order for all the compounds was observed for this type of column.

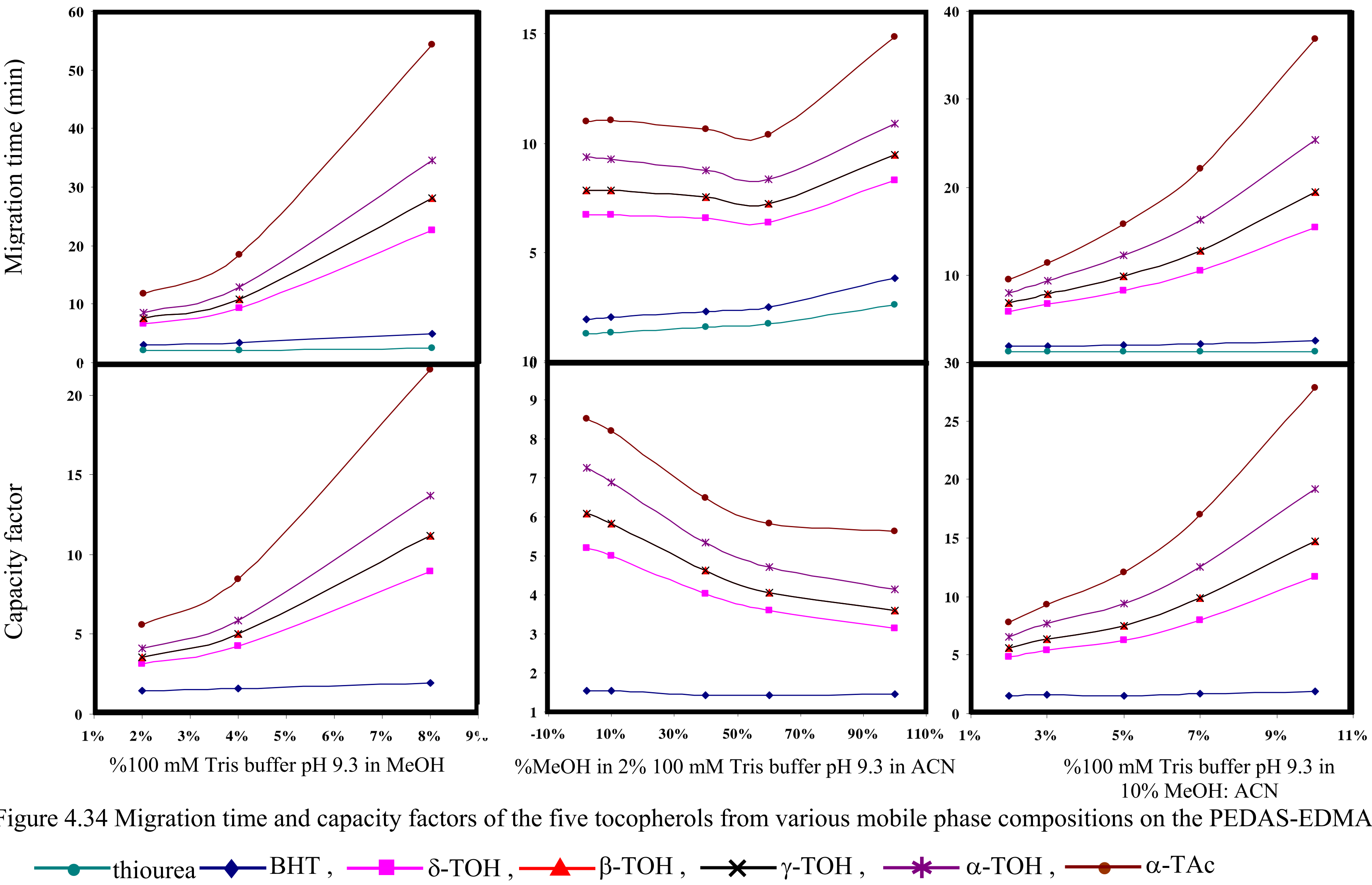


Figure 4.34 Migration time and capacity factors of the five tocopherols from various mobile phase compositions on the PEDAS-EDMA

