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DEVELOPMENT OF SENSITIVE ANALYTICAL METHODS USING LIQUID-LIQUID MICROEXTRACTION COMBINED WITH ONLINE PRECONCENTRATION TECHNIQUES IN CAPILLARY ELECTROPHORESIS

Ibrahim LUBBAD

Ph.D. THESIS

DEPARTMENT OF ANALYTICAL CHEMISTRY

GAZİ UNIVERSITY

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Supervisor: Prof. Dr. Nusret ERTAŞ
Analitik Kimya ABD, Gazi Üniversitesi
Bu tezin, kapsam ve kalite olarak Doktora Tezi olduğunu onaylıyorum

Başkan : Prof. Dr. Bengi USLU
Analitik Kimya ABD, Ankara Üniversitesi
Bu tezin, kapsam ve kalite olarak Doktora Tezi olduğunu onaylıyorum

Üye : Prof. Dr. Uğur TAMER
Analitik Kimya ABD, Gazi Üniversitesi
Bu tezin, kapsam ve kalite olarak Doktora Tezi olduğunu onaylıyorum

Üye : Prof. Dr. Nilgün GÜNDEN GÖĞER
Analitik Kimya ABD, Gazi Üniversitesi
Bu tezin, kapsam ve kalite olarak Doktora Tezi olduğunu onaylıyorum

Üye : Assoc. Prof. Burcu Doğan Topal
Analitik Kimya ABD, Ankara Üniversitesi
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bildirir, aksi bir durumda aleyhime doğabilecek tüm hak kayıplarını kabullendiğimi beyan ederim.

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SIVI-SIVI MİKROEKSTRAKSİYON VE KAPİLER ELEKTROFOREZDE ON-LINE ZENGİNLEŞTİRME İLE DUYARLI ANALİTİK METOD GELİŞTİRİLMESİ

(Doktora Tezi)

Ibrahim LUBBAD

GAZİ ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ

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ÖZET

Kapiler elektroforez güçlü bir ayırma tekniği olmasına rağmen duyarlılığının düşük olması ve gerçek numunelerde örnek matriksinin girişim etkileri nedeni ile mevcut kromatografik yöntemler ile rekabet edememektedir. Bu çalışmada biyolojik önemi olan bazı moleküllerin girişimlerden bağımsız ve duyarlı tayinleri için, yakın zamanda geliştirilmiş mikroekstraksiyon yöntemleri ile kapiler elektroforezde on-line zenginleştirme yöntemleri birleştirilmiştir. Çalışma üç ana başlıkta toplanabilir. İlk kısımda bir endokrin bozucu olan ve şişelenmiş su veya içecek kaplarının üretiminde kullanılan bisfenol A'nın (BPA) su ve idrar numunelerinde tayini çalışılmıştır. Yüzen organik damlacıkların katılaştırılmasına dayanan dispersif sıvı-sıvı mikroekstraksiyon (DLLME-SFO) yöntemi ile kapiler elektroforezde (CE) yükseltilmiş alan numune istifleme (FASS) modu ile ilk defa birleştirilerek su ve idrar örneklerinde bisfenol A'nın zenginleştirilmesi ve tayini için kullanılmıştır. Kalibrasyon grafikleri, su için 2,5-100 ng/mL, idrar için 10-100 ng/mL arasında doğrusallık göstermektedir, bağıl standart sapma (BSS%) 1.9, teşhis sınırı (LOD) ise su ve idrar örneklerinde sırasıyla 0.8 ng/mL ve 2.5 ng/mL olarak bulunmuştur. Çalışmanın ikinci bölümünde, DLLME-FASS-CE kullanılarak kutu süt numunelerinde dört alkilfenol, bisfenol F (BHM), bisfenol A (BPA), 4-tert-bütilfenol ve 4-nonilfenol tayin edildi. Analitlerin doğrusal aralıkları BHM ve BPA için 100-180 ng/mL ve TBP ve NP içinse 140-220 ng/mL arasında, %BSS 5'ten küçük ve LOD değerleri sırasıyla 25.6, 29.7, 48.1 ve 40.2 ng/mL olarak hesaplandı. Çalışmanın üçüncü bölümünde, balık dokusunda trifenilmetan grubuna ait, malaşit yeşili (MG), kristal menekşe (CV) ve bunların löko formları, löko malaşit yeşili ve löko kristal menekşe tayin edilmiştir. Analitlerin matristen ayrılması ve zenginleştirilmesi için bir mikroekstraksiyon yöntemi geliştirildikten sonra tayinler yalancı izotakoforez (P-iTP) yöntemi ile gerçekleştirilmiştir. Optimize edilmiş koşullarda, MG, CV, LMG ve LCV için doğrusal aralıklar 10-100 ng/g arasında, LOD değerleri ise sırasıyla, 2.3, 1.0, 1.6 ve 3.2 ng/g bulunmuştur. Geri kazanımlar %80-103 arasında ve BSS %8'den küçüktür. Geliştirilen yöntemlerin analitlerin biyolojik, çevre ve gıda numunelerinden ekstrakte edilmesi ve tayini için basit, duyarlı, yüksek verimli, ekonomik ve çevre dostu yöntemler olduğu gösterilmiştir.

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DEVELOPMENT OF SENSITIVE ANALYTICAL METHODS USING LIQUID-LIQUID MICROEXTRACTION COMBINED WITH ON-LINE PRECONCENTRATION TECHNIQUES IN CAPILLARY ELECTROPHORESIS
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Ibrahim LUBBAD

GAZI UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
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ABSTRACT

Although capillary electrophoresis (CE) is a powerful separation technique, it suffers from low sensitivity and matrix effects in real sample analysis. In this study, recently developed liquid-liquid microextraction and on-line preconcentration methods in CE have been combined for interference free and sensitive determination of different molecules having biological and environmental importance. The study included three main parts. In the first part, bisphenol A (BPA), an endocrine disrupter used in the production of bottled water or beverage containers, was determined in water and urine samples. Dispersive liquid–liquid microextraction method based on solidification of floating organic drop (DLLME-SFOD) was combined for the first time with field amplified sample stacking (FASS) in CE for preconcentration and determination of BPA in water and urine samples. Calibration curves were linear in the range of 2.5- 100 ng/mL for water and 10-100 ng/mL for urine samples. The relative standard deviation (RSD %) was 1.9 and the LOD was 0.8 ng/mL for water and 2.5 ng/mL for urine samples. In the second part of the study, four alkylphenols namely, bisphenol A, bisphenol F, 4-nonylphenol and 4-tert-butylphenol were determined in bottled milk using DLLME-FASS-CE. The linear ranges for the analytes were between 100-180 ng/mL for BHM and BPA and 140-220 ng/mL for TBP and NP. The RSD was less than 5% and the LOD values 25.6, 29.7, 48.1 and 40.2 ng/mL for BHM, BPA, TBP and NP respectively. In the third part of the study, triphenylmethane (TPM) dyes namely malachite green (MG), crystal violet (CV) and their leuco forms were determined in aquaculture fish samples. A novel method of sample pretreatment and microextraction was developed and combined with pseudo-isotachophoresis (P-iTP) stacking technique in CE for preconcentration and sensitive determination of TPM. Under the optimized conditions the linear range for the analytes was 10-100 ng/g with LOD values 2.3, 1.0, 1,6 and 3.2 ng/g for MG, CV, LMG and LCV, respectively. The recoveries ranged between 80-103% and the RSD was less than 8%. The developed methods required very simple and cheap devices and minimal consumption of organic solvents making them affordable, efficient and convenient methods for offline and online preconcentration and determination of trace analytes in a wide category of sample matrices including biological, environmental and food samples.

Science Code	:	201.1.004
Key Words	:	Capillary electrophoresis, preconcentration, microextraction, stacking, alkylphenols, bisphenol A, triphenylmethane, malachite green.
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SYMBOLS AND ABBREVIATIONS

Bu çalışmada kullanılmış simgeler ve kısaltmalar, açıklamaları ile birlikte aşağıda sunulmuştur.

Acronym	Definition
ACN	Acetonitrile
AP	Alkylphenols
APEOs	Alkylphenol polyethoxylates
ASE	Accelerated Solvent Extraction
BGE	Background electrolyte
BPA	Bisphenol A
CE	Capillary electrophoresis
CE−UV	Capillary electrophoresis−Ultraviolet
CFM	Chloroform
CV	Crystal violet
CZE	Capillary zone electrophoresis
DI	Deionized
DLLME	Dispersive liquid-liquid microextraction
DLLME-SFOD	Dispersive liquid-liquid microextraction based on solidification of floating organic drop
DNA	Deoxyribonucleic acid
EDCs	Endocrine disrupting chemicals
EOF	Electroosmotic flow
EPA	Environmental Protection Agency
EU	European Union
FASI	Field-amplified sample injection
FASS	Field-amplified sample stacking
GAC	Green analytical chemistry
GC	Gas chromatography
HF−LPME	Hollow fiber-liquid phase microextraction
HPLC	High-performance liquid chromatography

Acronym	Definition
HSDC	High-sensitivity detection cell
ITP	Isotachophoresis
LC	Liquid chromatography
LCV	Leuco crystal violet
LLE	Liquid-liquid extraction
LMG	Leuco malachite green
LOD	Limit of detection
LOQ	Limit of quantitation
LPME	Liquid-phase microextraction
LVSS	Large-volume sample stacking
MEKC	Micellar electrokinetic chromatography
MG	Malachite green
NP	Nonylphenol
NSM	Normal-stacking mode
OP	Octylphenol
PAGE	Polyacrylamide gel electrophoresis
pITP	Pseudo Isotachophoresis
PF	Preconcentration factor
RSD	Relative standard deviation
S/N	Signal to noise ratio
SBME	Solvent bar microextraction
SD	Standard deviation
SDME	Single-drop microextraction
SDS	Sodium dodecyl sulfate
SFOD−ME	Solidification of floating organic drop microextraction
SPE	Solid phase extraction
SPME	Solid-phase microextraction
tITP	Transient Isotachophoresis
TPM	Triphenylmethane
UV	Ultraviolet

1. INTRODUCTION

Capillary electrophoresis (CE) is one of the main separation techniques for analytical purposes. CE features several advantages, such as high separation efficiency, short analysis time, low solvents and buffers consumption and environmental friendliness. Unfortunately, the main inconvenience of this technique is the relative low sensitivity compared to the competitive liquid chromatography (LC). However, studies conducted in recent years have shown that application of on-line preconcentration techniques can make CE even more sensitive than LC. It reveals the potential of in-capillary preconcentration techniques (Dziomba et al., 2014).

CE is a field that continues to grow. All areas of CE including theory, separation modes, instrumentation, and applications remain active areas of research (Harstad, et al, 2016b). With a separation based on physical phenomena different from those used in chromatography, CE has been the focus of attention for developing new analytical methodology. The analysis of samples that cannot be separated by more common reversed phase LC are often resolved by CE. Unfortunately, the benefits from the high number of theoretical plates obtained with CE have been overshadowed by the poor detection limits achieved with UV detection (Osbourn et al., 2000). One of the drawbacks of CE equipped with direct UV detection is the poor concentration sensitivity resulting from minute injection volumes needed to maintain high separation efficiency and a short optical path length equal to the capillary diameter (Kim & Terabe, 2003). Solutions to this problem include: the use of capillaries designed for extended detection path length (e.g. multi-reflection and bubble cell, Z-shaped high sensitivity cell), the use of highly sensitive detectors (e.g. laser-induced fluorescence (LIF), mass spectrometer (MS) and electrochemical detectors), and offline sample preconcentration techniques, e.g., liquid-liquid extraction (LLE) and solid-phase extraction (SPE). However, all these techniques require rather expensive and somewhat complex hardware and/or time-consuming procedures (Kim & Terabe, 2003).

On the other hand, the online preconcentration techniques such as sweeping (Kim, et al., 2001) and sample stacking (Chien & Burgi, 1992a) have been used to overcome the sensitivity problem. Stacking techniques include (i) field-amplified sample stacking (FASS) (Quirino & Terabe, 2000a), (ii) large volume sample stacking (LVSS) with polarity switching (Chien & Burgi, 1992b), and (iii) the less often applied field-amplified sample injection (FASI) with electroosmotic flow (EOF) reversal (FASI/EOF reversal) (Sun et al., 2003). CE was originally considered as a powerful analytical tool for the analysis of biological macromolecules. It has though, over the years, been extensively used for the separation of other compounds, such as chiral drugs, food additives, pesticides, inorganic ions, organic acids, and others (Christodoulou, 2012b).

In the present study, novel analytical methods to achieve sensitivity enhancement as compared to conventional capillary zone electrophoresis (CZE) have been developed. The developed methods included sample pretreatment, cleanup and off-line preconcentration (liquid-liquid microextraction) procedures followed by online preconcentration techniques in capillary electrophoresis. The developed methods were optimized and applied to different groups of target molecules in different sample matrices. The developed methods in this study were on three parts taking into consideration the nature of target analytes and the sample matrix. In the first part, the target molecule was bisphenol (BPA) and the sample matrices were water and urine. A dispersive liquid–liquid microextraction method based on solidification of floating organic drop (DLLME-SFOD) was combined for the first time with field amplified sample stacking (FASS) in capillary electrophoresis (CE) for preconcentration and determination of BPA in water and urine samples (Alshana et al., 2013). In the second part, the target molecules were four alkyl phenols (Nonylphenol, 4-tertiary butylphenol, Octylphenol and BPA) and the sample matrices were urine and bottled milk. DLLME method was combined with the normal stacking in micellar electrokinetic chromatography mode NSM-MECK in CE for preconcentration, separation and determination of four APs in urine and bottled milk samples. In the third part, the last group of the studied target molecules were triphenylmethane dyes, malachite green (MG), crystal violet (CV) and their leuco metabolites, leucomalachite green (LMG) and leucocrystal violet (LCV) and the sample matrix was culture fish. A simple and rapid method was developed for

extraction, preconcentration and quantification of ultratrace residues of triphenylmethane dyes in culture fish samples. The developed method based on liquid-liquid extraction procedures followed by capillary electrophoresis (CE) determination. Pseudo Isotachophoresis (p-ITP) stacking technique was used for the separation and online concentration of MG, CV and their leuco metabolites.

In developing these methodologies, we had two main objectives: (1) the development of universal analytical methods for different analytes in different matrices using systems based on ultraviolet absorption and sample stacking and (2) the development of rapid and high-sensitivity determination methods for specific classes of analytes having importance in our daily life in different matrices. Initially we started with one analyte (BPA) in a simple matrix (water), then followed by the analysis of more analytes in more complex matrices (urine, milk and fish). The investigation and application of the developed procedures are reported herein.

2. GENERAL INFORMATION

2.1. Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore (20-200 μm i.d.) capillaries to perform high efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. The properties of the separation and the ensuing electropherogram have characteristics resembling a cross between traditional Polyacrylamide Gel Electrophoresis (PAGE) and modern high-performance liquid chromatography (HPLC) (Altria et al., 2001). CE offers a novel format for liquid chromatography and electrophoresis that:

- employs capillary tubing within which the electrophoretic separation occurs;
- utilizes very high electric field strengths, often around 500 V/cm;
- uses modern detector technology such that the electropherogram often resembles a chromatogram;
- has efficiencies on the order of capillary gas chromatography or even greater;
- requires minute amounts of sample;
- is easily automated for precise quantitative analysis and ease of use;
- consumes limited quantities of reagents;
- is applicable to a wider selection of analytes compared to other analytical separation techniques (Beckman Coulter, 1993: 1).

2.1.1. General Information about CE

Capillary electrophoresis (CE) is electrophoresis performed in a capillary tube. It is the most efficient separation technique available for the analysis of both large and small molecules. The transformation of conventional electrophoresis to modern CE was spurred by the production of inexpensive narrow-bore capillaries for gas chromatography (GC) and the development of highly sensitive on-line detection methods for high performance liquid chromatography (HPLC) (Y. XU, 1996a).

CE has become a mature technique for analytical separation and has been increasingly important in separation science. It has been widely utilized in biological, environmental, pharmaceutical, clinical, and food analysis. Furthermore, CE is superior over other separation techniques in terms of fast separation, high solution and minimal requirements of sample and reagent amounts (Aranas et al., 2009).

CE has emerged as a versatile and robust separation technique but there is still much to be learned about CE and further innovation is needed to improve the technique for the greater scientific community (Harstad et al., 2016b). One of the most challenging issues facing the technology of capillary electrophoresis (CE) is sensitivity. In general, when compared with high performance liquid chromatography (HPLC), CE is a less sensitive technique. This arises from the small dimensions of the capillary used in CE, which restrict both the volume of sample that can be injected and the optical path length during spectrophotometric detection. However, since the volume or length of the injected sample zone in capillary is very small or short, CE suffers from poor concentration sensitivity especially when on-line Ultraviolet (UV) detection is used. Several strategies have been developed to solve this problem (Chen et al., 2012). Besides the use of high sensitivity detectors such as laser-induced florescence (LIF), chemiluminescence or electrochemical detectors, electrophoresis-based and extraction preconcentration techniques are used widely (Aranas et al., 2009).

2.1.2. Capillary electrophoresis system

The basic instrumental configuration for CE is relatively simple. All that is required is a fused-silica capillary with an optical viewing window, a controllable high voltage power supply, two electrode assemblies, two buffer reservoirs, and an ultraviolet (UV) detector. The ends of the capillary are placed in the buffer reservoirs and the optical viewing window is aligned with the detector. After filling the capillary with buffer, the sample can be introduced by dipping the end of the capillary into the sample solution and elevating the immersed capillary a foot or so above the detector-side buffer reservoir. Virtually all of the pre-1988 work in CE was carried out on homemade devices following this basic configuration. While relatively easy to use for experimentation, these early systems were inconvenient for routine

analysis and too imprecise for quantitative analysis. The basic instrumental set-up, which is illustrated in Figure 2.1, consists of a high voltage power supply (0 to 30 kV), a fused silica (SiO_2) capillary, two buffer reservoirs, two electrodes, and an on-column detector (Y. XU, 1996a).

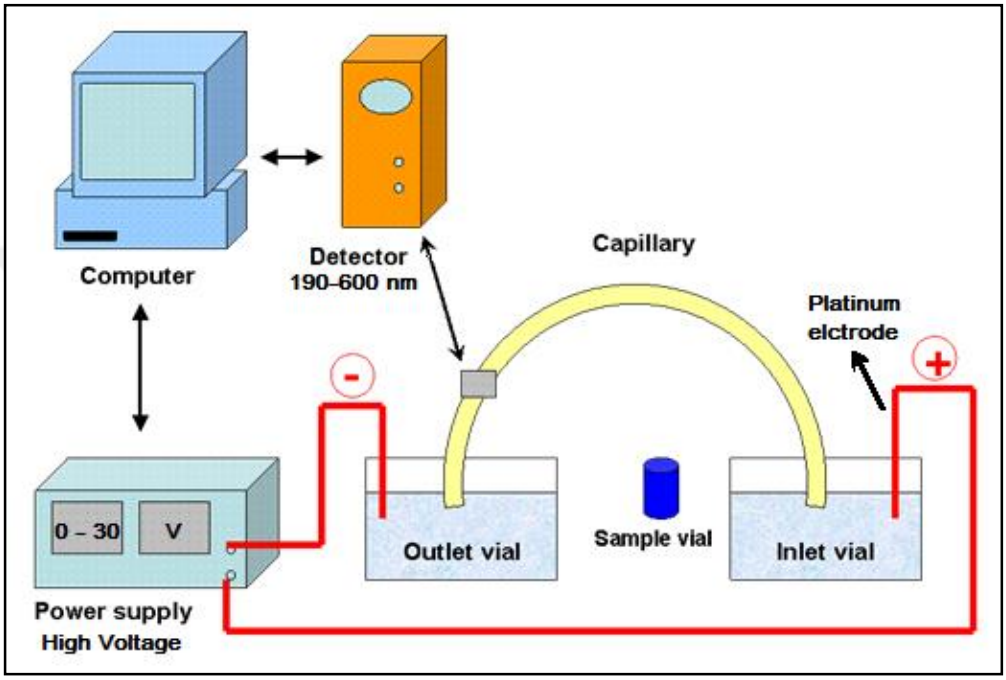


Figure 2.1.Instrumental setup of a CE system

The heart of capillary electrophoresis (CE) is electroosmotic flow (EOF). This is the mobile phase “pump” in CE. Unlike gas chromatography (GC), there is no pressurized gas acting as the mobile phase in CE. Neither as high-performance liquid chromatography (HPLC) there is no (high pressure) pumped mobile phase. And unlike paper chromatography, there is no capillary action that pulls the solvent through the stationary phase. Instead, the electrical potential maintained across the CE’s capillary tube by the electrical circuit of the 1) capillary, 2) buffer, 3) reservoirs, 4) electrodes, and 5) power supply sets up some pretty interesting conditions that makes the buffer solution flow from one buffer reservoir to the other, just as if it were being pumped. This flow is called electroosmotic flow (Chasteen, 2005) and it is described in the next section.

2.1.3. Separation mechanisms and modes

CE has become one of the most useful, versatile and robust technique in separation science because of its high separation efficiency, low cost, versatility, ease of sample preparation and automation (Harstad et al.,2016a) It has been widely utilized in biological, environmental, pharmaceutical, clinical, and food analysis. Furthermore, CE is superior over other separation techniques in terms of fast separation, high solution and minimal requirements of sample and reagent amounts (Chen et al., 2012).

The main advantages of CE are its analysis speed, its extraordinary high efficiency of separation, the minimal amounts of sample and reagent that it requires, and its environmentally friendly nature as it uses aqueous separation buffers (Aranas et al., 2009). With a separation based on physical phenomena different from those used in chromatography, capillary electrophoresis (CE) has been the focus of attention for developing new analytical methodology. The analysis of samples that cannot be separated by more common reversed phase liquid chromatography (LC) are often resolved by CE (Osbourn et al., 2000).

In CE, separation is driven by two factors. The first is the movement of the solute in capillary under the influence of an electric field, also called electrophoretic velocity. The second is the bulk flow of the buffer solution due to the surface charge on the capillary wall, also called electroosmotic flow (EOF). A detailed description of these theoretical aspects is given by Jorgenson and Lukacs (Jorgenson & Lukacs, 1983).

The movement of a charged solute through a conductive solution toward or away from an electrode is dependent upon the mobility of the solute and the magnitude of the applied electric field. This movement is called the electrophoretic velocity (V_{ep}) and is given by: $V_{ep} = \mu_{ep} E$

where $\boldsymbol{\mu_{ep}}$ is the electrophoretic mobility and **E** is the field strength (obtained by dividing the applied voltage by the length of the capillary). Electrophoretic mobility is dependent on the solute and the buffer properties and is given by: $\mu_{ep} = q / 6\pi\eta r$,

where ***q*** is the charge of the analyte, **η** is the buffer viscosity, and **r** is the solute radius. This means that the actual elution order for cations and anions is based on their charge-to-size ratio. Thus, cationic solutes with the largest charge-size ratio have the highest net mobility and elute first. For anions the opposite occurs; that is, solutes with the largest charge-size ratio elute last because of their greater attraction to the anode. Finally, neutral solutes, being uncharged, elute as a single peak. The form of CE just described is free-zone capillary electrophoresis.

Electroosmotic flow is the main factor that affects the movement of solute through the capillary. EOF describes the movement of ions through a solute under the control of an applied potential. In CE, the capillary columns consist of silica with silanol (Si-OH) groups exposed on the inner surface. The exposed silanol groups are ionized above pH 3, therefore creating a negatively charged silanoate ($Si\text{-}O^{-}$) groups inner capillary surface. Cations present in ionic solutions (buffer solution) will migrate toward the negatively charged wall forming an electric double layer. These cations are not of sufficient density to neutralize all the negative charges, so a second, outer layer of cations forms. While the inner layer is tightly held by the $Si\text{-}O^{-}$ groups, the outer layer of cations is not tightly held because of its larger distance from the silanoate groups. Under the influence of an electric field, the outer layer of cations is pulled toward the negatively charged cathode. Since these cations are solvated, they drag the bulk buffer solution with them, thus causing EOF, as represented in Figure 2.2.

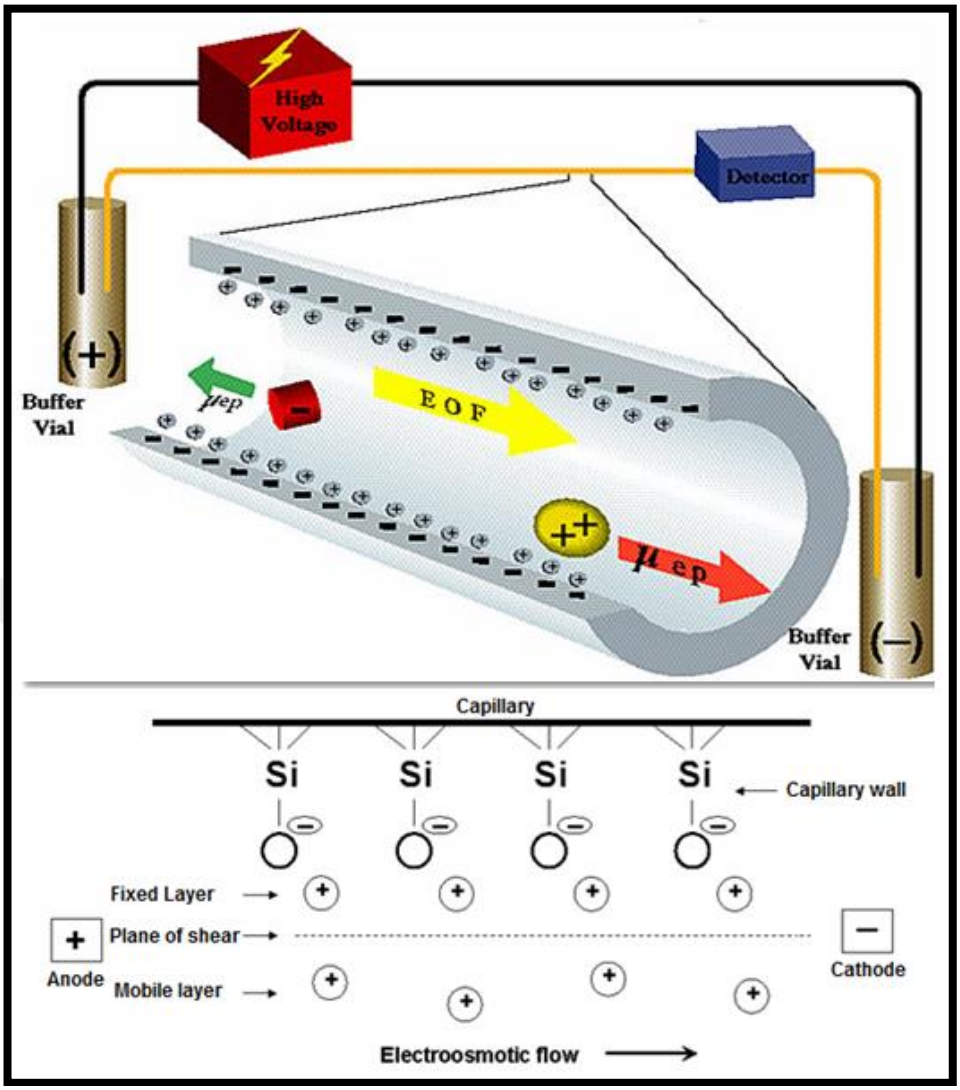


Figure 2.2. Representation of electroosmotic flow in a capillary

The electroosmotic flow can be described in terms of velocity, V_{EOF}, or mobility, μ_{EOF}:

$$V_{EOF} = \varepsilon\zeta E / 4\pi\eta$$

$$\mu_{EOF} = \varepsilon\zeta / 4\pi\eta$$

where **ε** is the dielectric constant of the buffer, **ζ** is the zeta potential that arises on the surface of the capillary, and **η** is the viscosity of the buffer. The overall mobility, that is, apparent mobility, $\boldsymbol{\mu_a}$, of a solute is the sum of the electrophoretic mobility and the electroosmotic mobility (Figure 2.3):

$$\mu_a = \mu_{ep} + \mu_{EOF}$$

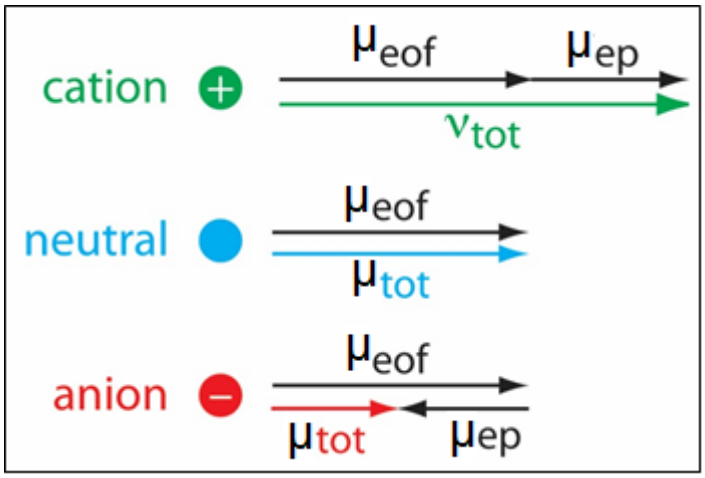


Figure 2.3. Visual explanation for the general elution order in CE

As illustrated in Figure 2.3, each species has the same electroosmotic flow, μ_{eof}. Cations elute first because they have a positive electrophoretic velocity, μ_{ep}. Anions elute last because their negative electrophoretic velocity partially offsets the electroosmotic flow velocity. Neutrals elute with a velocity equal to the electroosmotic flow.

As mentioned in the previous sections CE encompasses a family of related separation modes that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Depending on the types of capillary and electrolytes used, the technology of CE can be segmented into several separation modes. Examples of these include:

- *Capillary Zone Electrophoresis (CZE)*, also known as free-solution CE (FSCE), is the simplest form of CE. The separation mechanism is based on differences in the charge-to-mass ratio of the analytes. Fundamental to CZE are homogeneity of the buffer solution and constant field strength throughout the length of the capillary. The separation relies principally on the pH controlled dissociation of acidic groups on the solute or the protonation of basic functions on the solute.

- *Capillary Gel Electrophoresis (CGE)*, is the adaptation of traditional gel electrophoresis into the capillary using polymers in solution to create a molecular sieve also known as replaceable physical gel. This allows analytes having similar charge-to-mass ratios to be resolved by size. This technique is commonly employed in SDS-Gel molecular weight analysis of proteins and the sizing of applications of DNA sequencing and genotyping.

- *Capillary Isoelectric Focusing (CIEF)*, allows amphoteric molecules, such as proteins, to be separated by electrophoresis in a pH gradient generated between the cathode and anode. A solute will migrate to a point where its net charge is zero. At the solutes, isoelectric point (pI), migration stops and the sample is focused into a tight zone. In CIEF, once a solute has focused at its pI, the zone is mobilized past the detector by either pressure or chemical means. This technique is commonly employed in protein characterization as a mechanism to determine a protein's isoelectric point.

- *Isotachophoresis (ITP)*, is a focusing technique based on the migration of the sample components between leading and terminating electrolytes. Solutes having mobilities intermediate to those of the leading and terminating electrolytes stack into sharp, focused zones. Although it is used as a mode of separation, transient ITP has been used primarily as a sample concentration technique.

- *Electrokinetic Chromatography (EKC),* is a family of electrophoresis techniques named after electrokinetic phenomena, which include electroosmosis, electrophoresis and chromatography. A key example of this is seen with cyclodextrin-mediated EKC. Here the differential interaction of enantiomers with the cyclodextrins allows for the separation of chiral compounds. This approach to enantiomer analysis has made significant impact on the pharmaceutical industry's approach to assessing drugs containing enantiomers.

- *Micellar Electrokinetic Capillary Chromatography (MECC OR MEKC),* is a mode of electrokinetic chromatography in which surfactants are added to the buffer solution at concentrations that form micelles. The separation principle of MEKC is based on a differential partition between the micelle and the solvent. This principle

can be employed with charged or neutral solutes and may involve stationary or mobile micelles. MEKC has great utility in separating mixtures that contain both ionic and neutral species, and has become valuable in the separation of very hydrophobic pharmaceuticals from their very polar metabolites.

- *Micro Emulsion Electrokinetic Chromatography (MEEKC),* is a CE technique in which solutes partition with moving oil droplets in buffer. The microemulsion droplets are usually formed by sonicating immiscible heptane or octane with water. SDS is added at relatively high concentrations to stabilize the emulsion. This allows the separation of both aqueous and water-insoluble compounds, and is used effectively by the pharmaceutical industry as generic methodology to analyze a broad spectrum of pharmaceuticals.

- *Non-Aqueous Capillary Electrophoresis (NACE)*, involves the separation of analytes in a medium composed of organic solvents. The viscosity and dielectric constants of organic solvents affect both sample ion mobility and the level of electroosmotic flow. The use of non-aqueous medium allows additional selectivity options in methods development and is also valuable for the separation of water-insoluble compounds.

- *Capillary Electrochromatography (CEC)*, is a hybrid separation method that couples the high separation efficiency of CZE with HPLC and uses an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. Because there is minimal backpressure, it is possible to use small-diameter packings and achieve very high efficiencies. Its most useful application appears to be in the form of on-line analyte concentration that can be used to concentrate a given sample prior to separation by CZE (Tagliaro et al., 1998).

2.1.4. Injection modes used in capillary electrophoresis

The way in which analytes are injected in CE is substantially different than in HPLC. In HPLC, a fixed-volume injection is made and ‘inserted’ into the flowing mobile phase once the injection valve is actuated. Because CE is performed in capillaries

which have an internal diameter of 50-100 μm and separation is performed by application of voltage, the ‘insertion’ approach used in HPLC is not usually used. Instead, a ‘hydrodynamic’ or ‘electrokinetic’ injection is used to place a small amount of sample at the beginning of the capillary. In both cases, the capillary is removed from the separation electrolyte to the sample, sample injected, and then the capillary placed back in the separation electrolyte and the voltage applied. While both methods introduce sample to the capillary, they do this in different ways (Dawod et al., 2008).

In a hydrodynamic injection (Figure 2.4) sample enters because of a pressure difference between the inlet and outlet vials. This can be done by applying a positive pressure to the inlet, a negative pressure to the outlet, or as was done in the very early days of CE, by raising the inlet vial to a certain level about the outlet vial to siphon sample into the capillary. While this latter approach may appear to be very crude, it is very reproducible and is also much simpler to practically implement than a pressure or vacuum injection. However, because the height differential is only about 10 cm, the pressure generated is small meaning that it is only useful for very small injections in low viscosity electrolytes and is not suited for use with highly viscous separation electrolytes such as those for size separations of nucleic acids and proteins. This may explain why it is not used in most commercially available instruments found today. Hydrodynamic injection is typically limited to about 1-3% of the capillary volume as larger injections result in an increase in peak broadening due to different electroosmotic flow in the sample and separation electrolyte (Burgi & Chien, 1991).

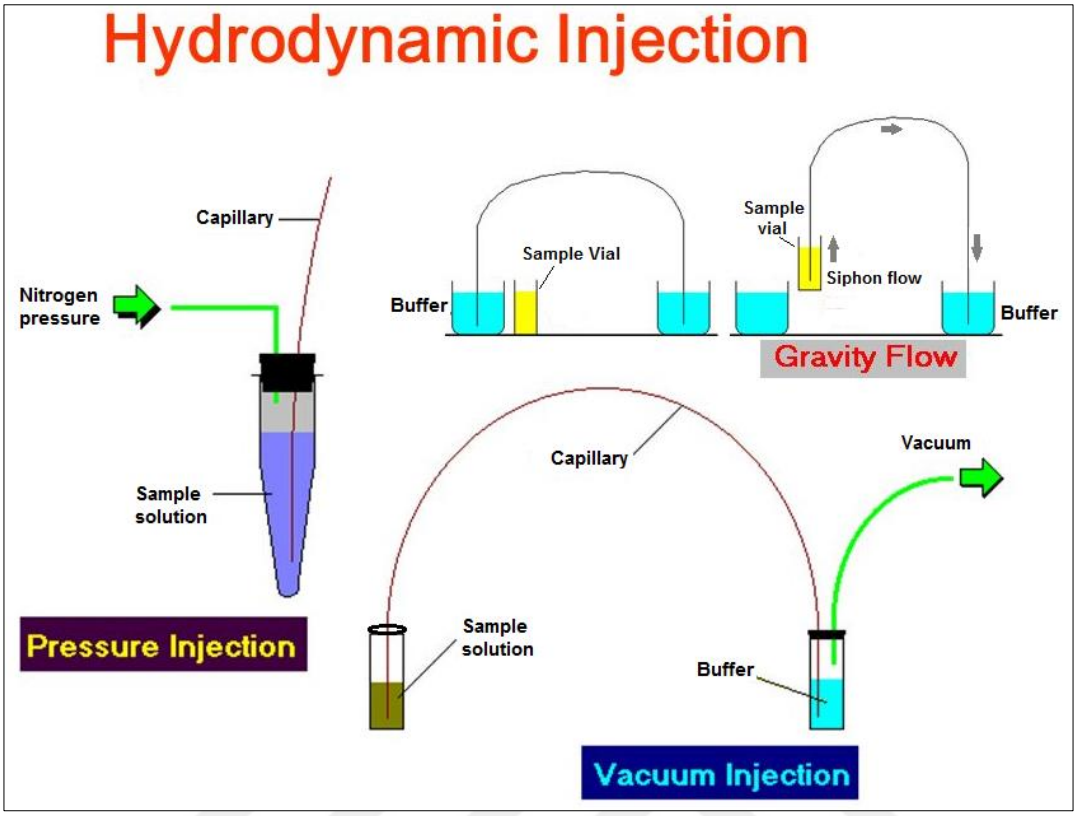


Figure 2.4. Hydrodynamic injection techniques

In contrast, electrokinetic injection uses the phenomena of electrophoresis to inject the sample into the capillary. Analytes migrate into the capillary through a combination of their own electrophoretic mobility and the electroosmotic flow, while the sample matrix and neutral components enter the capillary only through electroosmosis. This approach is also very simple to implement as it is performed in exactly the same way as a separation, with the exception that the inlet vial is the sample instead of the background electrolyte. As such electrokinetic injection can be performed in any CE instrument and is the simplest form of injection to implement. While there is no such thing as a 'typical' electrokinetic injection, 1-10 kV for 3-10 s is usually not a bad place to start (Chien & Burgi, 1991).

2.1.5. Online preconcentration techniques

Methods with a high sensitivity and high separation efficiency are goals in analytical separation techniques. On-line sample concentration techniques in CE separations have rapidly grown in popularity over the past few years because they achieve this

goal (Lin & Kaneta, 2004). Irrespective of the method of detection, detection sensitivity of CE can be maximized by performing sample preconcentration prior to CE. While offline preconcentration techniques such as liquid–liquid extraction and solid-phase extraction are routinely used for sample pretreatment, these methods are often laborious, are time consuming, can require significant experimental skills, and are difficult to automate (Bahga & Santiago, 2013). Several approaches to improve CE sensitivity have been developed. The use of different detection schemes, such as fluorescence (Paez et al., 1996), mass spectrometry (Pacifici et al., 1995), and electrochemistry (Mason et al., 1991) have been reported that enhance sensitivity for compounds that are amenable to these types of selective detection. Extended path length detector cells, such as bubble-shaped flow cells and Z-shaped flow cells that are part of the fused silica capillary (Figure 2.5) have also been employed; however, they provide only a 3-10 fold sensitivity enhancement and the sensitivity improvement is offset by a reduction in separation efficiency (Tsuda et al., 1990).

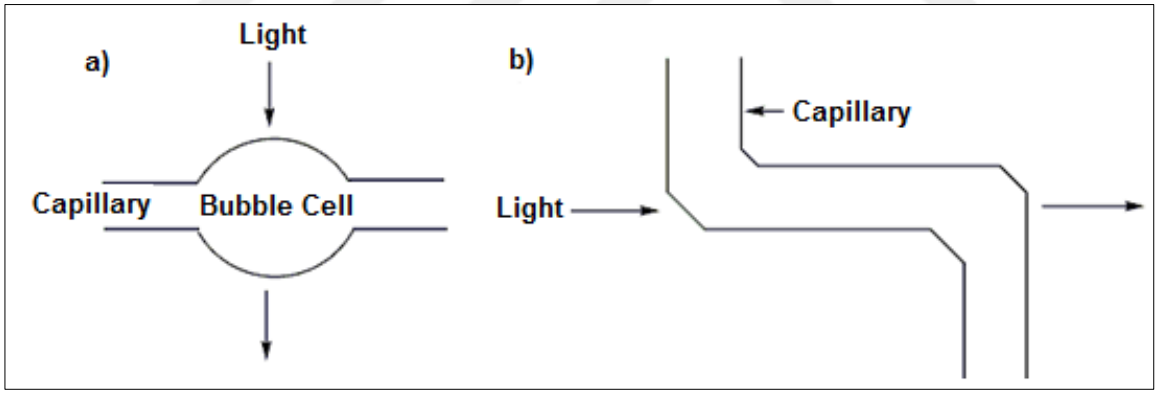


Figure 2.5. a) Bubble and b) z-shaped flow cells to increase the internal diameter of the capillary at the point of detection.

Sample pre-concentration methods are another possibility for increasing sensitivity. Pre-concentration can be combined with the CE in different ways. Four types of interfaces between sample pre-concentration and CE separation can be used: (1) off-line, where pre-concentration and CE separation are performed independently using methods that include manual solid-phase extraction (SPE), manual liquid-liquid extraction (LLE) (Bishop et al., 2004) and manual solid- phase microextraction (SPME) (Lord & Pawliszyn, 1997); (2) at-line, where a robotic system joins the

preconcentration and the separation steps (Veraart et al., 1998); (3) on-line, with direct transport taking place by connecting capillaries (Guzman et al., 1991); and (4) in-line, where concentration takes place in the CE capillary (Alnajjar & McCord, 2003).

While the use of an alternative detector can offer better sensitivity, a more universal approach is sample preconcentration. Numerous preconcentration techniques based on electrophoretic principles, chromatographic principles, or their combinations have been proposed recently (Breadmore & Haddad, 2001). Both charged and neutral analytes can be concentrated. These approaches can be categorized into two groups based on the physical phenomena used to concentrate analytes. One method involves manipulating the electrophoretic velocity of the analyte and includes techniques such as field-amplified sample stacking, large-volume sample stacking, isotachophoresis, pH-mediated stacking, and matrix switching. The other group utilizes partitioning into a stationary or pseudostationary phase to affect the analyte preconcentration, including chromatographic preconcentration and sweeping (Osbourn et al., 2000). Below there is a description of both approaches.

Electrophoretic

Electrophoresis based online preconcentration techniques include transient isotachophoresis preconcentration (Kaniansky & Marak, 1990) (Krivankova et al., 1995), field amplified sample stacking (Chien & Burgi, 1991) (Jacobson & Ramsey, 1995), dynamic pH junction (Aebersold & Morrison, 1990) (Britz-McKibbin & Chen, 2000), pH mediated stacking and sweeping (Kim et al., 2001) and. These techniques are well suited for increasing CE sensitivity as they require little or no changes in the existing CE setups, and their coupling with CE can be mostly or completely automated (Bahga & Santiago, 2013). Electrophoresis based techniques rely on differences in the velocity change of the electrophoretic mobilities of the analytes in different zones. The differences in migration velocity are caused by the change in electric field strength between the sample solution and separation zone or the change in effective charge on the analyte. This is very useful in on-line sample

preconcentration techniques in CE, where a larger volume of the sample solution is injected into the capillary and the analytes in a long sample zone can be focused into a narrow zone before separation and detection.

Electrophoretic pre-concentration methods for CE are usually easier to implement than other pre-concentration methods, as most do not require instrumental modification. There are three main electrophoretic preconcentration mechanisms: sample stacking, field-amplified sample injection, and isotachophoresis. A brief discussion of these techniques follows:

(a) *Sample stacking:* is the most straightforward method by which pre-concentration can be achieved in CE and was first explained by Mikkers et al in 1979 (Mikkers et al., 1979). In this method, the sample is prepared such that it has a lower conductance than the buffer solution. The sample is injected hydrodynamically into the capillary and the pre-concentration effect occurs when voltage is applied after injection. In general, if a sample is dissolved in pure water, solvent, or diluted buffer, then the ionic strength of the sample zone will be considerably lower than that of the rest of the capillary. The low conductivity sample zone will, therefore, have a higher resistance than the rest of the capillary. When a voltage is applied across the capillary, the field strength experienced in the sample zone is higher than the rest of the capillary. The sample ions will then initially move rapidly, but will slow down when they reach the buffer interface in the capillary because of the decrease in field strength. Therefore, when the voltage is applied, the contents of the sample zone are electrically focused (stacked), which reduces the length of the sample zone and produces on-capillary concentration Figure 2.6. In Figure 2.6, the circles represent a cationic solute. Top: the sample plug is injected. Middle: voltage is applied and since the electric field in the sample solution is higher than in the rest of the capillary, the cations rapidly migrate through the sample solution until they reach the low electric field in the buffer, where they slow down and become stacked at the boundary between the solutions. Bottom: the stacked ions migrate through the capillary as a zone that is narrower than the sample plug.