There have been reports on the successful reversed-phase separation of these tocopherol analogues by CEC using packed columns [141-143]. Warner *et al.* used a non-polar monomeric C30 material, with 1% (v/v) water in methanol as mobile phase [141]. Abidi *et al.* employed a polar reversed-phase stationary phase, pentafluorophenylsilica (PEPS) and 8% (v/v) 25 mM Tris buffer (pH 8) in methanol as the mobile phase [142]. Another approach developed by Carabias-Martinez *et al.* involved the use of a polar-embedded phase, called ULTIMA C18 [143], which contains amide and C15 functional groups with a mobile phase containing 5 mM Tris buffer that was prepared in 5% (v/v) water in methanol.

The elution order of TOH's (δ-, β-, γ- and α-TOH's, see Figure 4.28) for the PEDAS-EDMA, is the same as that reported for the ULTIMA C18 material [143] and also for the polar pentafluorophenylsilica stationary phase [142] indicating similar selectivity for these columns. However, the elution order of β- and γ- TOH's for the PEDAS-EDMA is reversed to that for the monomeric C30 column which is a pure reversed phase column.

In addition, the non-polar compound, α-TAc, eluted earlier (between δ- and β-TOHs, see Figure 4.32), for the monolith, compared to that for the monomeric C30 [141] and C18 columns (Figure 4.33). This could be explained by the different separation mechanisms of the stationary phases since the chromatographic mechanism for the monomeric C30 and C18 material is by hydrophobic interaction only. The C17 ligand and the –OH and ester groups on the amphiphilic PEDAS-EDMA material can contribute to the separation mechanism, involving both hydrophobic and polar interactions as in the ULTIMA C18 and PEPS phases.

4.2.6 Analytical performance of the method

Chromatograms of the standards mixtures for the construction of calibration curves of the five compounds are shown in Figure 4.35. Calibration curves for the five compounds were constructed by plotting the peak areas observed from injection of standards mixtures against the concentration (Figure 4.36). Linear

calibrations for all the compounds were obtained in the ranges of ppm levels with correlation coefficients of 0.999 (Figure 4.36 and Table 11).

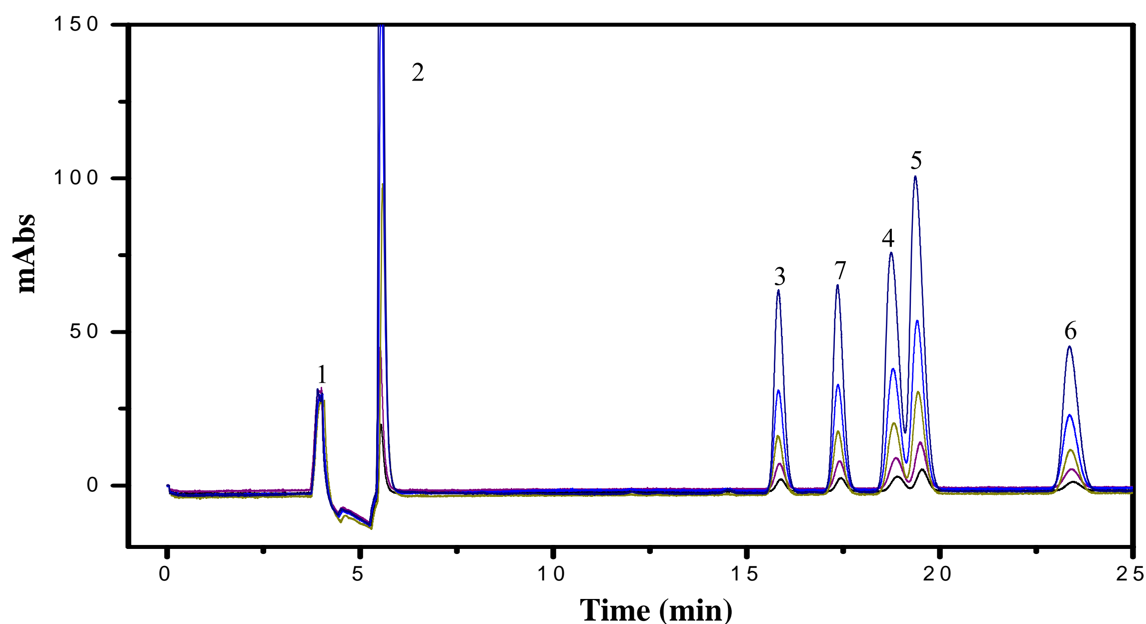


Figure 4.35 Separation of the five tocopherols for constructing of calibration curve. 1, thiourea; 2, BHT; 3, δ-TOH; 4, β-TOH; 5, γ-TOH; 6, α-TOH; 7, α-TAc. Other experimental conditions are given in Figure 4.32

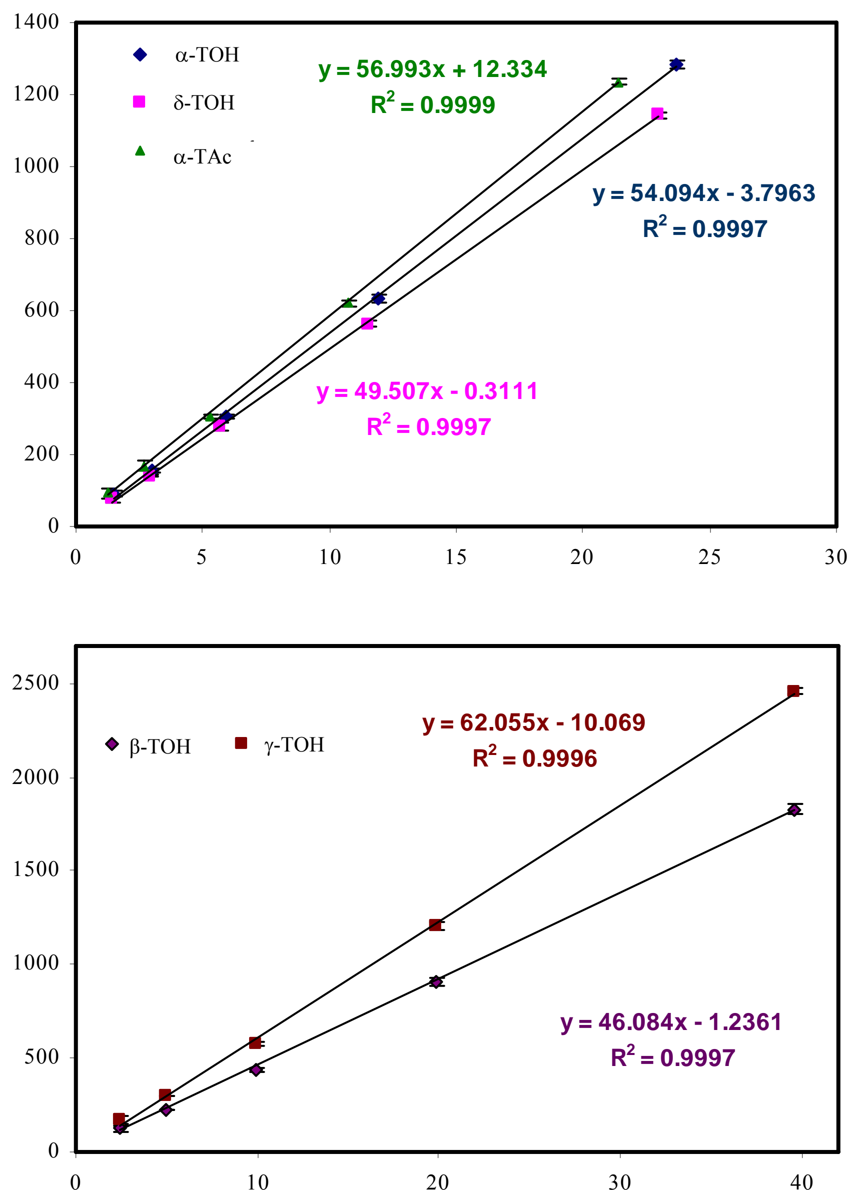


Figure 4.36 Calibration curves for the TOHs and TAc. Conditions as shown in Figure 4.32.