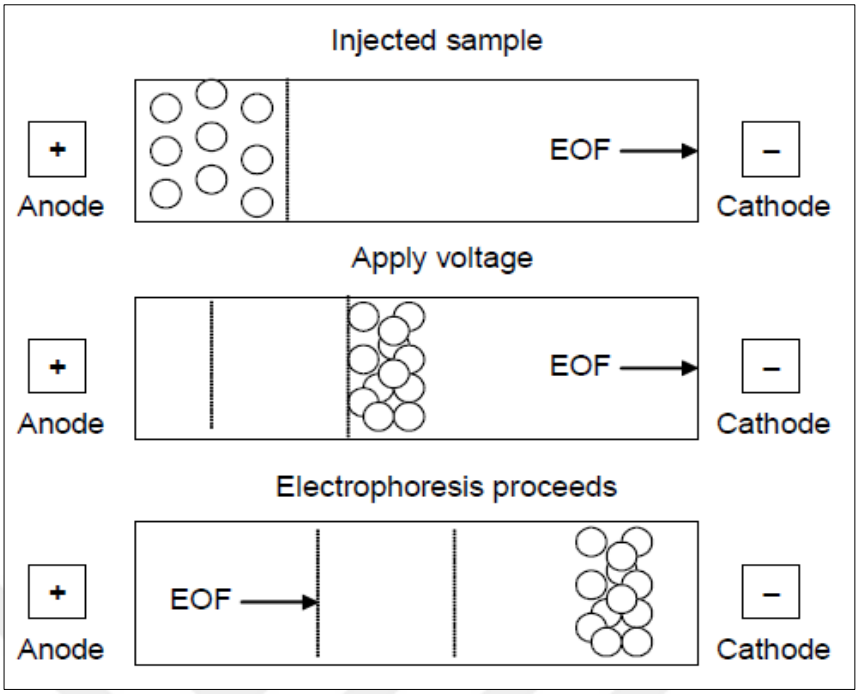


Figure 2.6. Sample stacking in a sample dissolved in a solution has a lower conductivity than BGE.

(b) *Field-amplified sample injection (FASI)* is similar to that of field amplified sample stacking (FASS) (Beckers & Bocek, 2000). The only differences are the injection procedure and the focusing process. In FASS, hydrodynamic injection is used and the focusing process occurs when the separation voltage is applied while in FASI, electrokinetic injection is used and the focusing process occurs during injection. Therefore, in this technique, both electrophoretic migration of the charged sample ions and electroosmotic flow of the sample solution contribute to the introduction of the sample into the capillary. This mode of injection increases the introduction of charged compounds while the introduction of non-charged compounds decreases. The low conductivity of the sample solution enhances the amount of charged analytes introduced into the capillary. Therefore, the ions are concentrated into a thin zone of the electrolyte front that possesses higher conductivity in the capillary. It has been reported from measurements on antimalarial drugs that electrokinetic injection results in increased sensitivity over hydrodynamic injection (Taylor & Reid, 1993) (Taylor & Reid, 1995).

(c) I*sotachophoresis (ITP)*, in which the analyte is positioned between two different buffers where the leading electrolyte which contains ions with a higher mobility than the analytes (at the detector side) and the terminating buffer which contains ions with a lower mobility than the analytes (at the injection side) (Peterson et al., 2003). When a high voltage is applied, a potential gradient is created throughout the capillary. Analytes are then distributed into zones on the basis of their mobilities. ITP is performed mainly in two modes, coupled-capillary ITP and transient ITP. In the first mode, two on-line coupled capillaries are utilized, where the first capillary is used for the ITP procedure and the second is used for the CE procedure. In transient ITP, both the ITP and CE procedures are completed in the same capillary.

Chromatographic

Several methods based on different chromatographic mechanisms are available for on-line pre-concentration (Guzman et al., 1997). These methods have at least one advantage over the previously described electrophoretic methods which is the ability not only to enrich but also to clean-up the sample. This is extremely useful in the analysis of biological samples, such as blood, urine, or saliva. Using this methodology, the sample can be purified from interfering and clogging components, such as proteins and salts, which can disturb the electrophoretic process. In addition, with appropriate modification it is possible to use an electrophoretic pre-concentration method (such as FAI) after chromatographic pre-concentration, thus providing further enhancement in sensitivity. A brief discussion of the most used techniques follows:

Solid-phase extraction (SPE) is commonly used off-line for the extraction and separation of a wide variety of compounds in biological mixtures (Veraart et al., 1998). This is a useful technique that allows a large volume of a low concentration analyte to be loaded onto the solid-phase and eluted into a smaller volume, providing concentrations that can be easily detected. Since this technique obviously consumes more analysis time, on-line methods have been investigated for CE (Beattie et al., 1995) (Strausbauch et al., 1995). One method is to pack a short segment, about 2 mm, from the injection end of the capillary with a liquid chromatography stationary phase Figure 2.7. This material is kept in place by using

frits at each side of the packing. Therefore, the preconcentration column is directly connected to the CE capillary.

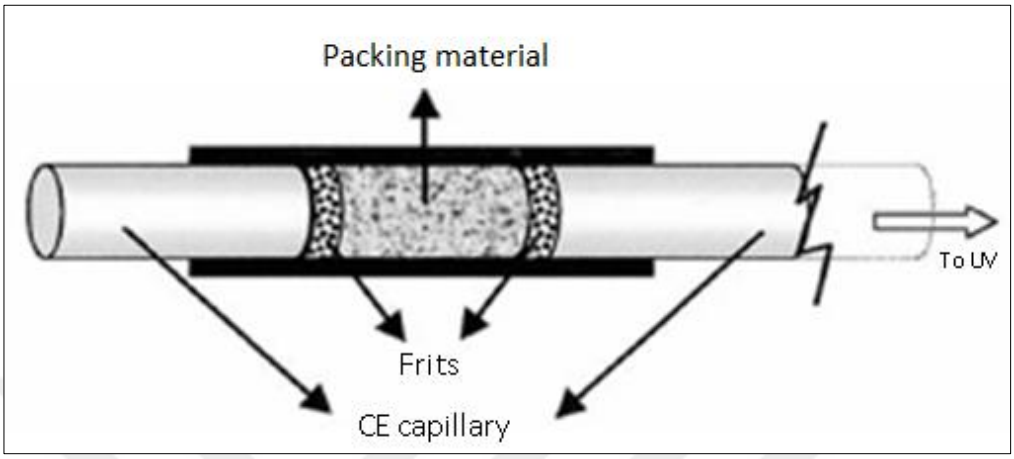


Figure 2.7. Configuration of the on-line SPE tip and its attachment to the inlet of the CE capillary.

The sample is loaded onto the stationary phase by hydrodynamic injection and then eluted from the packing by the injection of a small amount of organic solvent--usually 50-100 nL (Figure 2.8). Subsequently, the CE separation process is carried out. While this technology is very useful for cleaning and concentrating analytes from biological samples, a number of problems may arise (Tomlinson et al., 1996), including tailing, loss of CE efficiency, peak broadening, interference between the organic elution solvent and the CE electric field, and disturbance of the electroosmotic flow.

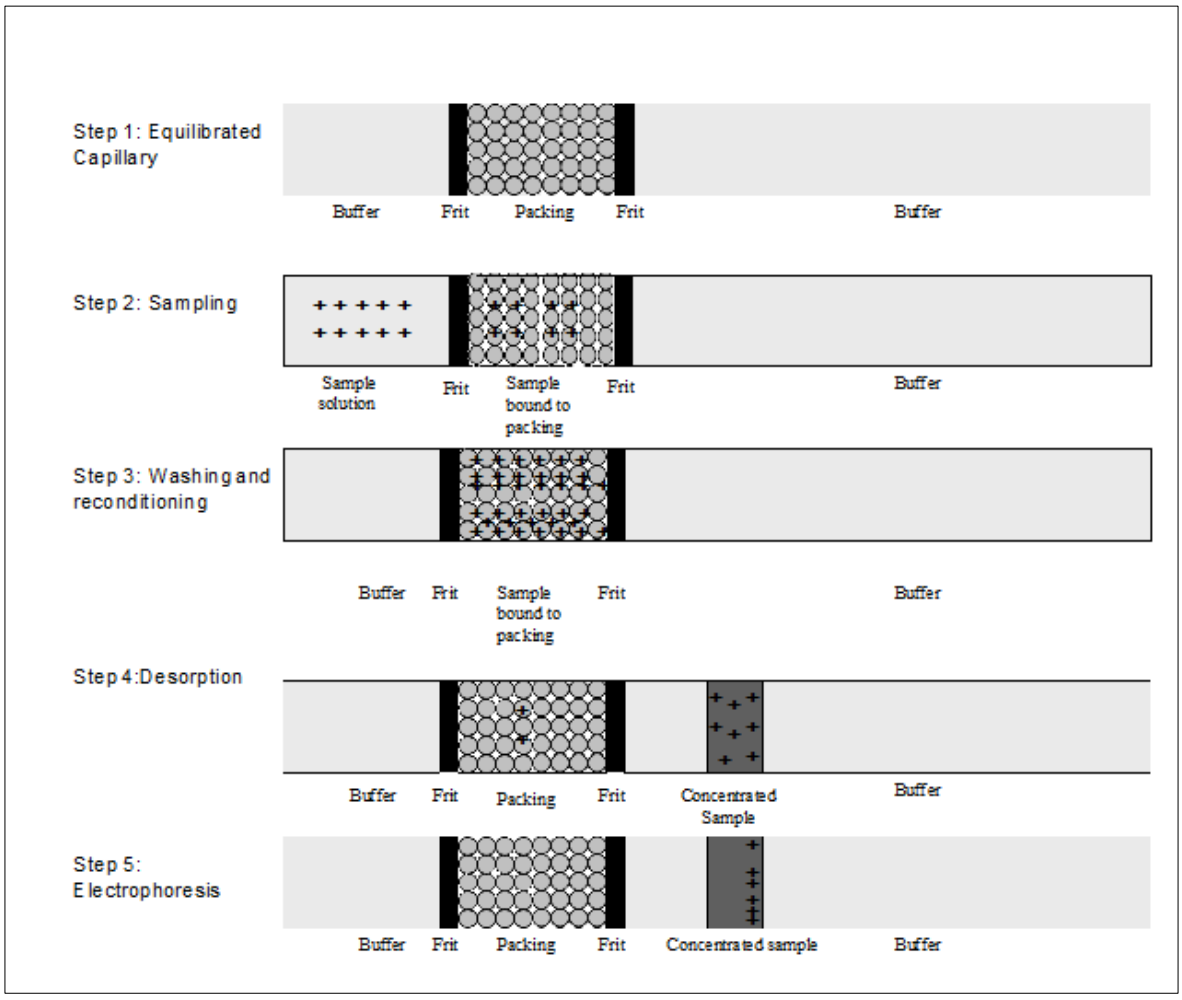


Figure 2.8. Steps of on-line pre-concentration using chromatographic techniques.

Analysis time is also longer with this method than with normal CE. These limitations are mostly caused by the packing material and the frits and can be, at least partially, solved by reducing the size of the solid-phase or completely removing the adsorptive phase from the CE capillary during electrophoresis by means of a switching valve.

Membrane pre-concentration (mPC) was first developed by Naylor and co-workers in (1995) (Tomlinson et al., 1995) to remove, or at least decrease, some of the problems arising from the use of large packed beds in SPE-CE. This technique is designed to improve the CE efficiency by minimizing the bed volume of the adsorptive phase at the inlet of the pre-concentration capillary. In mPC, a thin polymer membrane is installed in the center of a cartridge. Two pieces of fused silica capillary are inserted into each end of the cartridge and subsequently sealed with a

solvent-resistant epoxy resin Figure 2.9. Polymeric phases, such as styrene- divinyl benzene, C2, C8, and C18 have all been used for protein analysis (Tomlinson et al., 1996), (Tomlinson et al., 1995), (Tomlinson & Naylor, 1995) and (Naylor et al., 1996). Although, the application of this pre-concentration method partially improves the detection limit for on-line CE analysis, the CE efficiency is also greatly affected. Furthermore, the analysis time is longer and the capillary is subjected to clogging, especially when urine samples are analyzed.

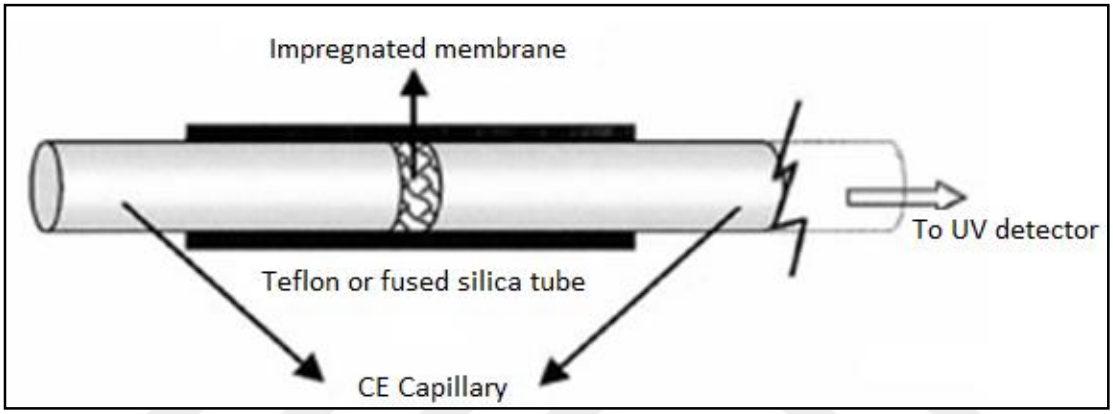


Figure 2.9. Configuration of the on-line mPC tip and its attachment to the inlet of the CE separation capillary.

Flow injection systems (or automated SPE-CE): Unlike the previous on-line chromatographic pre-concentration techniques, flow injection system (Figure 2.10) are among the most powerful tools for implementing pretreatment and conditioning samples in an automated fashion (Debets et al., 1992) (Arce, Rios, & Valcarcel, 1997). Various automated SPE-CE on-line assemblies have been used for this purpose with excellent results (Kuban et al., 2003) (Kuban et al., 2004). As can be seen in Figure 2.10, the system consists of a multi-channel peristaltic pump and an injection valve. The injection valve allows adsorption of the sample onto a C18 cartridge for its pre- concentration and clean-up. In the load position, samples are loaded onto the C18 column and cleaned with an organic solvent, followed by a water rinse. Analytes can then be eluted with a small amount of organic solvent and transferred directly to the CE system for analysis.

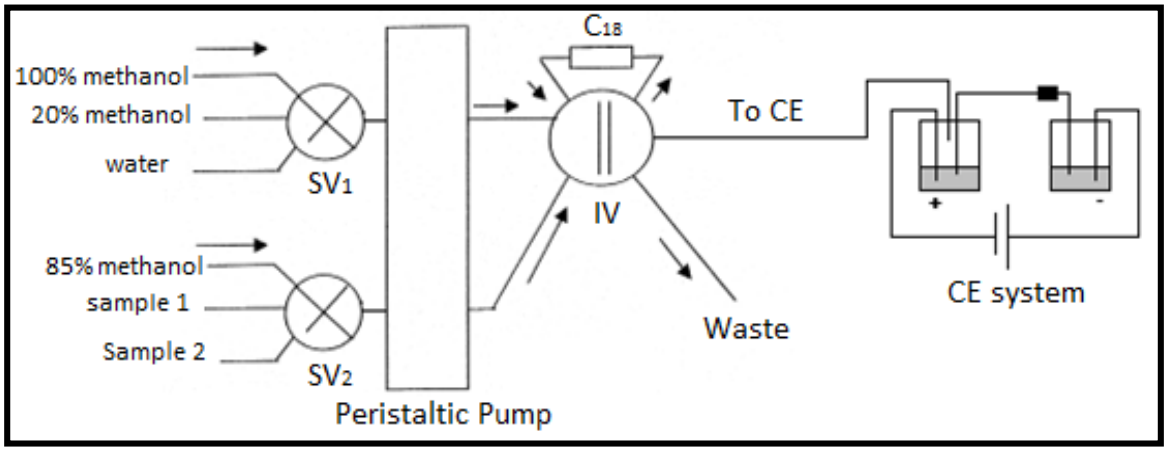


Figure 2.10. Automated SPE-CE designed for the pre-concentration and clean-up of urine samples.

a) *Solid-phase microextraction (SPME)* was developed in the late 1980s by Arthur and Pawliszyn (Eisert & Pawliszyn, 1997) (Pawliszyn, 1999). A sample (or its headspace for volatile analytes) is exposed to a coated fiber for a finite period of time, and analytes can then partition between the sample and the fiber phase. Depending on the length of exposure, the amount of analyte extracted is either based on its equilibrium distribution between the two phases or, when there is insufficient time for equilibrium to be reached, it is proportional to the initial concentration and the time of extraction. The strongest features of SPME are its simplicity, rapid extraction time, solvent-free nature, and its ability to be automated. This technique is most commonly used in environmental research for the extraction of organic compounds from water samples (Arthur et al., 1992). However, there are a few reports of the use of SPME coupled with GC for the analysis of abused drugs, such as amphetamine and cocaine, in biological matrices (Yashiki et al., 1995) (Watanabe et al., 2003). The reported techniques proved to be very sensitive; however, they are less sensitive when applied to the detection of polar drugs such as morphine and codeine especially since derivatization reagents are not available.

Furthermore, online sample preconcentration can be performed just by injecting a large volume of the sample solution without modification of the instrument and the analyte can be focused (through stacking or sweeping) into a minimum volume inside the capillary. Therefore, online sample preconcentration is a useful technique to improve the concentration sensitivity of the detector by taking advantage of the

small sample volume requirement in CE (Simpson & Terabe, 2008). These include sweeping and stacking, the latter can be performed through several modes such as (i) FASS (Quirino & Terabe, 2000), (ii) LVSS with polarity switching (Chien & Burgi, 1992b), and (iii) FASI/EOF reversal (Sun et al., 2003).

2.1.6. Why capillary electrophoresis

The unprecedented resolution of CE is a consequence of the technique's extremely high efficiency. CE analyses are usually very fast, use little sample and reagents, and cost much less than chromatography or conventional electrophoresis. Although modern CE is still in its teenage years, it has demonstrated tremendous potential for a wide range of applications, from small molecules that include inorganic ions, organic acids, amino acids, peptides, drugs, nucleosides, nucleotides, vitamins, steroids, and carbohydrates, to larger molecules, such as hormones, proteins, nucleic acids, and even living cells (Y. Xu, 1996b).

CE is useful for the analysis of the wide variety of solutes found in illicit drug seizures (Weinberger & Lurie, 1991), especially for those compounds which are otherwise difficult to analyze via GC and high-performance liquid chromatography (HPLC). GC can be problematic for the analysis of nonvolatile, thermally labile, and highly polar drugs, while HPLC often lacks sufficient resolving power for complex mixtures. In addition, although drugs of forensic interest can be analyzed by either GC or HPLC, derivatization and/or the use of expensive, specialized columns are usually required. CE offers high efficiency, high selectivity, and low-cost operation. Therefore, it has great potential for the forensic chemist. The economy of operation arises from the low flow rates (nL/min) and capillary low costs (Lurie, 2002).

In addition to saving labor and time (most separations can be done in minutes), CE has several other advantages over traditional gel electrophoresis. Since the capillaries are so small (usually 10 to 100 centimeters in length, with an inner diameter of 50 to 100μm), the Joule heat generated by the electric current is quickly dissipated. This eliminates the potential problem of overheating samples; in addition, it prevents convection currents in the sample that can lead to poor

resolution. Faster heat dissipation also allows researchers to use higher voltages than those that are normally used in gel electrophoresis (Y. Xu, 1996b). Higher voltages (as high as 30 kilovolts) can lead to shorter run times. The tiny capillaries also mean that scientists can use very small sample sizes (on the order of nanoliters) to do an analysis. Small sample size, can be a real benefit to those researchers who work with limited amounts of sample to begin with, such as geneticists who perform restriction mapping studies of valuable DNA material. Perhaps the most notable advantage of CE over other electrophoretic methods is its ability to give researchers high-resolution separations. Also, because the system is small and can be cooled quickly, we don't get band or sample spreading due to heating. The time frame for each run, also plays a role in resolution. The longer you take, the greater the spreading. Better resolution coupled with sensitive detectors increase the wealth of information a researcher can draw from one sample (Palmieri, 1990) (Y. Xu, 1996b).

The narrow tubes used in capillary electrophoresis help to give the technique good resolution. When a sample is introduced in a tube and an electric field applied, the components move at different rates leading to separation. The advantages of using capillary tubes are that lateral diffusion effects are reduced and temperature differences across the tube are decreased. The properties of the tube, and other properties set by the technician, lead to what is known as plug flow where the velocity of the fluid is considered constant across the tube’s cross-section, perpendicular to the flow. Under plug flow, axial diffusion is the only factor leading to dispersion, so the separation efficiency using CE is very high.

Using narrow capillaries also helps to reduce band-broadening seen in the peaks generated in other techniques such as HPLC. In CE, the velocity of the liquid as it travels along the tube is uniform across the tube. In the wider tubes and using pumped flow in other techniques, the velocity is not uniform across the tube. This is known as laminar flow, the velocity is slower at the interface between tube wall and liquid, giving a velocity profile with a bulge at the center of the tube. This leads to band-broadening and the wider peaks seen in HPLC for example Figure 2.11. In CE, two terms in Van Deemter Equation (shown below) are zero; the multiple-path term (A) which is known as “Eddy diffusion” and the resistance to mass-transfer

coefficient (C), and this is because in the capillary of CE the separation is carried out in a single phase of uniformly flowing carrier liquid. Thus, the only source of band broadening under ideal conditions is originated from the longitudinal diffusion term (B).

$$H = A + \frac{B}{u} + \mathrm{C}u$$

Where, ***H*** is the Height equivalent to a theoretical plate and ***u*** is the linear velocity.

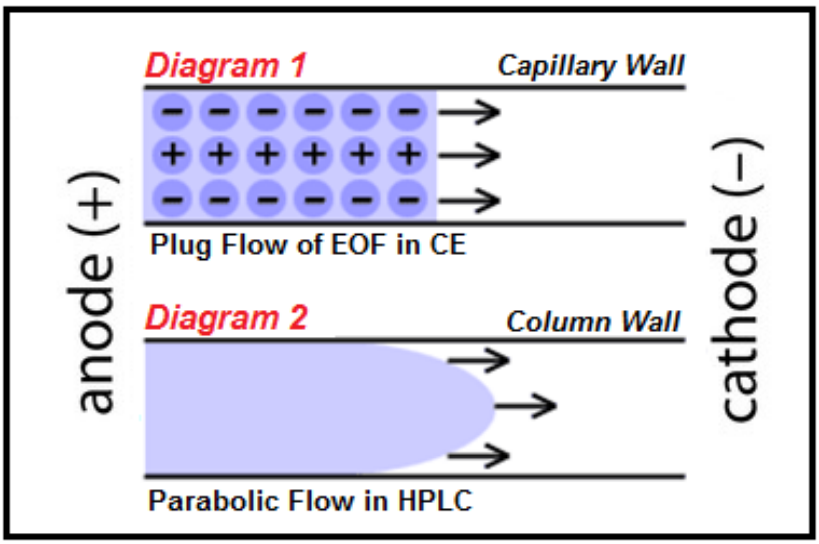


Figure 2.11. HPLC versus CE flow profiles.