Limits of detections (LOD) were below 1 ppm. Precision of the method was examined by interday and intraday repeatedly injecting standard mixtures of the TOHs and TAc. %RSD for retention time and peak area for intraday and interday variation are shown in Table 4.7. Good precision (%RSD < 2.33 for both interday and intraday precision) show that that the developed method has satisfactory precision. The good precision for retention times of the EOF marker (thiourea) and compounds in Table 4.7 indicate a stable EOF. Performance of the developed method is summarized in Tables 4.8.

Table 4.7 Precision data (%RSD) for retention time (t_r) and peak area[a] (PA) of tocopherol isomers (TOH's), and thiourea (EOF maker) separated on the PEDAS-EDMA monolithic column, using the selected condition shown in Figure 4.32.

Compound	Intraday precision (n=7)		Interday precision (3 days)	
	t_r	PA	t_r	PA
1. α-TOH	0.73	0.37	2.33	1.28
2. β-TOH	0.73	0.62	2.15	1.10
3. γ-TOH	0.73	0.51	2.18	0.72
4. δ-TOH	0.69	0.39	1.95	0.72
5. thiourea	0.85	-	1.46	-

[a] %RSD for ratio of peak area to peak area of α-TAc

Column efficiencies (plates/m) for α-, β-, γ- and δ-TOH's and α-TAc were 79532, 62770, 91881, 86923 and 99387, respectively which is better than when using reversed-phase HPLC. The lifetime of the monolithic column has not been fully investigated in this work. However the column used for this study has been employed for more than 250 injections without loss of performance.

Table 4.8 Analytical performance of the developed CEC method for tocopherol compounds.

Feature	α-TOH	β-TOH	γ-TOH	δ-TOH	α-TAc
Studied linearity range (ppm)	1.5-23.7	2.5-40.0	2.5-40.0	1.4-23.0	1.3-21.4
Calibration equation[a]	Y = 54X – 6	Y = 46X –2	Y = 62X -12	Y = 49X	Y = 56X + 12
Linear regression (r^2)	r^2 = 0.999	r^2 = 0.999	r^2 = 0.999	r^2 = 0.999	r^2 = 0.999
LOD[b] (ppm)	0.5	0.7	0.5	0.4	0.4

[a]Y is peak area (mAbs*s), X is concentration (ppm)

[b]S/N = 3, (S is peak height, N is noise)

4.2.7 Applications for vitamin E pharmaceutical drugs

The developed method was successfully applied to the analysis of vitamin supplements, 'Natural Mixed Tocopherols', which is claimed to contain total vitamin E of 268 mg/capsule and 'Natural vitamin E-Oil' containing 335 mg α-TAc/g. Figures 4.37 and 4.38 show chromatograms for the Natural Mixed Tocopherols and Natural vitamin E-Oils obtained from the developed method, respectively. Results in Figure 4.37 shows that the Natural Mixed Tocopherols contains four tocopherol isomers, including δ-, β-, γ- and α-TOH's at 0.57±0.01, 1.51±0.01, 1.65±0.02, 262±5.2 mg/capsule respectively. Therefore the total vitamin E content of the sample is 265±5.2 mg/capsule. The UV spectrum from the diode array detector showed that the peak at 17.1 min which has the same retention time of TAc, was not in fact TAc. The label of the sample also did not state the inclusion of TAc in the capsule. Figure 4.37 clearly shows that α-tocopherol is the major form for this sample. The recoveries for δ-, β-, γ- and α-TOH's were 99±2, 93±3, 98±1 and 98±3%, respectively.

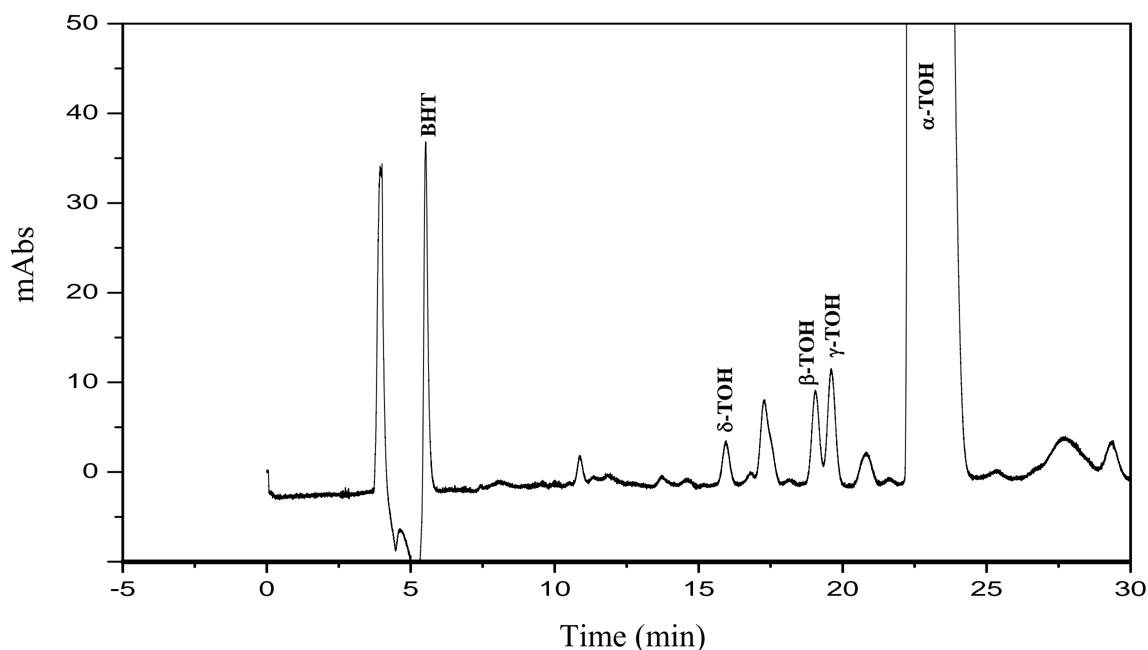


Figure 4.37 Chromatogram of Natural Mixed Tocopherols for the PEDAS-EDMA monolithic column, separation conditions are shown in Figure 4.32.

For Natural vitamin E-Oils sample, only α-TAc found in this sample at a concentration of 342±5.9 mg per g with % recovery of 98±2. All analytical results for the two samples are summarized in Table 4.10.

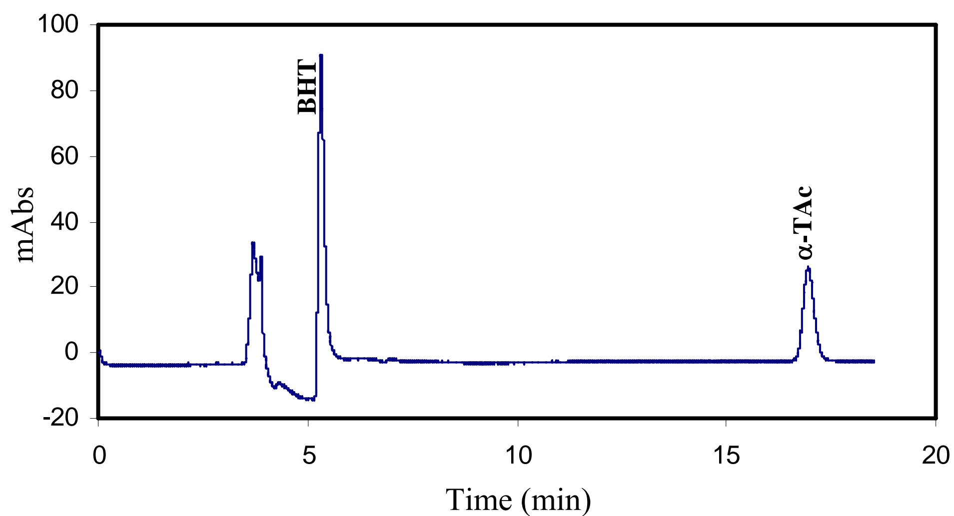


Figure 4.38 Chromatogram of Natural Mixed Tocopherols for the PEDAS-EDMA monolithic column, at the selected separation condition as shown in Figure 4.32.