2.2. Analytical Considerations

Qualitative and quantitative analysis of analytes in real samples is often difficult due to interruptions caused by different interfering substances found in the sample matrix. Therefore, a sample-preparation procedure is a necessary step prior to the electrophoretic analysis, in order to isolate the analytes under study from real samples. Different matrix separation and preconcentration methods have been used over the years, including solid-phase extraction (SPE) with C-18, silica, or other cartridges and liquid-liquid extraction (LLE) with different organic solvents (Kapnissi-Christodoulou, 2012a).

2.2.1. Qualitative analysis in CE

Qualitative analysis in CE provides information about the identity of a peak in an electropherogram. The simplest way to identify a CE peak is to compare its migration time with that of a known compound. As with other separation techniques, however, the migration time alone is not always reliable for confirming peak identity and purity; final confirmation requires additional information. One method of confirmation entails comparing the ratio of absorbances at different wavelengths in the unknown with that ratio in the suspected compound using spectrophotometric detection. Using diode array detectors (DAD) in CE analysis can provide qualitative information about the sample plug during passing through the detection window of the capillary. This happens through recording the whole spectrum of the sample plug by the DAD then comparing the produced spectrum with stored library for matching. Another method is to compare the ratio of currents obtained from two different electrical potentials using amperometric detection.

2.2.2. Quantitative analysis in CE

Quantitative analysis provides information about the amount or concentration of a substance in a given sample. Although quantitative analysis by CE is still under research under development to improve detection limits, the following aspects deserve special attention:

(a) *External (direct) calibration:* Solute concentration is directly related to peak height or peak area, while residence time in the detector is directly related to peak width. Therefore, the amount of solute is directly related to peak area and quantitative information can be obtained in CE by directly comparing an analyte's peak area or height with those of calibration standards.

(b) *Internal calibration*: Internal calibration usually results in better precision compared to methods that rely on direct calibration because neither the quantity injected nor the detector response needs to remain constant. In internal calibration, a known amount of internal standard is added to each sample prior to the sample pretreatment procedure, be it the calibration standard or unknown. After pretreatment, the solution of sample and internal standard is subjected to

electrophoresis. The calibration graph consists of ratios of peak area (or height) of the calibration standards to that of the internal standard plotted against the concentration of the standard. The unknown concentration is determined from the peak area (or height) ratio of the unknown mixture.

2.2.3. Sample matrix effect

The sample matrix strongly influences quantitative precision and accuracy in CE, especially when electrokinetic injection is used. The matrix effect can be accounted for by using matrix-matched standards combined with internal calibration (Y. Xu, 1996b). The effective electrophoretic mobility of an ion is determined not only by the solution parameter, pH, ionic strength (conductivity), and viscosity, but also by the substance parameters effective charge and hydrodynamic radius, which in return depend on the composition of the solution (Leube & Roeckel, 1994).

2.2.4. Limit of detection

Spectrophotometric detectors used in CE may be an order of magnitude less sensitive compared to those available for HPLC because the former detectors use shorter path-lengths. The reduced sensitivity is partially compensated by the high separation efficiency of CE, which allows for more precise integration of peak areas. Other methods for improving detection limits include the use of a short detection wavelength (down to 185 nm), where many solutes have greater absorptivities, and by using sample stacking or a packed-inlet capillary for on-column concentration of samples. Without sample concentration, limits of detection (LODs) in the low μM (or μg/mL) range can be obtained with UV detection; with sample preconcentration, LODs as low as a few nM (or ng/mL) in the original sample can be achieved. LODs at sub-μM and sub-pM levels are typical with amperometric detection and laser-induced fluorescence detection, respectively. In Table 2.1, the LODs of commonly used detectors in CE are shown. It’s clear that lower LODs can be obtained in terms of mass concentration than those obtained in molar concentrations, indicating that CE have a good mass sensitivity.

Table 2.1. LODs of different detectors used in CE.

	LOD	
Detector	**Mol**	**Mol/L**
UV-VIS	10^{-13}-10^{-16}	10^{-5}-10^{-7}
Fluorescence	10^{-15}-10^{-17}	10^{-7}-10^{-9}
LIF	10^{-18}-10^{-20}	10^{-13}-10^{-16}
Mass spectrometer	10^{-16}-10^{-17}	10^{-8}-10^{-10}
Amperometer	10^{-18}-10^{-19}	10^{-7}-10^{-10}
Conductometer	10^{-15}-10^{-16}	10^{-7}-10^{-9}

2.2.5. Range of linearity and reproducibility

The linear dynamic range of CE applications has been as narrow as one order of magnitude or as wide as six orders of magnitude, depending on the analyte and type of the detector used. Reproducibility is typically in the range of 1-2% for peak area and 3-7% for peak height (Y. Xu, 1996b).

2.2.6. Sample preparation

Many CE applications do not require sample pretreatment (e.g. derivatization) other than a possible dilution. Other applications require the sample to be treated before injection, especially those dealing with the analysis of biological samples. Sample preparation has substantial objectives before sample injection (Kohler, Schappler, & Rudaz, 2013), including:

- Reducing or eliminating matrix interferents or undesired endogenous compounds;
- Increasing selectivity for targeted analyte(s);
- Preconcentrating the sample to enhance sensitivity; and
- Stabilizing the sample by reconstituting it in an inert solvent.

Although great improvements have been made in the development of fast separation techniques, sample pretreatment remains the most time-consuming step, accounting for about two thirds of the entire analytical procedure (Hyotylainen, 2009). In addition, because of the lack of automation of several offline procedures,

sample preparation is also regarded as a primary source of analytical errors that can significantly affect the throughput (Ramos, 2012).

Sample preparation can be based either on selective methods, e.g., the widely used solid-phase extraction (SPE) and liquid–liquid extraction (LLE), or non-selective methods, e.g., using membrane techniques or protein precipitation (PP). A common feature of all these conventional sample-preparation techniques is the relatively high consumption of solvents that are environmentally hazardous and health risks for humans.

2.3. Extraction Methods

In recent years, sensitivity and specificity of analytical instruments have been achieved, but most of them cannot directly handle the complex matrices such as biological, environmental and food samples yet (Kataoka et al. , 2009). Among the analytical processes such as sampling, sample preparation, separation, detection and data analysis, sample preparation is important for isolating desired components from complex matrices and greatly influences their reliable and accurate analysis. The sample preparation step in an analytical process typically consists of an extraction procedure that results in the isolation and enrichment of components of interest from a sample matrix (Rezaee et al., 2006). The selective extraction of analytes is based on their different chemical and physical properties, including molecular weight, charge, solubility, polarity, and volatility (Vinas et al., 2014). Below there is a description of the most well-known extraction techniques that are used in analytical determinations and chemical analysis.

2.3.1. Liquid-liquid extraction (LLE)

Liquid–liquid extraction (LLE) is among the oldest of the preconcentration and matrix isolation techniques in analytical chemistry (Liu & Dasgupta, 1996). LLE or solvent extraction is a separation process which is based on the different distribution of the components to be separated between two liquid phases. It depends on the mass transfer of the component to be extracted from a first liquid phase to a second one.

However, LLE suffers from major drawbacks, for example emulsion formation at the interface of the immiscible phases, lack of selectivity (co-extraction of endogenous interferents), lack of automation, time-consuming and use of large sample volumes and large amounts of toxic organic solvents that are environmentally harmful (up to 10 mL per mL of sample). New methods based on the LLE principle or with original set-ups have been developed during the last two decades to overcome these drawbacks. Miniaturization of LLE has led to several new liquid-based microextraction techniques in which the total volume of organic solvent required has been reduced to the sub-mL level (Kohler et al., 2013).

2.3.2. Solid phase extraction (SPE)

Solid phase extraction (SPE) is an effective alternative to liquid-liquid extraction (Zief & Kiser, 1990). SPE involves absorbing the analyte from the sample onto a modified solid support. The analyte is then desorbed either by thermal means or by using a solvent. The primary advantage of SPE is the reduced consumption of high-purity solvents, thereby reducing laboratory costs and diminishing the need for solvent disposal. The time required in SPE methods to isolate the analyte of interest is greatly reduced when compared to classical liquid-liquid extraction methods. However, solid phase extractions often suffer from high blank values (Green & Lepape, 1987), there is considerable variation between the products offered by different manufacturers, and lot to lot variation can be a problem. SPE cartridges are normally constructed from plastic, which can adsorb the analyte and increase interferences in the analysis (Arthur & Pawliszyn, 1990).

2.4. Microextracions

Analytical microextractions (MEs) represent an important development in the field of sample preparation, addressing issues of simplicity, miniaturization and time efficiency (Pawliszyn, 2006). Miniaturized sample preparation methods combined with chromatographic techniques provide several advantages: high analysis speed with great efficiency, low operational costs since solvent consumption is very low,

environmentally friendly analytical procedures, and highly selective analysis (Saito & Jinno, 2003).

ME techniques are defined as non-exhaustive procedures that use very small volumes of the extracting solvent and for which the volume of sample is relatively large compared with that of the extracting phase. MEs reduce or eliminate the consumption of solvents while simultaneously reducing sample volume, analysis time, and operating costs. Many techniques have been developed over the last few decades for a variety of applications, i.e., in environmental analysis (pesticides, hormones), food analysis, and bioanalysis for clinical, toxicological and forensic purposes or doping analysis. In bioanalysis, often only small amounts of the sample are available, typically in the mL range for urine and in the µL range for serum or plasma or alternative matrices, for example sweat, saliva, or tears. Because of the complexity of theses matrices and the low concentrations of the target analytes compared with endogenous interferents, sample preparation is mandatory, and MEs are particularly well adapted for this purpose (Kohler et al., 2013).

A variety of analytical techniques, including separation based approaches, can be implemented in combination with MEs in bioanalysis. Non-polar and volatile compounds are conveniently analyzed by gas chromatography (GC), whereas liquid chromatography (LC), including ultra-high- pressure liquid chromatography (UHPLC), is extensively used in bioanalysis for both quantitative and qualitative purposes, because of its wide applicability to a large number of compounds with different physicochemical properties. As very small amounts of (µL range) or no organic solvents are required for CE analysis, its use in combination with ME techniques is regarded an attractive and environmentally sustainable analytical tool. Extracts can be directly injected for analysis, or evaporated and reconstituted in a very small volume. Because a few nL of sample is injected in CE, very high preconcentration factors can be achieved, enhancing the overall sensitivity, which is a disadvantage of the capillary format. Applications of ME techniques before to CE analysis have been reported over the past few decades in bioanalysis of low-molecular-weight compounds or small peptides. MEs are classified according to their extraction principle and improvement of extraction performance.

2.4.1. Liquid phase microextraction (LPME)

LPME has emerged as a powerful technique for preconcentration and matrix separation in the last decade, featuring simple operation, low cost, and high efficiency. In terms of the operation mode, LPME can be divided into different techniques and mainly including single drop microextraction (SDME), dispersive liquid–liquid microextraction (DLLME), ultrasound-assisted emulsification microextraction (USAEME) and hollow-fiber LPME (HF-LPME). Below there is a description of some of these techniques.

Single-drop microextraction (SDME)

SDME was introduced in 1997 by Jeannot et al. (Jeannot & Cantwell, 1997) and He et al. (He & Lee, 1997). In the first study, a 1-µL drop of n-octane was suspended in a stirred aqueous sample from the tip of a micro-syringe needle (Figure 2.12). After a few minutes, the drop was retracted into the needle and injected directly for gas chromatographic (GC) analysis. He and Lee used the same method with a 1-µL drop of toluene that was immersed in the aqueous sample for 15 min before retraction and injection (He & Lee, 1997). SDME uses very small amounts of organic extraction solvents, which enables important preconcentration factors (PFs) to be achieved. Since single-drop microextraction (SDME) was introduced as an effective sample-pretreatment technique (He & Lee, 1997), LPME was developed quickly and applied to chromatography and CE analysis. LPME possesses advantages (e.g., high enrichment factor, fine purification capability, low running cost, simple operation set-up, and trace-solvent consumption). With a single micro syringe of several µL, which serves as both solvent holder and sample injector to perform the extraction procedure and extract injection, SDME has become a crucial, successful microextraction technique (Jeannot & Cantwell, 1997).

The main problems with this method are lack of droplet stability at high stirring speeds and the high manual dexterity required. Moreover, SDME is only suitable for relatively non-polar analytes and suffers from low recovery and repeatability. Therefore, SMDE was regarded as be not suitable for biological matrices, in which

an extra filtration step is necessary. Many derived techniques based on SDME were thus proposed, solidified floating organic drop microextraction (SFODME), dispersive liquid– liquid microextraction (DLLME), and used in combination with CE to obtain sufficient selectivity, sensitivity, and repeatability in bioanalysis (Kohler et al., 2013).

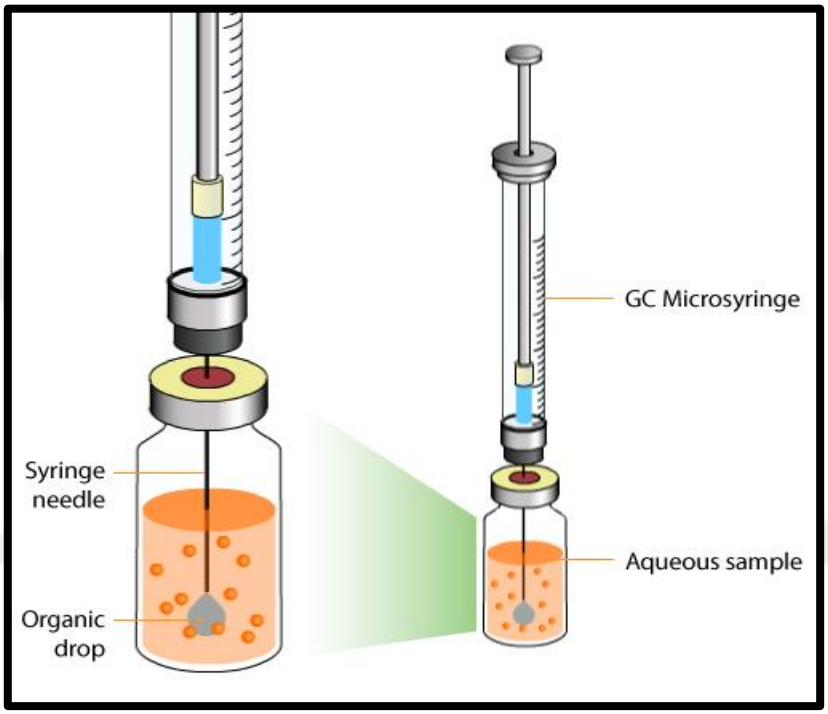


Figure 2.12. Single-Drop Microextraction (SDME) in direct immersion mode.

Solidified floating organic drop microextraction (SFODME)

In 2007, Yamini and coworkers (Zanjani et al., 2007) developed a new mode of dispersive LPME termed solidified floating organic drop microextraction (SFODME), which was then successfully applied to polycyclic aromatic hydrocarbon analysis in water samples. This technique is similar to SDME, but a specific holder is not required for supporting the organic microdrop and the sample solution can be agitated at high speed. The aqueous solution that contains the analytes is transferred into a vial. A small volume of an organic solvent with a melting point near room temperature (in the range of 10–30 ^{o}C) is floated on the surface of the aqueous solution. The aqueous phase is stirred for a prescribed period of time, and then the

sample is transferred into an ice bath. When the organic solvent is solidified, it is transferred into a small conical vial, and the melted organic solvent is used for subsequent analysis. SFODME is a modified solvent extraction method, and has the advantages of simplicity, low cost, minimum organic solvent consumption, and achievement of a high enrichment factor. Since its introduction, SFODME has been successfully applied to the analysis of organic analytes, trace metals and inorganic species in various environmental samples (Liu et al., 2015).

Dispersive liquid-liquid microextraction (DLLME)

DLLME is a very simple and rapid method for extraction and preconcentration of organic compounds from water samples. It is based on ternary component solvent system such as homogeneous liquid–liquid extraction (HLLE) and cloud point extraction (CPE) (Rezaee et al., 2006). In DLLME (Figure 2.13), the extracting solvent is mixed with a dispersing solvent that is miscible both with the former and with the aqueous sample. The mixture is rapidly injected into the sample with a syringe, producing high turbulence that leads to the formation of tiny droplets (cloudy solution is formed). Because of the large surface area between the extracting droplets and sample, the extraction time is drastically reduced. After centrifugation, the sedimented phase at the bottom of the tube is collected and either injected directly or evaporated to dryness before reconstitution and injection (Kohler et al., 2013).

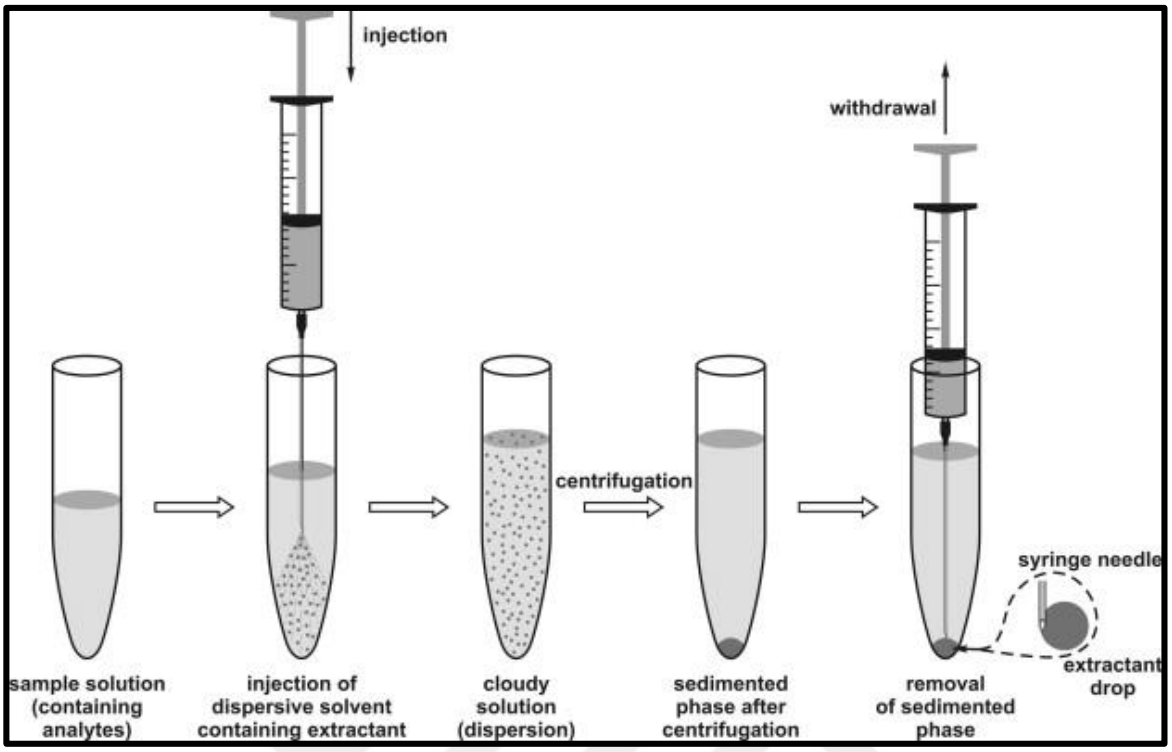


Figure 2.13. DLLME procedures.

The advantages of DLLME method are simplicity of operation, rapidity, low cost, high recovery, and enrichment factor (Rezaee et al., 2006).

In our laboratory at the department of Analytical Chemistry in Gazi University, different applications of DLLME including ultrasound-assisted emulsification microextraction (USAEME) and solidification of floating organic drop microextraction (SFODME) along with online preconcentration techniques in CE as efficient techniques for the analysis of ephedrines in human urine (U. Alshana, et al. 2012), non-steroidal anti-inflammatory drugs (NSAIDs) in milk and dairy products (Alshana, Göğer et al., 2013), bisphenol A in water and human urine (Alshana, Lubbad, et al., 2013) and beta(2)-agonists in bovine urine (Us, Alshana, et al., 2013) were introduced.

2.4.2. Solid-phase microextraction (SPME)

SPME was introduced in 1990 by Arthur and Pawliszyn (Arthur & Pawliszyn, 1990). In SPME, a small amount of sorptive, homogenous, non-porous extracting phase dispersed on the surface of or inside a solid support is exposed to the sample for a specific period of time until equilibrium is reached. The main commercially used sorbents are polydimethylsiloxane (PDMS) for rather non-polar or volatile compounds and polyacrylate (PA), PDMS–divinylbenzene (PDMS–DVB), or Carbowax– divinylbenzene (CW–DVB) for polar compounds. Extraction can be performed in two main formats: fiber SPME and in-tube SPME.

Fiber solid-phase microextraction (fiber SPME)

In fiber SPME, the sorbent (variable film thickness) is coated on the external surface of a fused-silica fiber tip as an appropriate polymeric stationary phase. The device, a modified syringe, consists of a fiber assembly with the built-in fiber inside the needle and an assembly holder. A plunger is used to move the coated fiber inside or outside the needle. Two extraction modes can be used with fiber SPME: a) direct immersion of the fiber in the aqueous sample which is known as direct-immersion fiber solid-phase microextraction (DI-SPME). It entails direct immersion of the fiber into the aqueous sample with consequent stirring, enabling transfer of non-volatile analytes into the coating (Theodoridis et al., 2000), b) headspace extraction which is known as headspace fiber solid-phase microextraction (HS-SPME), which was first described in 1993 (Jinno et al., 1997). HS-SPME has been shown to be advantageous, mainly for volatile compounds, because of its higher speed, higher recovery, greater selectivity, longer fiber lifetime, and lower fiber contamination than for DI-SPME, but it is only suitable for highly volatile compounds (Kohler et al., 2013).

In-tube SPME

In-tube SPME, which was introduced in 1997, was primarily developed to overcome the inherent problems of fiber SPME, i.e., fiber fragility, low sorption capacity, and

bleeding of fiber coatings, and to provide an automation option (Kataoka & Saito, 2011). In this method, targeted compounds are directly extracted into the internally coated stationary phase of a fused-silica capillary, enabling on-line coupling with CE. In-tube SPME is a type of so-called capillary MEs, which also include open tubular trapping, wire-in-tube SPME, fiber in-tube SPME, sorbent-packed capillary in-tube SPME, and monolithic capillary in-tube SPME. Capillary MEs are distinguished from the composition of the extraction stationary phase (fiber, polymer, sorbent) and its packing and can be used on-line with CE (Kohler et al., 2013).

Dispersive solid phase extraction (DSPE)

DSPE has been a widely used technique since its invention around 2000 and has been successfully applied as a method of extraction, isolation, and cleaning in the analytic treatment of a wide variety of veterinary drugs employed in the livestock industry. DSPE simplifies SPE clean-up, allows more samples to be analyzed at one time, is quite rapid, and requires low solvent consumption.