Table 4.9 Quantification of commercial vitamin E supplements (three capsules for Mixed tocopherols supplement and three replicate analysis for Natural vitamin E-Oils).

Samples	Quantification[a] / % recovery						Label claim
	α-TOH	β-TOH	γ-TOH	δ-TOH	α-TAc	total	
1. Natural Mixed tocopherols capsule[b]							
capsule I	255 ± 12.4	1.13 ± 0.1	1.22 ± 0.1	0.49 ± 0.04	-	257	260 mg/capsule
capsule II	262 ± 5.2	0.97 ± 0.1	1.01 ± 0.1	0.38 ± 0.02	-	264	
capsule III	253 ± 10.8	1.51 ± 0.0	1.65 ± 0.0	0.57 ± 0.00	-	256.	
% recovery	98 ± 3	93 ± 3	98 ± 1	99 ± 2	-		
2. Natural vitamin E-Oils[c]	-	-	-	-	342 ± 5.9	342±5.9	335 mg/g
% recovery	-	-	-	-	98 ± 2		

[a]average ± SD, three injections
[b]mg/capsule
[c]mg/g

4.2.8 Advantages of the developed method

The method developed in this work, with PEDAS-EDMA monolith, has several advantages over former methods. As summarised in Table 4.10, the monolith is technically simpler to produce compared to microparticulate columns. PEDAS-EDMA monoliths can be prepared without the need of high technical skill or use of complex apparatus. Packing of microparticulates (stationary phase no. 1 to 4 in Table 4.10) requires skilled personnel to carry out the procedure. The monolithic stationary phase (No. 5) is a fritless column and therefore has no serious problem from bubble formation, which is often found with packed columns when using CEC.

The monolithic column has been shown to have a better selectivity for β- and γ-TOHs compared to pure reversed-phase C8 and C18 column, which are commonly used in reversed-phase chromatography. The monolith is applicable to the separation of all the important forms of vitamin E, including tocopherols (α-, β-, γ- and δ-TOH's) and tocopherol acetate (α-TAc). Resolution of β- and γ-TOH's on our column was similar to that for a packed-particle ULTIMA C18 column but slightly lower than that for the C30 stationary phase.

TAc is the least polar amongst the compounds studied and typically elutes last from reversed-phase columns and hence this compound governs the separation time. However for the amphiphilic PEDAS-EDMA monolith, the TAc elutes earlier as discussed in section 4.2.5. The separation time for the monolith is therefore shorter (data in Table 4.10). The analysis time on this monolithic column is 12 min less than using a C30 stationary phase.

Table 4.10 Comparison of properties of the PEDAS-EDMA monolith for separation of TOH's and TAc to other columns from literature.

Stationary phase	Type of column	Column Preparation	Analyte	Separation time (min)	$R_s(\beta\text{-}\gamma)^a$	Ref.
1. C8, C18	microparticulate	Difficult	α-,[β+γ]-,δ-TOHs, α-TAc	2.5-18[b]	0.0[c]	118-120, 124-135
2. Monomeric C30	microparticulate	Difficult	α-,β-,γ-,δ-TOHs, α- TAc	37	1.5	141
3.Pentafluoropheny-lsilica	microparticulate	Difficult	tocopherols and tocotrienols	30	-[d]	142
4. ULTIMA C18 [e]	microparticulate	Difficult	α-,β-,γ-,δ-TOHs	28	1.2	143
5. PEDAS-EDMA[f]	monolith	Simple	α-,β-,γ-,δ-TOHs, α-TAc	25	1.2	This work

[a]resolution (R_s) between β- and γ-TOH, $R_s = 2[(t_r)_\gamma-(t_r)_\beta]/(W_\beta+W_\gamma)$, t_r = retention time and W = baseline peak width

[b]depend on applications and techniques (CEC and LC)

[c]cannot separate β- and γ-TOHs

[d]not reported

[e]bifunctional groups of amide and C15

[f]bifunctional groups of hydroxyl and C17

CHAPTER V
CONCLUSIONS

In this thesis, studies have been carried out on the investigation of monolithic material for capillary electrophoresis and capillary electrochromatography for pre-concentration and separation, respectively. The work can be concluded into two parts as given below.