DSPE consists of the addition of a solid sorbent, usually silica or polymer based, directly into the sample solution. The dispersion process increases the contact area between the sorbent and the analyte. The sorbents employed in DSPE in the determination of residues of specific analytes are solids chemically modified by the addition of several chemical compounds that modify their affinities. These modifications ensure the selectivity for the analytes of interest, which allows the maximal retention, minimizing the interferences in the analytical matrix. After the dispersion, the sorbent is isolated by a centrifugation, filtration process or by a magnet. Once the solid phase is isolated, the analytes or interferences adsorbed on the surface of the sorbent could be easily eluted or eliminated with the addition of adequate organic solvents. Figure 2.14 shows a scheme of the DSPE procedure.

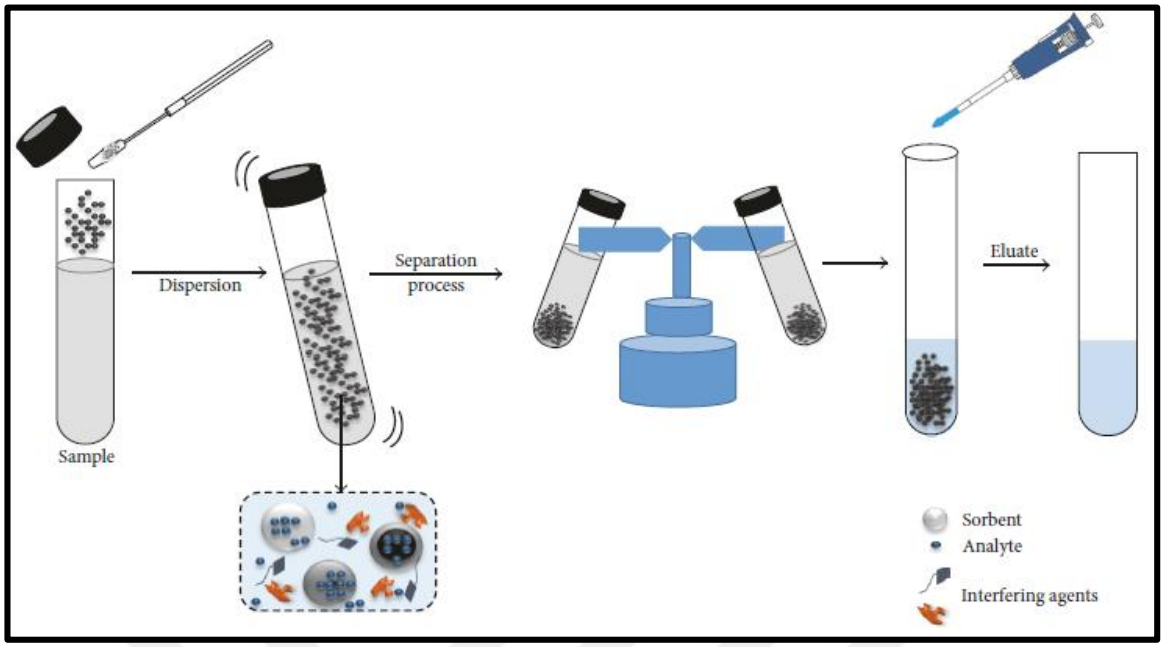


Figure 2.14. DSPE procedures.

DSPE is considered to be a micro- and macroscale method of extraction and cleaning, employed in different analytical methodologies as a procedure for the elimination of potential interferences (clean-up) that could affect the subsequent determination of the analytes. However, one of the critical steps in DSPE is the selection of the sorbent, and it is necessary to consider chemical and physical characteristics that allow maximal interaction between the sorbent and the analytes, ensuring selectivity extraction, removal, or preconcentration of analytes present in analytical matrices. DSPE technique achieves adequate limits of detection (LOD), with the additional advantage of low consumption of solvents in the treatment of the sample. Therefore, it is considered to be a low-cost technique in comparison with classical techniques LLE and SPE (Islas et al., 2017).

2.5. Green Analytical Chemistry

Primary proposals for green analytical chemistry (GAC) relate to minimizing analytical reagents and wastes, replacing toxic reagents and introducing reagent-free methodologies (Armenta et al., 2008). Capillary electrophoresis (CE) is a green analytical technique, due to its low consumption of samples and reagents, high separation efficiency, fast analysis speed, multiple separation mode and excellent biocompatibility. Its consumption of samples and reagents is extremely low [e.g., the

injected sample volume ranges from picolitre (pL) to nanolitre (nL) and the buffer solution consumed is less than 1 µL for each sample analysis]. However, most commercial CE apparatus are equipped with on-column ultraviolet visible (UV-Vis) spectrometers with low sensitivity detection, because of the trace injection volume and the short light path. It is therefore difficult to determine trace analytes in real samples directly. To overcome this deficiency, sample-pretreatment steps are often adopted to concentrate analytes and clean up sample matrices before CE analysis (Xie & He, 2010).

Sample-pretreatment can be based either on selective methods, e.g., the widely used solid-phase extraction (SPE) and liquid–liquid extraction (LLE), or non-selective methods, e.g., using membrane techniques or protein precipitation (PP). A common feature of all these conventional sample-preparation techniques is the relatively high consumption of solvents that are environmentally hazardous and health risks for humans. The advent of the concept of “green chemistry” at the beginning of the 1990s emphasized the need for non-toxic and environmentally friendly analytical procedures. The concept also promoted the use of environmentally sustainable sample-preparation methods with the development of solvent-free or miniaturized extraction methods (Kohler et al., 2013).

Green sample-pretreatment techniques are noticeable trends in analytical chemistry. They can eliminate or reduce toxic reagents and minimize analytical wastes that are hazardous to health and environment. Solid-phase microextraction (SPME) and liquid-phase microextraction (LPME) were developed in 1990s. They not only enhance the extraction efficiencies compared with traditional solid-phase extraction (SPE) and liquid–liquid extraction (LLE), but also conform to the requirements of GAC.

2.6. Alkyl Phenols

The chemical structure of alkyl phenols (APs) is based on the phenol ring with multi-carbon moieties: Nonylphenol is a 9-carbon side chain alkyl phenol; octylphenol has an 8-carbon alkyl chain; butyl phenol has a 4-carbon side chain, and dodecyl phenol

is a 12-carbon side chain alkylphenol (Figure 2.15). Nonylphenol and octylphenol are the most widely used alkylphenols and have the broadest range of application (Kayama, 2003b).

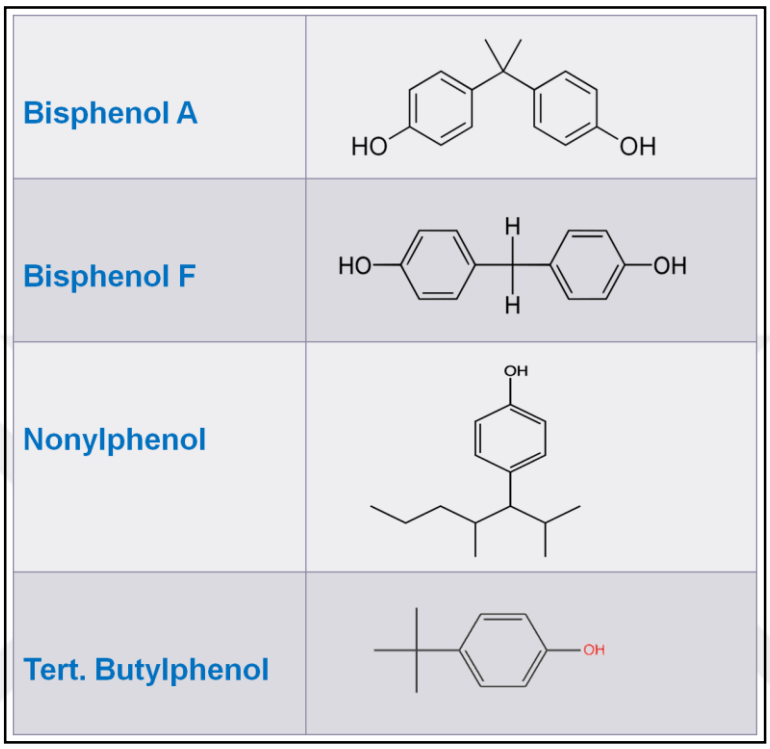


Figure 2.15. Structure of alkylphenols.

APs are considered estrogenic endocrine-disrupting compounds (EDCs) which could have negative effect in human reproduction at low concentrations, acting on the estrogens receptors. The trace-level determination of these compounds with similar structures contained in complex sample matrices requires the development of analytical methods with high sensitivity, selectivity, and resolution. These methods have been applied to soil, sediment, water, and other environmental samples, such as biological samples (Sosa-Ferrera et al., 2013).

2.6.1. Usage and consumption volumes

Alkylphenols (AP) and bisphenol A (BPA) are widely used industrial chemicals including the manufacture of plastics, textiles, paper, and agricultural chemical products (D. Benanou, 2000). BPA is used in the production of polymers and AP

are widely used to make alkylphenol ethoxylates, widely used as nonionic surfactants (Li et al., 2005). Furthermore, BPA is one of the most commonly produced industrial chemicals in the world and is a component of polycarbonate plastics and epoxy resins, the uses of which include lining food cans (Nerin et al., 2002). APs are mostly used as alkylphenol ethoxylates, that is, alkylphenols binding an ethoxy chain through their hydroxyl groups. The wide range of alkylphenol ethoxylate applications includes surfactants, ethylcellulose stabilizers, hydrophobic phenol resins, which are utilized in detergents, oil varnishes, synthetic rubber vulcanization accelerators, antioxidants of petroleum products and pesticide additives in agriculture (Kayama, 2003a). Alkylphenol polyethoxylates (APEOs) are a group of non-ionic surfactants that find widespread use as detergents, emulsifiers, wetting agents, stabilizers, de-foaming agents and intermediates in the synthesis of anionic surfactants (Takasu et al., 2002).

As an example, on the consumption of APs, more than twenty thousand tons of alkylphenols and alkylphenol ethoxylates are used annually in Japan in synthetic rubber industry, plastic, fabric, and metal processing industries as surfactants, cleaners, stabilizers for ethylcellulose, plasticizes, and phenol resins (Kayama, 2003b).

2.6.2. Health effects

Alkylphenols are industrial chemicals used in the production of detergents and other cleaning products, and as antioxidants in products made from plastics and rubber. Alkylphenols are also found in personal care products, especially hair products, and as an active component in many spermicides. As such, people are exposed to these compounds in a variety of ways, including through the skin and from contamination of both air and water. In the Silent Spring Institute study of household contaminants, alkylphenols — especially 4-nonylphenol (4-NP) and its breakdown products were found in all samples of house air and 80 percent of house dust samples (Rudel et al., 2003). Substantial concentrations of these chemicals have also been found in wastewater associated with domestic sewers and municipal landfills (Rudel et al., 2002). These compounds have been closely studied in recent years because they

have been shown to have estrogenic effects in fish, avian, and mammalian cells and are suspected of having endocrine-disrupting toxicity (Li et al., 2005).

EDCs are residual chemicals that can cause adverse effects on human and wildlife via interactions with the endocrine system. EDCs have shown to have a profound influence on the reproduction of some species and their offspring as well, for example, the occurrence of reproductive and developmental disruptions in snails, fishes, piscivorous birds, alligators, and sea mammals. Effects on human health have been linked to reduced sperm counts in human males experiencing occupational exposure to these chemicals. Various reports suggest possible involvement of EDCs in lower sperm counts, undescended testicles, early puberty, and thyroid dysfunction (Wu et al., 2008). The chemical, physical and toxicological properties of APEOs are governed by the number of EO units synthesized. For example, APEOs with short ethoxy chains (EO number <4) are lipophilic, while those with long chains (EO number >10) are hydrophilic. Toxicity increases as the number of EO decreases. Therefore, there is a need for an analytical method for the determination of the EO number of APEOs for product quality control, toxicological evaluation and environmental monitoring (Takasu et al., 2002). For the accurate assessment of human exposure of these chemicals, it is important to develop simple analytical methods for those chemicals (Takeda et al., 2003).

2.6.3. Environmental effects

The global production of chemical products has increased in the last decades, and although many of the products have been beneficial for mankind, many of them are also toxic because they exhibit a long environmental persistence and can accumulate within organisms (Daughton, 2004). Currently, many of the problems of pollution are due to intermittent spillage of these substances into the environment. In addition to their toxicity, persistence, and risk of bioaccumulation, these substances also clearly affect biological processes both in plants and in animals, including humans (Sosa-Ferrera et al., 2013). During their manufacture, APs and BPA can enter the environment by leaching or metabolism. Three AP: 4- tert-butylphenol (4-tBP), 4-nonylphenol (4-NP), and 4-tert-octylphenol (4-tOP) and BPA have frequently been found in waste water, natural water, and sewage effluents.

The compounds have been listed in ‘Strategic Programs on Environmental Endocrine Disruptors ’98’ (SPEED’98) by the Environmental Agency of Japan (Li et al., 2005).

2.7. Triphenylmethane Dyes

Triphenylmethane (TPM) dyes, any member of a group of extremely brilliant and intensely colored synthetic organic dyes. They are mainly derivatives of colorless triphenylmethane and diphenylnaphthyl methane characterized by a central carbon atom joined to three aromatic rings. (EPA, 1999).

Malachite green (MG) and crystal violet (CV) are triphenylmethane dyes. There have been many reports of the inappropriate use of MG and CV as veterinary drugs. They are readily absorbed into fish tissue from water exposure, and are reduced metabolically by fish to leucomalachite green (LMG) and leucocrystal violet (LCV). MG and CV have been banned for use as fungicides and antiseptics in aquaculture and fisheries because of their carcinogenic and mutagenic properties. Thus, it is necessary to develop a sensitive, rapid, inexpensive, and reliable method for the determination of MG, CV, and their leucometabolites in aquatic products (Sun & Qi, 2013).

2.7.1. Production and occurrence

The triphenylmethane derivatives are among the oldest man-made dyes, a practical process for the manufacture of fuchsine having been developed in 1859. Several other members of the class were discovered before their chemical constitutions were fully understood. Crystal violet, the most important of the group, was introduced in 1883. The range of colors is not complete but includes reds, violets, blues, and greens. They are applied by various techniques, but most belong to the basic class, which are adsorbed from solution by silk or wool, but have little affinity for cotton unless it has been treated with a mordant such as tannin.

These dyes are synthesized industrially by one of four processes: the aldehyde, ketone, diphenylmethane, and benzotrichloride methods. These processes are named for the manner in which the central carbon atom is incorporated into the dye. The choice of process is determined by the structure of the dye manufactured (EPA, 1999).

2.7.2. Uses

Triphenylmethane dyes are used extensively in textile industries for dying nylon polyacrylonitrile modified nylon, wool, silk, and cotton. But, because of their poor light and wash fastness, they are less marketable than other dye classes. They have poor resistance to light and to chemical bleaches and are used chiefly in copying papers, in hectograph and printing inks, and in textile applications for which lightfastness is not an important requirement (EPA, 1999). Some of the triphenylmethane dyes are used as medicine and biological stains. Paper and leather industries are also major consumers of triphenylmethane dyes. This group of dyes are also used for coloring plastics, gasoline, varnish, fats, oil, and waxes. Food and cosmetic industries also use different types of triphenylmethane dyes. Triphenylmethane dyes are some of the most widely used dermatological agents. Gentian Violet has been used in oral consumption for the treatment of pinworms and in topical applications in humans and domestic animals; it has been shown to be effective in controlling fungal growth under varying conditions. Gentian Violet has also been added to poultry feed to control fungus, thus exposing the human population directly or indirectly to Gentian Violet through its extensive medicinal and commercial use (Azmi et al., 1998).

MG is an extensively used as a biocide in the aquaculture industry world-wide. It is highly effective against important protozoal and fungal infections. Basically, it works as an ectoparasiticide: it has also been used to control skin flukes and gill flukes. Aquaculture industries have been using malachite green extensively as a topical treatment by bath or flush methods without paying any attention to the fact that topically applied therapeutants might also be absorbed systemically and produce significant internal effects. On the other hand, it is also used as a food coloring agent, food additive, a medical disinfectant and anthelminthic as well as a dye in

silk, wool, jute, leather, cotton, paper and acrylic industries (Srivastava et al., 2004). CV is a well-known toxic cationic dye and belongs to the class of triarylmethane dyes widely used for textile dying, food additives, pharmaceutical industries, cosmetic, plastic and paper printing (Ma et al., 2012).

2.7.3. Toxicity and health effects

The presence of triphenyl methane (TPM) dyes even in a very low concentration in water (less than 1 ppm for some) is highly visible and undesirable. In extreme cases, it may cause vomiting, shock, jaundice, and tissue necrosis and may lead to respiratory and kidney failure (Yao et al., 2015). Malachite green dye has generated much concern regarding its use, due to its reported toxic effects. The toxicity of this dye increases with exposure time, temperature and concentration. It has been reported to cause carcinogenesis, mutagenesis, chromosomal fractures, teratogenecity and respiratory toxicity. Histopathological effects of MG include multi-organ tissue injury. Significant alterations occur in biochemical parameters of blood in MG exposed fish.

Residues of MG and its reduced form, leucomalachite green (LMG) have been reported from serum, liver, kidney, muscles and other tissues as also from eggs and fry. Toxicity occurs in some mammals, including organ damage, mutagenic, carcinogenic and developmental abnormalities. However, despite the large amount of data on its toxic effects, MG is still used as a parasiticide in aquaculture and other industries (Srivastava et al., 2004). The cytogenic toxicity of Gentian Violet in Chinese hamster CHO cells in vitro has been studied. It was stated that this compound is a mitotic poison as well as a clastogen in vitro. Its clastogenic properties were confirmed in five other different mammalian cell types. Unless *in vivo* studies prove otherwise, MG and CV may be regarded as biohazardous substances (Azmi et al., 1998).

3. METHODS AND MATERIALS

3.1. Instrumentation

The CE instrument used was an HP G1600AX 3D Capillary Electrophoresis (Agilent Technologies, Germany) Figure 3.1. Conventional CZE and stacking modes were performed using uncoated fused-silica capillaries (Agilent Technologies, USA) of 50 µm and 75 µm ID. Online UV diode-array detector (DAD) working at the wavelength range of 190 - 600 nm was used. Optimum wavelengths for the target analytes were determined using 'Isoabsorbance' and '3D' plots in the instrument's 'Data Analysis' software. Pressure and/or electrokinetic injections were employed throughout the experiments.

Figure 3.1. Agilent G1600AX 3D Capillary Electrophoresis instrument

Thermo Electron Orion 720A pH meter equipped with a glass electrode (measuring range: −2.000-19.999 pH, accuracy: 0.002, resolution: 0.001) and Hanna HI98103 Checker pH Tester (measuring range: 0.00 to 14.00 pH, +/-0.2 pH Accuracy, 0.01

pH Resolution) (Figure 3.2) were used for measuring the adjusted pH of all samples and aqueous solutions throughout the experiments.

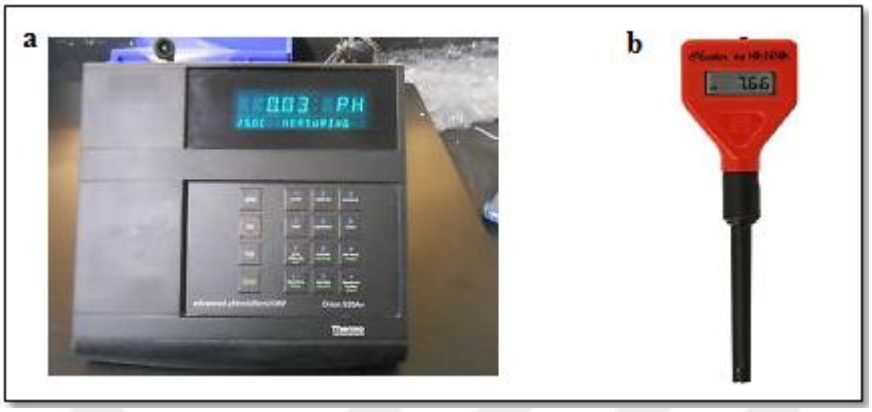


Figure 3.2. a) Thermo Electron Orion 720A pH meter and b) Hanna HI98103 Checker pH tester.

Deionized (DI) water (18.2 MΩ.cm) treated with Millipore (Simplicity, 185) Milli-Q water purification system was used for the preparation of all aqueous solutions and dilutions. FIRLABO laboratory vortex (rotational speed: min.: 500 rpm, max.: 2500 rpm) and NF 200 bench top centrifuge (max. capacity: 12x15 ml, max. speed: 5000 rpm, max. RCF: 2.822xg, programmable microprocessor control system) devices (Figure 3.3) were used in the experiments.

Figure 3.3. Centrifugation and vortex devices.

Kitchen blender was used to mash and homogenize the different fish samples to be used in experiments and analysis of triphenylmethane dyes. Techne sample concentrator (Figure 3.4) was used in conjunction with a Techne block heater to accelerate the evaporation process of solution samples (fish extracts) and make them ready for analysis. With the heat generated from the block heater and the steady flow of argon gas above the surface of the sample to carry away evaporated products, the rate of sample concentration is significantly increased. The blown inert gas in combination with the heat from the heater produce ideal conditions for fast efficient evaporation.

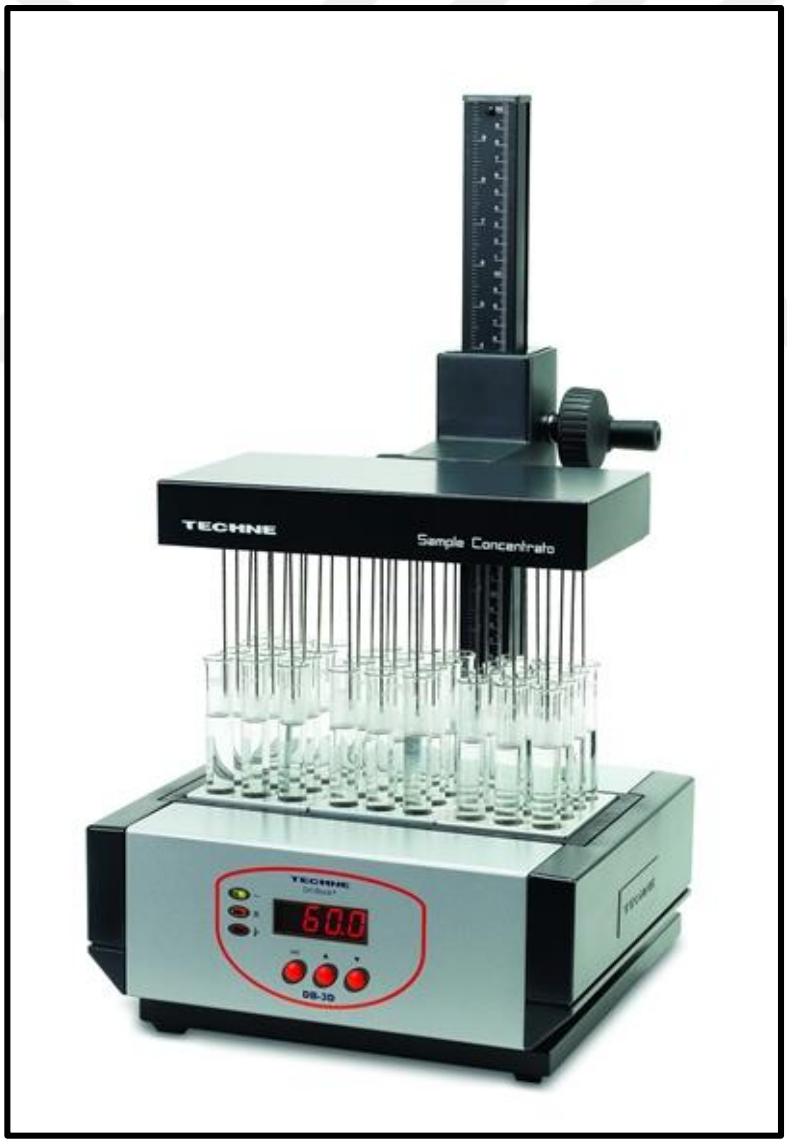


Figure 3.4. Techne sample concentrator equipped with a block heater.

3.1. Materials and Reagents

3.2.1. Bisphenol A

BPA (solubility in water at 25 ^{o}C < 0.1 g /100 g; log P = 4.0; pKa = 9.7) was purchased from Sigma-Aldrich (99.9 %, Munich, Germany). HPLC-grade methanol (Lab-Scan, Gliwice, Poland), acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) and acetone (Merck, Darmstadt, Germany) were used. Sodium chloride was purchased from Merck (Darmstadt, Germany). 1-undecanol (1-UN) (99.0 %), 1-dodecanol (1-DO) (98.0 %), and diphenyl ether (DPE) (99.0 %) were obtained from Sigma-Aldrich (Steinheim, Germany). A stock solution of the BPA was prepared by dissolving an appropriate amount in methanol to obtain a 1000 mg L^{-1} solution that was stored in the dark at 20 ^{o}C. Aliquots of this stock solution were daily diluted with deionized water to prepare standard solutions. All other reagents and solvents used were at least of analytical reagent grade unless otherwise specified. The sample solution for the liquid–liquid microextraction method based on solidification of floating organic drop (DLLME-SFOD) extraction experiments was prepared by spiking the analyte in deionized water. Samples of tap water were taken from Gazi University (Ankara, Turkey); spring and bottled water were purchased from the local market. Borate buffer was prepared from $Na_2B_4O_7.10H_2O$ obtained from Sigma-Aldrich (Steinheim, Germany). All background electrolytes (BGE) and solutions were prepared in deionized water and were stored in the dark at 4^{o}C. When necessary, pH of the solutions was adjusted with 0.1 M NaOH (Merck, Darmstadt, Germany) and 0.1 M HCl (Sigma-Aldrich, Steinheim, Germany). All solutions and samples were degassed using a sonicator (Sonorex Bandelin Electronic, Walldorf, Germany) and filtered through 0.20-mm filters (Econofilters, Agilent Technologies, Waldronn, Germany) before use.

3.2.2. Alkylphenols

Four alkylphenols were used in the experiments of this study. 2,2-Bis(4-hydroxyphenyl) propane (Bisphenol A), Bis(4-hydroxyphenyl)methane (Bisphenol F), 4-Nonylphenol and 4-tert-Butylphenol all are analytical standard grade and having assays above 99%. Liquid milk packet samples (200 mL) were obtained from

local markets and kept in refrigerator (4 ^{o}C) for analysis. Chemical reagents: Ortho-Phosphoric acid 85% MERCK, acetonitrile (Sigma-Aldrich, St. Louis, MO, USA), chloroform (≥99.8%, Sigma -Aldrich) and sodium chloride for analysis (Sodium chloride 99.99%, Suprapur - Merck) were used. Stock solutions 1.0 mg/mL of each of the four APs were prepared in methanol and stored at 4 ◦C in the dark when not in use. Working standards were prepared on daily base at the concentration levels of interest.

3.2.3. Triphenylmethane dyes

Malachite green (MG) in the form of Malachite green chloride (analytical standard grade ≥96.0%, water solubility 1.38 mg L^{-1}), Crystal violet (CV) in the form of Crystal violet chloride (analytical standard HPLC grade, water solubility 50 g/L at 27 °C), Leucomalachite green (LMG) (analytical standard, ≥98.0% HPLC grade) and LeucoCrystal violet (LCV) (assay 95.0%) were purchased from Sigma-Aldrich, Munich, Germany). The molecular structures of the four triphenylmethane dyes investigated in this study are shown in Figure 3.5.

Individual stock solutions of 50 mg L^{-1} for MG, LMG, CV and LCV were prepared in HPLC-grade acetonitrile. All were kept at –20°C (stable for three months). Standard solutions of all analytes were mixed and diluted with acetonitrile, and working solutions of all analytes and calibration concentrations were prepared by appropriate dilutions from the stock solutions on the day of analysis. All standards were stored at 4°C in the dark (the tubes of stock solutions were wrapped with aluminum folio and the standard solutions are stable for one month).

Sodium acetate anhydrous for analysis (HPLC grade, ≥99.5%) and ammonium acetate (reagent grade, ≥98%) were purchased from Sigma-Aldrich. To prepare 100 mL aqueous solution of 0.5M sodium acetate, 4.1 g CH3COONa Molecular weight = 82.0343) was weighed and transferred into a 250-mL conical flask, followed by addition of 80 mL deionized / Milli-Q water. The contents were mixed until the sodium acetate is completely dissolved. The pH was adjusted to 4.5 with glacial acetic acid then the solution was transferred to a 100-mL volumetric flask and the volume was adjusted to 100 mL with deionized / Milli-Q water. Ammonium acetate

(0.1 mol/L) was prepared by dissolving 3.85 g of ammonium acetate in a 500-mL volumetric flask. Formic acid 98-100% for analysis was purchased from Merck.

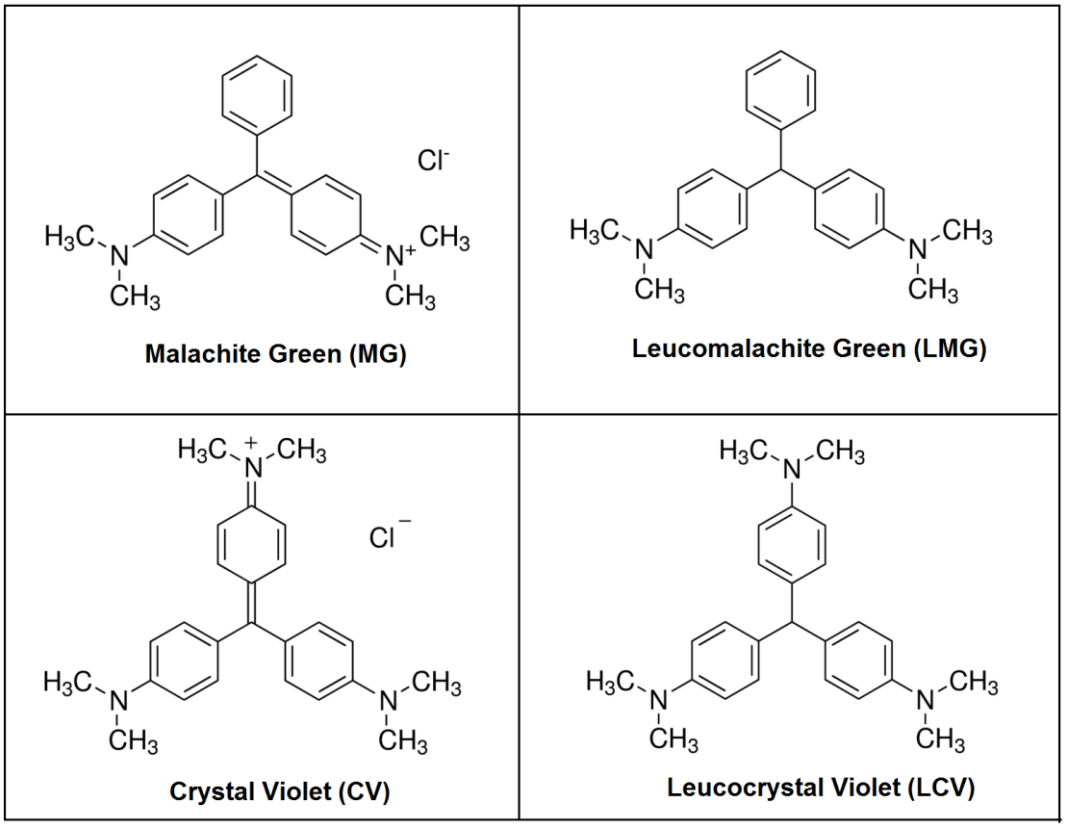


Figure 3.5. Molecular structures of Triphenylmethane dyes.

Three different types of aquaculture fish (Figure 3.6) where used in this study as follow:

1. Salmon (Oncorhynchus Tshawytscha), known in Turkey as “Somon”,
2. Gilt-head bream (Sparus Aurata) known in Turkey as “Çipura”, and
3. Flathead trout (Salmo Platycephalus), known in Turkey as “Alabalık”.

Fresh fish samples were purchased from the fish market in Ulus – Ankara, cleaned and prepared for further procedures as will be described in the following sections.

Figure 3.6. Fish types used in the study.

3.2. Procedures

3.2.1. Bisphenol A procedures

Method development for BPA analysis using online and offline preconcentration techniques combined with the CE technique included the development of liquid–liquid microextraction method based on solidification of floating organic drop (DLLME-SFOD) procedures and investigations to study the applicability of the developed method on 4 different sample types namely bottled water, spring water, tap water and human urine. Below there is a description of the developed procedures and its application to the different sample types.

DLLME-SFOD procedures

Experimental procedures for DLLME-SFOD were as follows (Figure 3.7): A 10 mL BPA-free sample of deionized water was placed in a glass test tube and spiked with BPA at a concentration of 20 $\mu g L^{-1}$. Next, pH of this solution was adjusted to 4.0 using 0.1 mol L^{-1} HCl solution; a mixture containing 90 µL 1-UN (used as the organic extraction solvent) and 1.5mL acetone (used as the disperser solvent) was rapidly pipetted into the sample solution using a micropipette; the tube was sealed and vortex mixed for 1 min. A cloudy suspension (consisting of water, acetone and 1-UN) that resulted from the dispersion of fine 1-UN droplets in the aqueous solution formed in the test tube. After centrifugation for 5 min at 5000 rpm, phase separation took place and the floating organic drop appeared on the top of aqueous solution in the test tube. The test tube was left in the freezer at -20^{o}C and the floating organic

drop was solidified after 5 min; the solidified drop was separated using a small medical spatula and became ready for the back-extraction step.

Water samples

The developed DLLME-SFOD procedures were applied directly to the three water sample types (bottled water, spring water and tap water) without the need for sample cleanup or pretreatment as the case of human urine. Because normally urine matrix is more complex and needed sample pretreatment before proceeding in extraction procedures and CE analysis.

Human urine samples

Human urine is a complex matrix and needed sample clean-up and pretreatment before proceeding in DLLME-SFOD procedures and analysis. Urine samples were collected from a healthy male volunteer (37 years old) and were frozen at – 20^{o}C. Samples were allowed to thaw at room temperature prior to analysis. 4.0 mL of the supernatant transparent solution were transferred into a test tube and were spiked with prescribed concentrations of BPA. pH of this solution was adjusted to 4.0 using 0.1 mol L^{-1} HCl solution. Next, the solution was mixed with acetonitrile at 2:1 (v:v) ratio and the ionic strength was increased by adding 1.0 g of NaCl in order to promote a salt-induced phase separation between acetonitrile and the aqueous phase after the solution was vortex mixed for 1 min and centrifuged for 1 min at 4000 rpm. The resultant 1.0 mL of acetonitrile was transferred into a glass test tube, completed to the total volume of 10 mL using deionized water, then the DLLME-SFOD procedure was applied. It is noteworthy that acetonitrile here served as the disperser solvent instead of acetone in the subsequent DLLME-SFOD procedure.

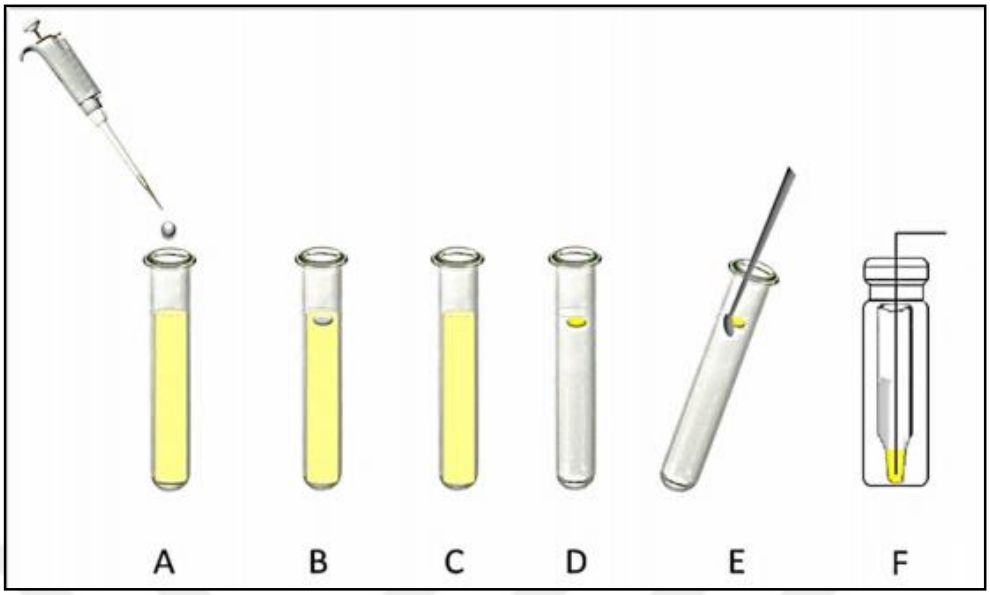


Figure 3.7. Schematic representation of the DLLME-SFOD procedure.

Back-Extraction procedures:

The resulted solidified organic drop melted rapidly at room temperature (normally melting points (m.p.) near room temperature in the range 10–30 ^{o}C) and was transferred into a glass insert inside a CE vial (Agilent Technologies, Waldbronn, Germany) as can be seen in Figure 3.7 (F). BPA was back-extracted into a 20 µL of 0.10 mol L^{-1} NaOH basic solution (hereafter referred to as back-extraction solution: BES) after vortex mixing for 1 min and centrifugation at 4000 rpm for 1 min. Finally, the aqueous phase containing the analyte was directly injected into CE without the need to separate the organic phase.

3.2.2. Alkylphenols procedures

Milk samples pretreatment and fortification

Carton-Box-Packed milk samples (200 mL) were purchased from the market. 2.0 mL milk were transferred into a glass test tube then spiked with different concentrations of the four alkyl phenols. 100 µl conc. phosphoric acid was added followed by vortex mixing for 1 minute. 4.0 mL acetonitrile followed by 0.2 g sodium

chloride were added. Again, vortex mixing for 1 minute. Then the tube containing mixture were centrifuged at 5000 rpm for 5 minutes. At this stage two phases were formed. 3.0 mL of the upper phase (acetonitrile) were pipetted into a new clean glass test tube followed by addition of 0.5 mL deionized water, then 0.2 g NaCl and 1.0 mL Hexane. The new mixture was vortex mixed for 1 minute, then centrifuged at 5000 rpm for 5 minutes. At this stage, three phases (from up to down, hexane, acetonitrile and aqueous) were formed in the glass tube. 1.5 mL of the middle phase (acetonitrile) were pipetted into a new glass tube. 150 µl Chloroform (CFM) was added, followed by volume completion to 10 mL using DI water. This last mixture was vortex mixed for 1 minute, then centrifuged at 5000 rpm for 5 minutes. 100µL of the organic layer (CFM) in the bottom of the tube was collected using a micropipette into a 1.0 mL plastic eppendorf and became ready for the back-extraction step.

Back-extraction procedures

To the eppendorf containing the organic layer (CFM, 100 µL) resulted from the previous extraction procedures, 60 µl of back extraction solution (pH=11.5) were added followed by vortex mixing for 1 minute and centrifugation at 10,000 rpm for 1 minute. The upper phase (aqueous) was transferred into a CE injection vial with glass insert to be injected for analysis.

3.2.3. Triphenylmethane dyes procedures

Fish samples preparation

Fish samples were firstly cleaned (gutted and scaled) in the fish shop then taken to the laboratory. In the laboratory, the three types of fish were skinned and deboned using a knife in the laboratory. At this stage, only the tissue part of the fish remained. The remaining part of each type of fish was cut into smaller parts and then each type of fish tissues was homogenized separately using a household blender. The homogenized fish tissues were kept in separate and labeled glass jars and left in freezer at (-20 ^{o}C) for future analysis.

Sample fortification and extraction

2.0 g of homogenized fish was weighed in a 10- mL plastic test tube. The weighed sample was spiked with 20 µL from 5ppm standard mixture of the four analytes in ACN, then the spiked sample was allowed to stand in the dark for 20 minutes to ensure the tissue-analyte interaction. To the spiked sample, 2.0 mL acetate buffer (pH=4.5), 3.0 mL of ACN and 0.5 g NaCl were added followed by vortex mixing for 1 minute, then centrifugation at 5000rpm for 5 minutes. The resulting mixture solution was left in freezer at -20 ^{o}C temperature for 5 minutes. At this stage two phases are formed in the test tube. 2.0 mL of the supernatant (supposed to be ACN containing the analytes) was collected and transferred into a 2.0 mL eppendorf. Evaporation to dryness under argon gas and using water bath was carried out using a sample concentrator equipped with a thermostat controlled block-heater. To the Eppendorf containing the dried sample at the bottom, 0.5 mL ACN and 0.5 mL saturated solution of NaCl (makes total volume of 1.0 mL) is added and followed by hand shaking to ensure that the dry content is dissolved in the solution. The eppendorf is left in freezer for 5 min. At this stage, again two phases are formed in the eppendorf (the upper phase - about 300 µL - colored bluish). 250 µL from the upper layer is transferred into a CE glass vial followed by addition of 250 µL ACN, 150 µL of 0.25M formic acid and 350 µL DI water (total volume of 1.0 mL). The obtained sample solution is ready for injection and analysis by CE.

In Figure 3.8, there is an explanatory graphical diagram showing the whole procedures of Triphenylmethane determination in fish samples including sample pretreatment, extraction and analysis.

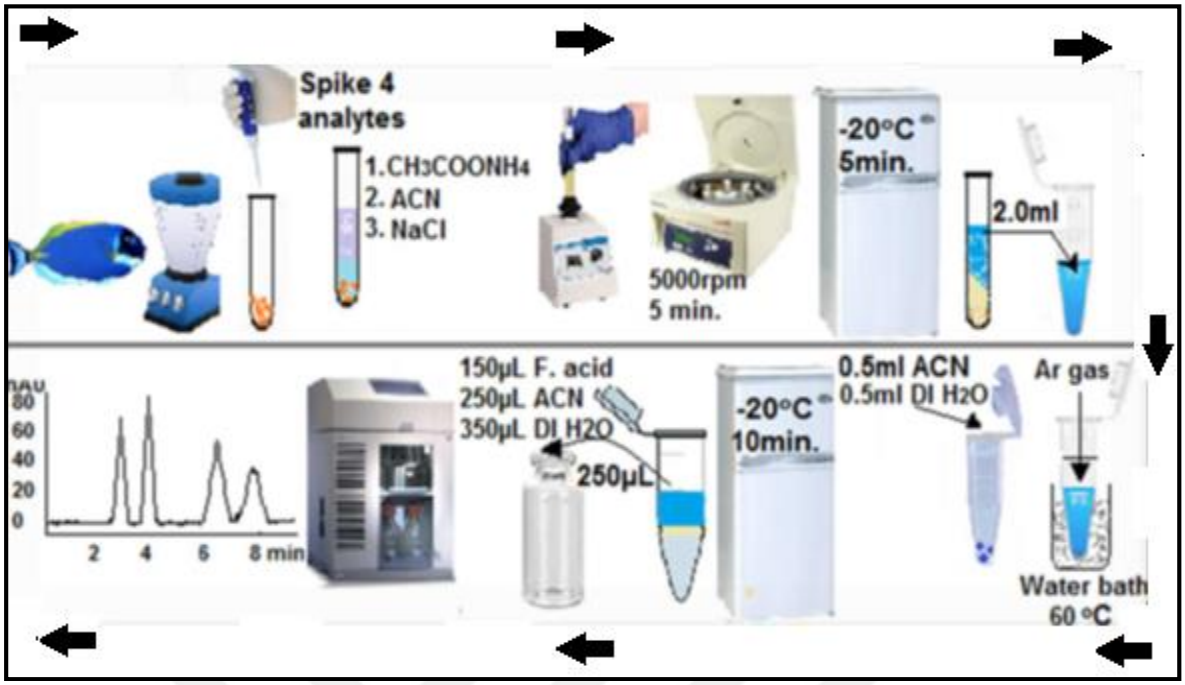


Figure 3.8. Procedures for determination of TPM dyes in fish samples.

4. RESULTS AND DISCUSSION

This study consisted of three parts. Different procedures were developed and applied to determine concentrations as low as the (μgL^{-1}) of different analyte groups in different matrices including food, biological and environmental samples using different offline (micro-extraction) and on-line preconcentration techniques combined with capillary electrophoresis technique. The studied analytes and matrices were as follow: In the first part, Bisphenol A in water and human urine, in the second part of the study, Alkyl phenols (BPA, BHM, NP and TBP) in commercial carton-box milk samples while in the third part of the study triphenylmethanes (MG, CV, LMG and LCV) in culture fish samples were studied. The investigated analytes are of great importance as they are proved to have impacts on human health and environment as well. Also, a variety of important matrices were chosen and treated in this study covering food (milk and fish), biological (urine) and environmental (water) matrices.

4.1. Bisphenol A

4.1.1. Optimization of parameters for CE analysis

New capillaries used for CE analysis were successively rinsed using deionized water (10 min), 1.0 M NaOH (20 min), deionized water (20 min) and finally with the BGE (20 min) prior to separation of analytes. To assure reproducibility, at the end of each run, the capillary was successively flushed with methanol (2 min), deionized water (1 min), 1.0 M NaOH (1 min), deionized water (2 min) and then with the BGE (2 min).

Conventional CZE

The capillary was conditioned with the BGE composed of 25mM sodium borate buffer and 5.0% methanol at pH 9.3, then the sample, prepared in the same BGE, was injected to the capillary using pressure injection at 50 mbar for 5 seconds. A

positive voltage of 20 kV was applied and the CE separation has started. The analyte migrated in a homogeneous conductivity medium and detected near the outlet end.