5.1 Part I: Investigation of polymer based monolithic and silica based packed particle phases for in-line pre-concentration/extraction steps in capillary electrophoresis

In this part, two synthetic monoliths namely methacrylic acid-ethylene dimethacrylate (MAA-EDMA) and butyl methacrylate- ethylene dimethacrylate (BMA-EDMA) have been investigated for in-line coupling with capillary electrophoresis (CE) for sensitivity enhancement in the analysis of benzyl alcohol (Bz), terbutaline (TER) and 4-hydroxy-3-methoxy-methamphetamine (HMMA). The coupling was perfomed by *in situ* preparation of the monoliths inside fused silica capillary. The two monoliths showed incompatibility with pure aqueous medium. Irreproducible results with shifting of retention time and peak areas for the three compounds were observed when pure aqueous solutions were used. Improvement in repeatability was observed when solutions used contained at least 10% (v/v) organic solvent such as methanol or acetonitrile. However since CE separation of the compounds with this solvent (10% (v/v) organic solvent:aqueous buffer) was poor. The CE separation was carried out in non-aqueous mode. The MAA-EDMA monolith was therefore coupled with non-aqueous CE.

Although a large improvement in sensitivity over the conventional CE technique was observed, poor repeatability was still observed between run. It was therefore concluded that the monolith was not suitable for such application which may be due to change in properties of the monolith. However, more work on properties and structural studies of the monolith before and after passing solvents need to be carried out

The work on coupling of SPE with CE was also carried on silica based C18 packed particle phase a conventional chromatographic phase. A large improvement in sensitivity was achieved with this type of material. In contrast to the polymer based monolithic phase, coupling of packed silica based SPE to a CE system showed good compatibility with both aqueous and non-aqueous solvents. The method has been applied for the three compounds with excellent repeatability. This shows that silica based material would be more suitable for these compounds than the polymeric-based monolith. The in-line SPE-CE method would possibly be useful for samples like human serum, in which the analytes are present at low levels.

5.2 Part II : Development of a monolithic capillary electrochromatographic method for separation and determination of tocopherols and tocopherol acetate

This part demonstrated the first use of monolith for separation of α-, β-, γ- and δ- tocopherols, and α-tocopherol acetate by capillary electrochromatography with UV detection. Performance of a pentaerythritol diacrylate monostearate - ethylene dimethacrylate monolith (PEDAS-EDMA), an amphiphilic column for the separation was demonstrated. The PEDAS-EDMA monolith showed a remarkably good selectivity for separation of the tocopherol isomers including the β- and γ-isomers which are not easily separated by standard C8 or C18 packed column. The separation was credited to its amphiphilic nature, having both polar hydroxyl/ester and non-polar C17 groups. Retention studies indicated a reversed-phase mechanism was involved in the separation on the PEDAS-EDMA column, but polar interactions with the

underlying ester and hydroxyl groups enhanced the separation of the problematic β- and γ-isomers. Results have shown that the methanol composition plays an important role in the separation and elution order. Hydrophobicity of the column can be tuned by adjusting the methanol composition. Addition of sufficient amount of methanol increases the hydrophobicity of the system by the formation of H-bonding between methanol and the –OH groups of PEDAS.

Separation of all the compounds was achieved within 25 min using 3:10:87 (v/v/v) 100 mM Tris buffer (pH 9.3): methanol: acetonitrile as the mobile phase. The method was successfully applied to vitamin E supplements with recoveries from 93% to 99%. Intraday and interday precisions (%RSD), for peak areas and retention times for all the compounds were less than 2.3. Limits of detection for all four tocopherols and tocopherol acetate were below 1 ppm.

Being an amphiphilic column, with –OH and C17 ligands, the monolith provides selectivity for the separation of β- and γ- tocopherols. These two tocopherol isomers are exceptionally difficult to separate using common reversed-phase materials (C8 or C18) unless C30 is employed. The ability to separate β- and γ- tocopherols on the PEDAS-EDMA monolith, which can be readily prepared in the laboratory, is noteworthy.

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AWARDS

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