Field amplified sample stacking (FASS)

In FASS, at first, the capillary was conditioned with the BGE composed of 25mM borate buffer and 5.0% methanol and having the pH 9.3. In this case, the sample was prepared in water (low-conductivity medium) to achieve the maximum stacking efficiency as the difference in conductivity between the injected sample plug zone and the BGE is relatively high. The sample was injected into the capillary for 50 s at 50 mbar. After the sample solution was injected, a positive voltage of 20kV was applied. At this point, BPA was stacked at the boundary between the low-conductivity sample plug and the high-conductivity BGE. Thus, enriched and sharp peak of BPA was obtained.

4.1.2. Optimization of DLLME-SFOD conditions

In order to obtain the most effective extraction procedures, it was important to determine the optimum DLLME-SFOD conditions for the analysis of BPA including type and volume of the extraction and disperser solvents, pH and volume of sample and back-extraction solutions, and ionic strength. Peak area was used to evaluate the influence of those variables on the extraction efficiency of the DLLME-SFOD technique.

Type and volume of the extraction solvent:

Organic solvents that are appropriate for microextractions based on solidification of the floating organic drop are selected according to the following characteristics: to have low volatility and low solubility in water for them to be stable during the extraction process; to have a high extraction efficiency for the analytes; to be separated from the analyte peaks in chromatographic applications; to have melting points (m.p.) near room temperature (preferably in the range 10 - 30 ^{o}C).

Accordingly, 1-UN (mp:13-15 ^{o}C; density:0.830 g.mL^{-1}) and 1-DO (mp: 24 – 27 ^{o}C; density: 0.833 g mL^{-1}) were investigated. In addition, DPE (m.p.: 25–27C; density: 1.060 gmL^{-1}, solubility in water: 0.002 g in 100 mL of water at 25 ^{o}C) seemed to be a promising extraction solvent for DLLME-SFOD applications. It is worthy to note, however, that DPE is denser than water and sediments at the bottom of the extraction tube or floats at the surface depending on salt content in the sample solution due to proximity of its density to that of water. 1-UN gave the highest extraction efficiency. In Figure 4.1, samples were spiked to 10 µg L^{-1} of BPA and the extraction conditions were as follows: an aqueous sample volume 10 mL extracted with each extraction solvent (1-Un, 1-DO and DE) and 1.5 mL acetone; the extraction time was 1 minute and there was no salt addition. The back-extraction solution consisted of 20 µL of 0.10 mol L^{-1} NaOH. Moreover, because of its stability, low vapor pressure and low water solubility at the extraction conditions, 1-UN was selected as the extraction solvent in the present study.

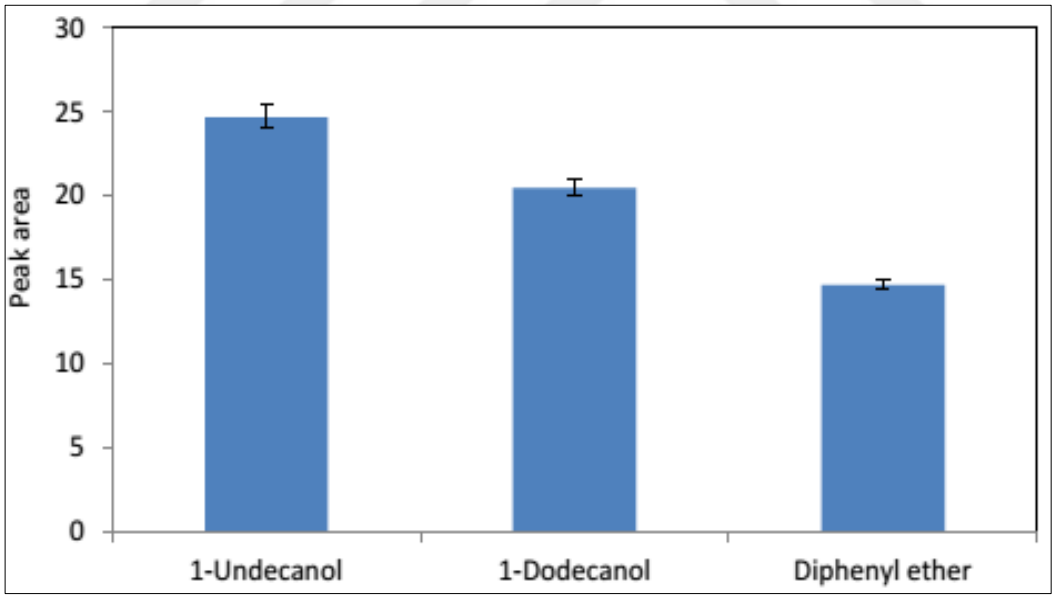


Figure 4.1. Effect of extraction solvent type on extraction efficiency.

In DLLME-SFOD, the volume of the extraction solvent is a key parameter that affects extraction kinetics and therefore enrichment factors. Its effect on the analytical signal of BPA was studied in the range of 10 –120 µL. As can be seen in Figure 4.2, the analytical signal of the target analyte increased by increasing the extraction

solvent volume in the range of 10–90 µL before it decreased again afterward. Based on these observations, the volume of 90 µL was set as the optimum value and used in further experiments.

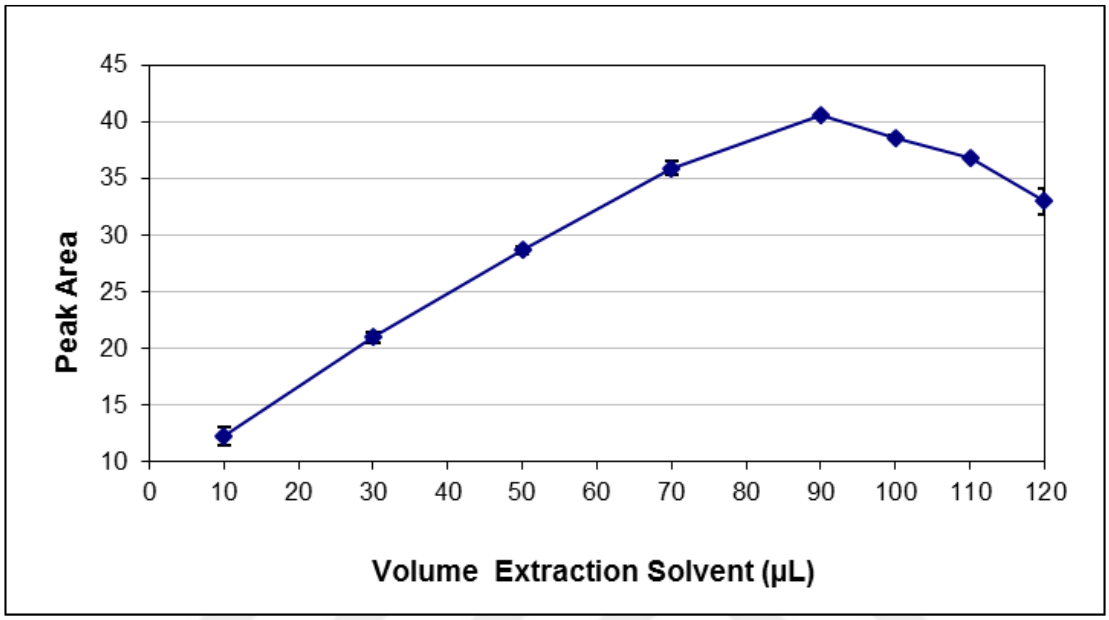


Figure 4.2. Effect of the volume of extraction solvent (1-Undecanol) on extraction efficiency.

Type and volume of disperser solvent

Miscibility of disperser solvent with extraction solvent and sample solution was one of the most important criteria when selecting the disperser solvent in DLLME-SFOD procedures. Thereby, acetone, acetonitrile, and methanol, which have this property, were suitable to be used as disperser solvents. To investigate the type of disperser solvent, a series of sample solutions was extracted using 1.5 mL of each of these disperser solvents containing 90 µL 1-UN. Acetone was found to give the highest extraction efficiency (Figure 4.3); it also has lower toxicity and is cheaper than the other two solvents methanol and acetonitrile.

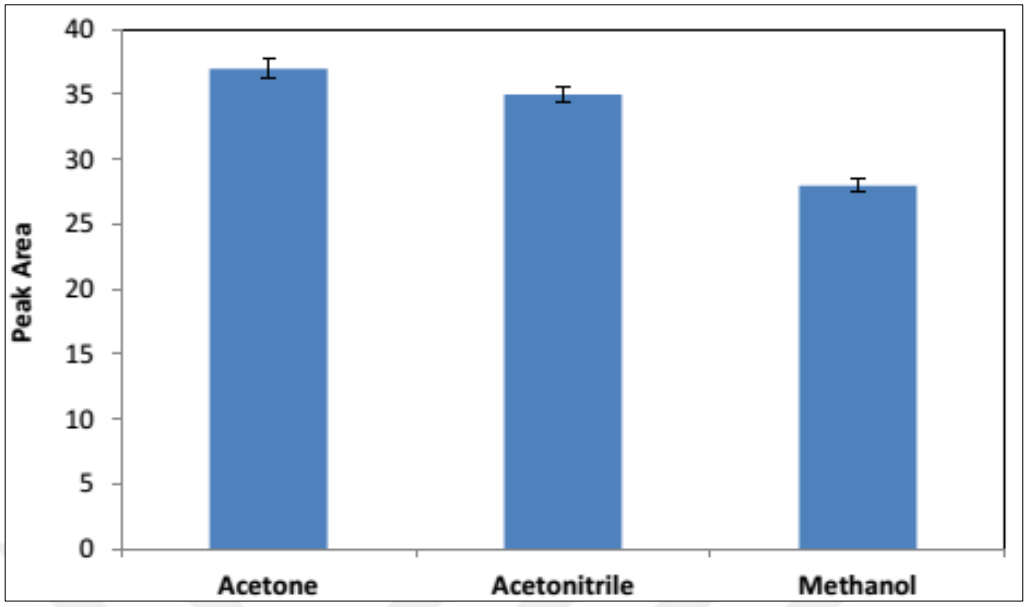


Figure 4.3. Effect of disperser solvent type on extraction efficiency.

Thus, acetone was used through the experiments followed to investigate the effect of disperser solvent volume on extraction efficiency and various volumes of acetone ranging between (0.5 - 2.5 mL) were used as shown in Figure 4.4. Increasing the volume of acetone from 0.5 to 1.5 mL resulted in a gradual increase in extraction efficiency, but increasing the volume beyond this point decreased the extraction efficiency steadily. This was thought to be due to the increase of the solubility of extraction solvent (1-Undecanol) in water-acetone mixture with the increase of the volume of acetone. The optimum sensitivity was achieved when 1.5 mL acetone was used.

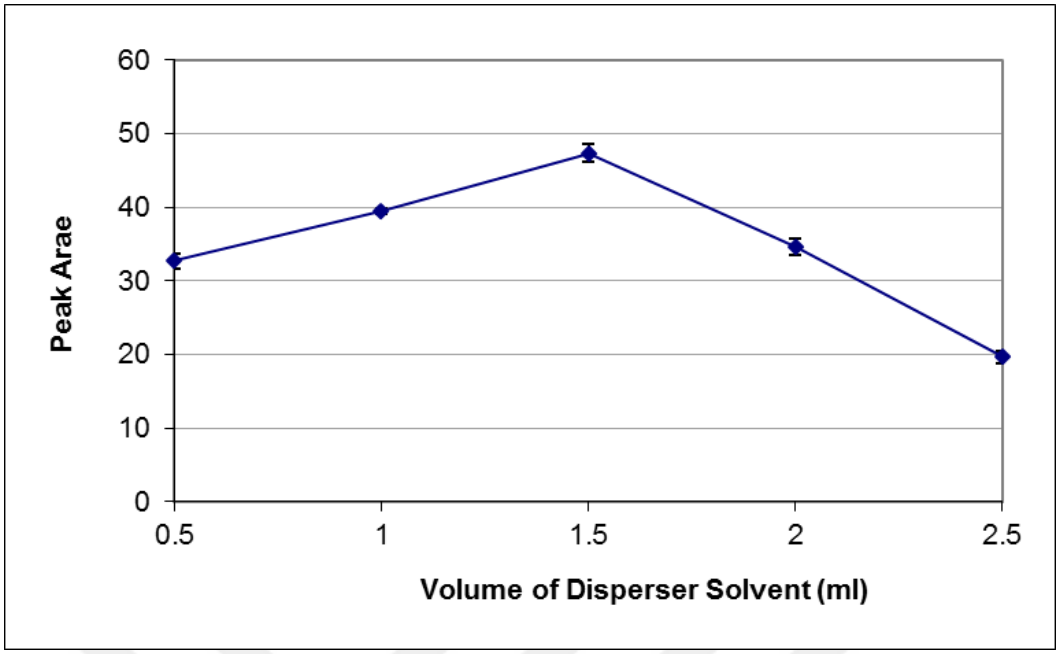


Figure 4.4. Effect of the volume of acetone on extraction efficiency.

During these experiments, samples were spiked to 10 µg L^{-1} of BPA and the extraction conditions were as follows: aqueous sample volume 10 mL; extracted with different volumes of 1-UN, and different volumes of acetone; extraction time of 1 minute without salt addition and the back-extraction solution used was 20 µL of 0.10 mol L^{-1} NaOH.

pH of sample and back-extraction solution

pH of sample solution played an important role since extraction efficiency was greatly affected by the charge on the studied analyte. Based on its pKa value of 9.7, BPA is completely present in its neutral form in acidic media (pH 5.4) and more than 97.6 % of it in its negatively charged form in highly alkaline media (pH 12.0). pH of sample solution was studied over the range 3.0 - 9.0 (Figure 4.5). The highest extraction efficiency was obtained at pH 4.0 for sample solution.

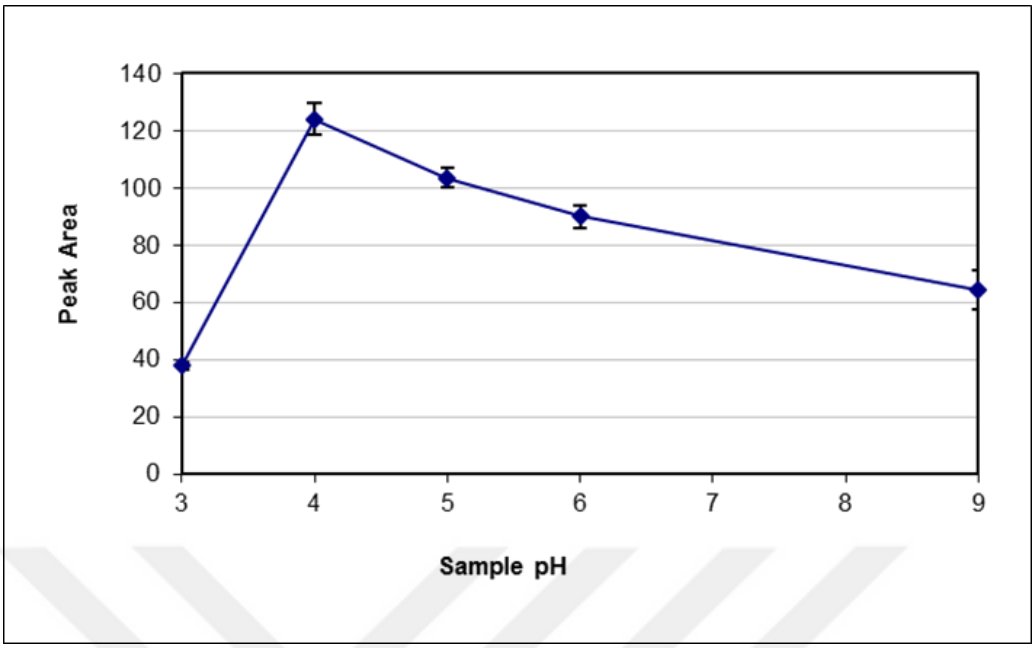


Figure 4.5. pH of Sample solution.

To investigate the optimum composition of back extraction solution (BES), BPA was back-extracted into an aqueous solution containing varying concentrations of NaOH in the range of 0.01–0.20 mol L^{-1} (Figure 4.6). It was decided to use a basic aqueous solution as (BES) taking into consideration that BPA is ionized (negatively charged) in basic medium. Maximum extraction efficiency was obtained at the concentration of 0.10 mol L^{-1}. as such these values (Sample solution pH = 4 and BES is composed of basic aquous solution with NaOH concentration = 0.10 mol L^{-1}) were set as the optimum values and thus used for subsequent experiments.

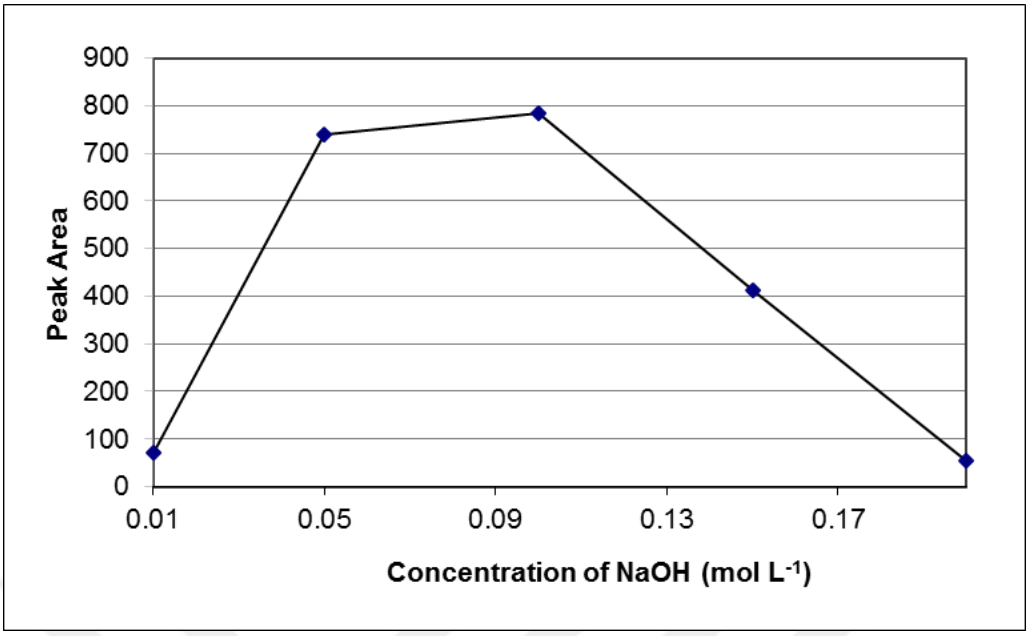


Figure 4.6. Optimum concentration of NaOH in BES

Volumes of sample and back-extraction solutions

In three-phase LPME, higher enrichment factors can be achieved by increasing the volume ratio of the aqueous sample to the back-extraction solution. However, in many cases at equilibrium the maximum recovery can be limited by the distribution coefficient of the analyte between the donor and acceptor phases (Farahani et al., 2009). Volume of sample solutions was increased from 5 to 15 mL while all the other parameters including the volume of 20 µL for BES were kept constant. The results showed that the largest analytical response was obtained at a sample volume of 10 mL (Figure 4.7).

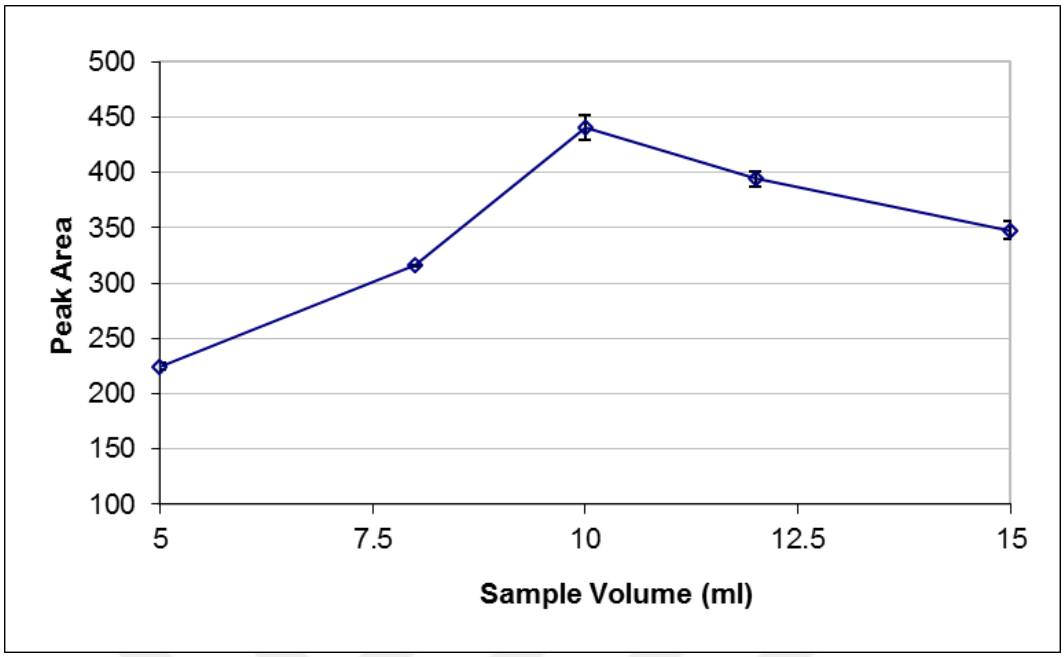


Figure 4.7. Optimization of sample volume

The effect of volume of back-extraction solution was investigated over the range 20 to 60 µL. As can be seen in Figure 4.8, the extraction efficiency gradually decreased with increasing the volume which was due to dilution. Lower volumes than 20 µL could have resulted in higher peak areas, but when lower volumes were used, it was practically difficult to collect and handle the resulted micro-drop as the aqueous phase was surrounded (contaminated) by the organic phase this resulted in a current drop during the CE analysis after injection when separation voltage was applied. Therefore, a volume of 20 µL was accepted and set as the optimum value for further experiments.

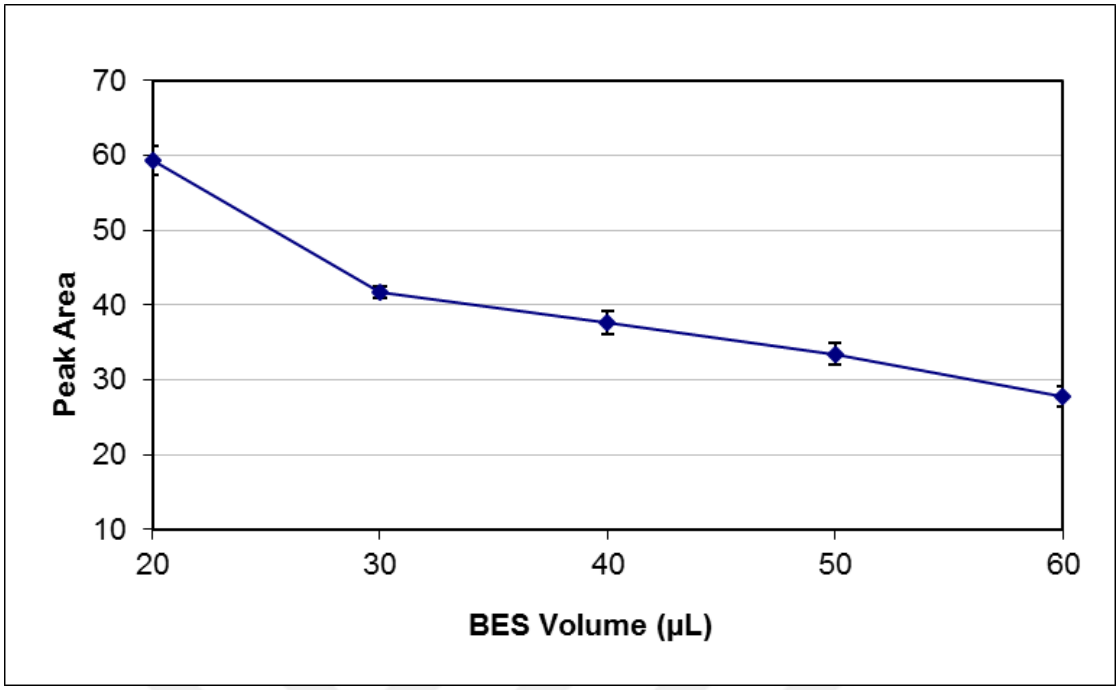


Figure 4.8. Optimization of back extraction solution (BES) volume.

During these experiments, samples were spiked to 10 µg L^{-1} of BPA and the extraction conditions were as follows: variable volumes of aqueous sample (5 – 15 mL) extracted with 90 µL 1-UN, and different volumes of acetone; extraction time of 1 minute without salt addition. Variable volumes (20 – 60 µL) of back-extraction solution 0.10 mol L^{-1} NaOH was used.

Salt addition

The addition of salt into the sample solution has been widely applied in LLE in order to improve the extraction efficiency of the analytes due to the salting-out effect (Penalver, Pocurull, Borrull, & Marce, 2000). However, it has shown no effect or even controversy results in DLLME-SFOD depending on the studied analyte(s) (Rezaee, Yamini, Khanchi, Faraji, & Saleh, 2010). The effect of increasing the ionic strength of the sample solution on the extraction efficiency of BPA was investigated by the addition of NaCl (0-0.4 mol L^{-1}) into the sample solution. It was observed that extraction efficiency decreased with increasing the salt content. Hence, further extractions were performed without salt addition.

Effect of extraction time

In DLLME–SFO, extraction time is defined as the time interval between the injection of the mixture of disperser and extraction solvents and the time at which the sample is centrifuged (H. Xu, Ding, Lv, Song, & Feng, 2009). In our study, this corresponded to the time of vortex mixing. The effect of extraction time on the extraction efficiency was studied in the range of 0–5 min under constant experimental conditions. The results obtained showed that the extraction time did not have any significant influence on the signal of BPA. This was due to the fact that in DLLME after formation of the cloudy solution, the surface area between extraction solvent and aqueous sample is infinitely large. Thereby, transition of the analyte from the aqueous sample into the extraction solvent is considerably fast. In fact, independence on time is one of the great advantages of DLLME. In this method, the time-consuming steps were centrifugation of the sample solution and solidification of 1-UN, which was about 5 min each.

4.1.3. Analytical performance and figures of merit

Limits of detection (LOD) of the target analyte generated by DLLME-SFOD combined with filed amplified sample stacking under optimized conditions in water and urine matrices are listed in Table 4.1. LOD calculated based on a signal-to-noise (S/N) ratio of 3; N: noise of the baseline calculated for eleven noise peaks chosen at different places of the baseline void of analytical peaks obtained using CZE as 1.0 mg L^{-1}. Applying FASS produced an LOD (145 µg L^{-1}) that was lower by 6.9 times as compared to CZE mode. In addition, application of DLLME-SFOD improved the CE sensitivity further by 181 times in water matrix and 58 times in urine matrix giving rise to LODs of 0.8 µg L^{-1} and 2.5 µg L^{-1} for BPA in water and urine, respectively (Table 4.1). Thus, overall improvement factors of CE sensitivity for the determination of BPA (Ratio of LOD in conventional CZE to that with DLLME-SFOD combined with FASS were 430 and 1,250 in urine and water, respectively.

Table 4.1. Figures of merit of DLLME-SFOD with FASS

	Linear Equation	Linear range (μg L^{-1})	R^2	RSD (%)[a] (n=5)		LOD (μg L^{-1})	IF[b]
				Intra-day	*Inter-day*		
Water	y = 8.4075x + 3.2701	2.5 –100	0.9992	0.5	1.2	0.8	1,250
Urine	y = 3.1873x + 10.867	10.0 –100	0.9989	0.9	1.9	2.5	430

[a]*Data were calculated based on extraction of 20 μg L^{-1} BPA.*
[b]*Overall improvement factor (Ratio of LOD in conventional CZE to that with DLLME-SFOD combined with FASS.*

Regression data and linearity of the calibration plots were investigated over a concentration range of 2.5-100 and 10-100 μg L^{-1} for water and urine, respectively. The calibration curves are shown in Figure 4.9 and Figure 4.10. Analytical figures of merit obtained for the method are presented in Table 4.1. BPA exhibited good linearity with a coefficient of determination greater than 0.998 in all calibrations. These calibration curves were obtained after performing the developed SFOD extraction procedures and using the FASS mode in CE analysis under the optimum parameters. Reproducibility of the proposed method was determined by intra-day and inter-day precision. As can be seen in Table 4.1, intra-day and inter-day (n = 5) precisions (RSD) for 20 μg L^{-1} BPA were equal to or less than 0.9 % and 1.9 %, respectively.

On the other hand, a calibration curve for BPA in conventional CZE (Figure 4.11) was constructed where the BPA for CE analysis was prepared in the BGE. The results obtained from this calibration was compared with calibrations obtained using DLLME-SFOD combined with FASS to calculate the overall improvement factor (IF) obtained from both the DLLME-SFOD microextraction method and the FASS mode as explained above and results are presented in Table 4.1 .

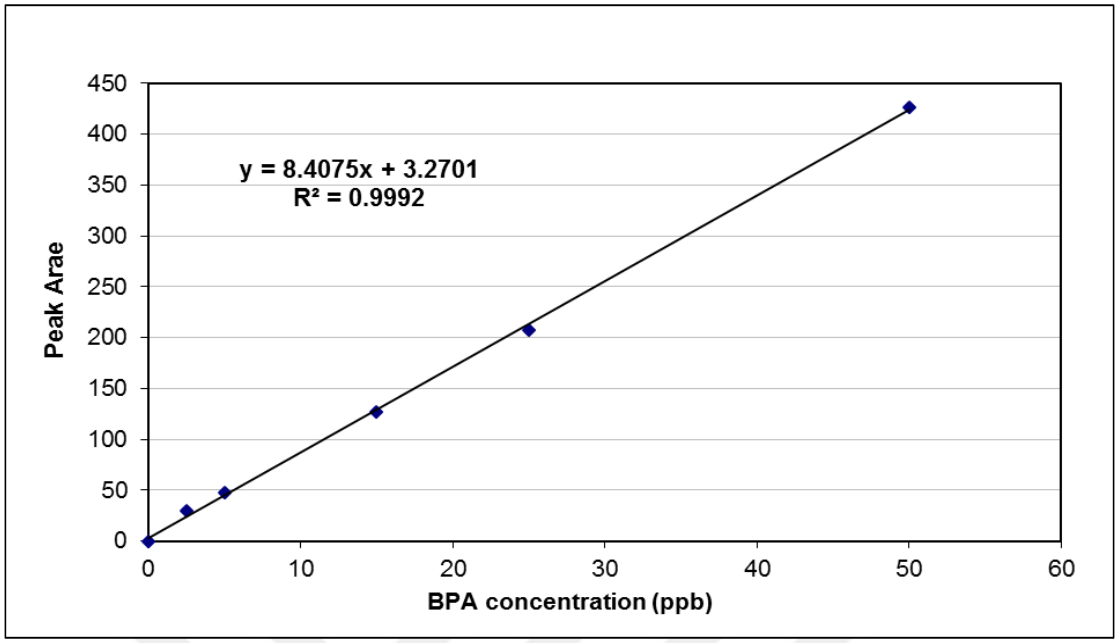


Figure 4.9. Calibration curve of BPA in water samples obtained after DLLME-SFOD combined with FASS

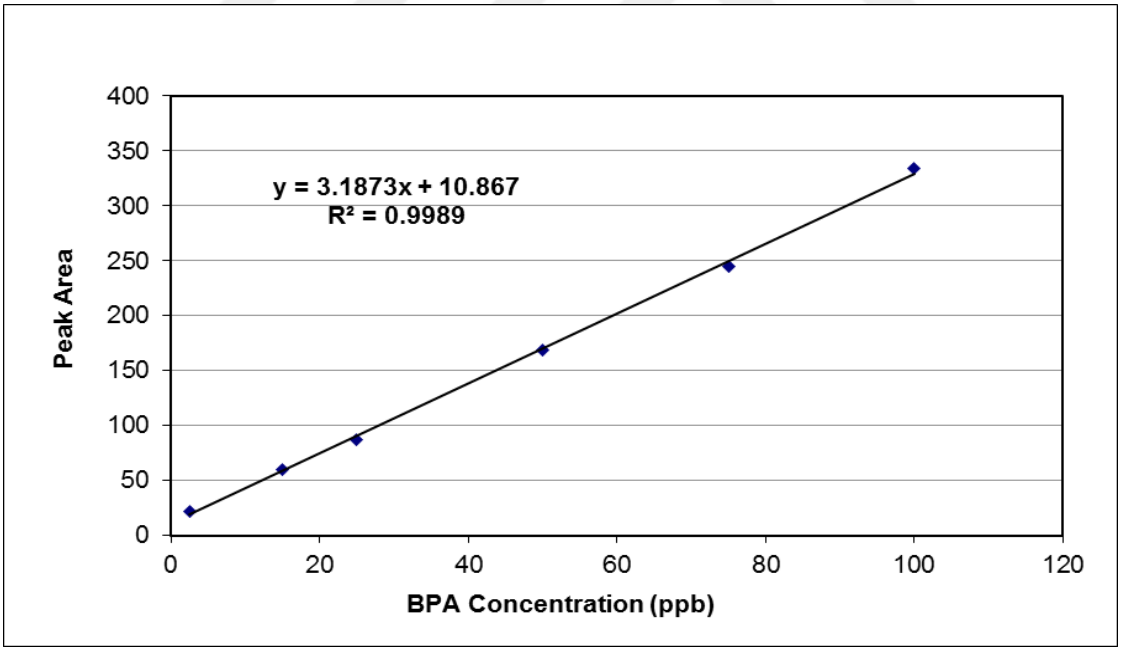


Figure 4.10. Calibration curve of BPA in urine samples obtained after DLLME-SFOD combined with FASS

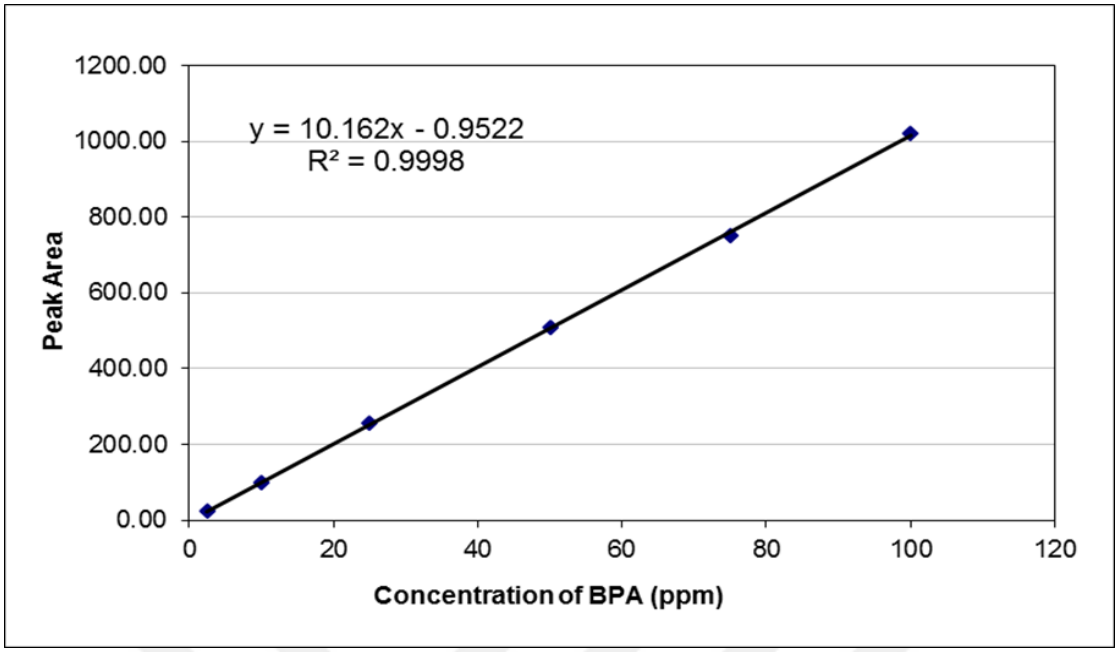


Figure 4.11. Calibration curve of BPA in water samples obtained after DLLME-SFOD and conventional CZE.

Representative electropherograms obtained from extracts of tap water and urine after performing extraction using the DLLME-SFOD procedures under optimum extraction and stacking conditions are provided in Figure 4.12, in which the electropherograms of (a) tap water spiked with BPA at 20 μg L^{-1}, (b) blank tap water (c) human urine spiked with BPA at 20 μg L^{-1} and (d) blank human urine are shown. Electrophoretic conditions used in CE analysis to obtain these electropherograms were as follows: separation temperature: 30^{o}C; separation voltage: 20 kV; BGE: 25 mM borate buffer containing 5.0 % methanol (pH 9.3); sample injection mode: pressure injection at 50 mbar for 50 s.

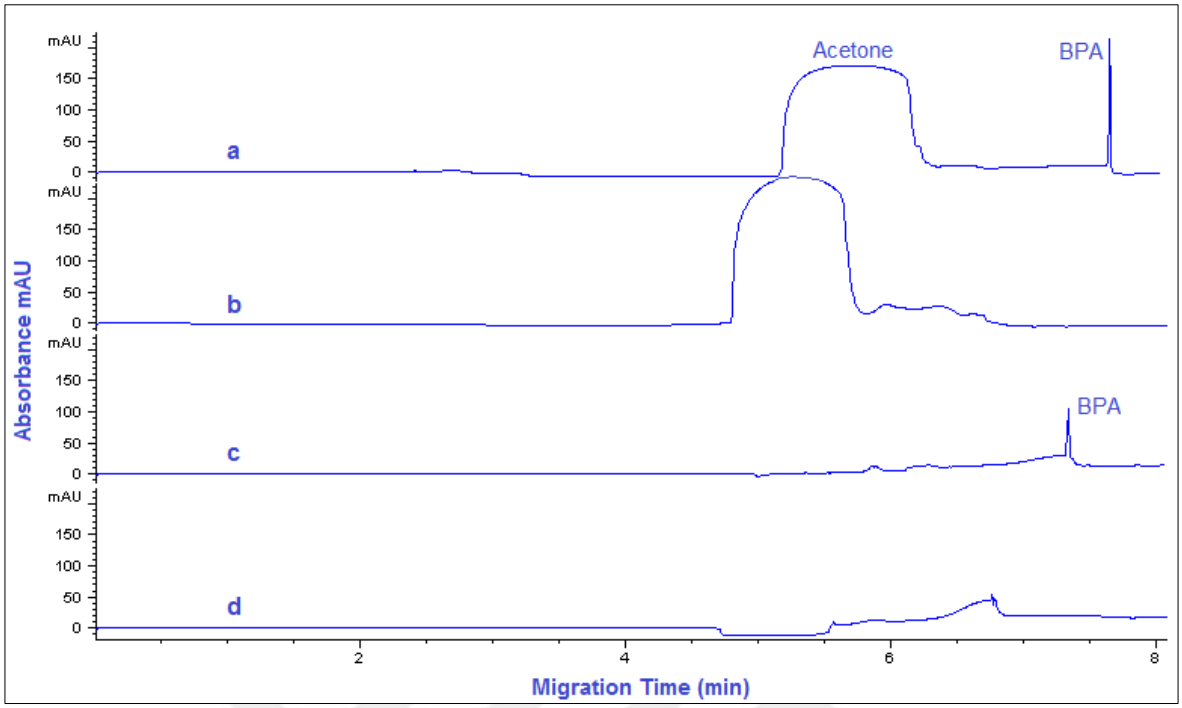


Figure 4.12. Electropherograms of extracts of tap water and urine after DLLME-SFOD procedures

4.1.4. Analysis of real water and urine samples

In order to investigate the possibility of matrix effects and investigate the applicability of the method to the analyses of real samples, the proposed method was used to determine BPA in three different water samples including tap, bottled and spring water as well as human urine. Water and human urine samples were spiked with the target compound at three concentration levels. The results are summarized in Table 4.2. Relative recoveries (RR) in water matrix were in the range of 92.4–104%. RRs in urine matrix were calculated using matrix-matched calibration and they were in the range of 99.5 – 100.3%.

Table 4.2. Relative recoveries of BPA from water and human urine samples spiked with the target analyte.

Sample Type	C_{Added} (µg L^{-1})	C_{Found}(µg L^{-1})	RR[a]	RSD (%)
TAP WATER	-	n.d.[b]	-	-
	20.0	20.8 ± 0.1	104.0	0.5
	40.0	40.8 ± 0.3	102.0	0.7
	70.0	69.6 ± 0.4	99.4	0.6
BOTTLED WATER	-	n.d.[b]	-	-
	20.0	18.9 ± 0.2	94.5	1.1
	40.0	37.2 ± 0.3	93.0	0.8
	70.0	64.7 ± 0.5	92.4	0.8
SPRING WATER	-	2.7 ± 0.5	-	-
	20.0	19.1 ± 0.2	95.5	1.0
	40.0	37.4 ± 0.4	93.5	1.1
	70.0	64.9 ± 0.5	92.7	0.8
URINE[c]	-	9.2 ± 0.4	-	-
	20.0	19.9 ± 0.2	99.5	1.0
	40.0	40.1 ± 0.5	100.3	1.2
	70.0	69.9 ± 0.1	99.9	1.6

[a] Relative recovery, percentage value obtained considering extraction yields in deionized water as 100%,
[b] Not detected and
[c] Relative recovery, percentage value obtained considering extraction yields from matrix-matched calibration.

4.1.5. Comparison with other preconcentration methods

The developed DLLME-SFOD-CE method was compared with other preconcentration methods used for the determination of BPA in terms of LOD, linearity, RSD%, volume of extraction solvent and extraction time for ionic liquid-dispersive liquid phase microextraction (IL-DLPME), liquid–liquid–liquid microextraction (LLLME), SDME, DLLME, and solid-phase microextraction (SPME). As can be seen in Table 4.3., the current method is most importantly much faster than the other microextraction methods. With the exception of DLLME which is also very fast (extraction time is less than 3 min), extraction times for IL-DLPME, LLLME,

SDME, and SPME ranged from 20 to 60 min, without equilibrium being reached in most cases (Us et al., 2013). As no specific holder is required for supporting the organic microdrop like in SDME, DLLME-SFOD, the developed method is considered much more robust. Also, this method had the lowest RSD among the other methods. This method provided an acceptable LOD (0.8 µg L^{-1}) and a good linear range (2.5 – 100 µg L^{-1}) without using derivatization reagents, which may complicate the extraction process and extend the extraction time, or applying more sensitive detectors such as MS which are expensive and are not affordable by many laboratories. In contrast to IL-DLPME, LLLME, SDME, and SPME, extraction time had no influence on the DLLME-SFOD efficiency. In addition to other advantages of the developed method, it is simple, rapid, inexpensive, and easy to apply.

Table 4.3. Comparison of the developed method with other methods for extraction and determination of BPA

Pre-concentration Method	Sample type	Detection System	LOD (μgL^{-1})	Linear Range (μgL^{-1})	RSD (%)	V_{ES}[a] (µL)	$T_{extraction}$ (min)	Reference
IL-DLPME[b]	Water	HPLC-FLD[c]	0.15	1.0–100	3.4	65	20	(Zhou, Gao, & Xie, 2011)
LLLME	Water	HPLC-FLD	0.014	0.1–200	4.7	15	50	(C. Y. Lin, Fuh, & Huang, 2011)
SDME	Seawater	HPLC-UV	4	15–125	4.1	2.5	60	(Lopez-Darias, German-Hernandez, Pino, & Afonso, 2010)
DLLME	Water	HPLC-UV	0.07	0.5–100	6.0	142	<3	(Rezaee, Yamini, Shariati, Esrafili, & Shamsipur, 2009)
SPME	Waste-water	GC-MS	0.04	0.027–195	10.0	–	60	(Braun et al., 2003)
DLLME-SFOD-FASS	Water	CE-UV	0.8	2.5–100	1.2	90	2	This study
	Urine		2.5	10–100	1.9			

[a] Volume of extraction solvent.
[b] Ionic liquid-dispersive liquid phase microextraction.
[c] Fluorescence detection.

4.2. Alkyl Phenols

The increased global concern about APs highlights the importance of developing sensitive analytical methods to detect trace amounts of this compound in environmental and biological samples. In this study, the dispersive liquid-liquid microextraction (DLLME) method was combined with field amplified sample stacking mode (FASS) in CE for preconcentration and determination of APs in bottled milk samples.

4.2.1. Milk samples clean-up and pretreatment

Most of the methods reported for determination of organic molecules in milk involve protein precipitation with an organic solvent in the presence of either an acid or a base. Commonly used organic solvents are ACN, ethanol, and methanol, with ACN being the most often used. Phosphoric acid is often used to acidify the sample, while in cases of protein precipitation from basified samples, either sodium hydroxide or ammonium hydroxide is used (Ei-Gindy, Sallam, & Abdel-Salam, 2008).

In this study, at the beginning a combination of ACN and sodium hydroxide was studied and found to be ineffective in cleaning-up (precipitating proteins) from milk spiked samples with alkylphenols and this procedure gave poor recovery of analytes data. Meaning that ACN – base combination did not completely precipitate the protein from the milk samples. The second approach for precipitation of protein was used, in which a combination of ACN with phosphoric acid was used. To ensure the removal of fats from milk samples, n-hexane was used.

4.2.2. Optimization of extraction and analysis parameters:

Type of organic extractant

The required characteristics for selecting organic solvents that are suitable for microextractions based on SDME method were mentioned in BPA study. However, in this part of the study, different organic solvents (chloroform, CFM and carbon tetra chloride CTC) were added and investigated, which are denser than water and sediments at the bottom of the extraction tube. As shown in Figure 4.13, four different organic solvents were studied to extract the target analytes from milk samples and actually three of them showed good and comparable extraction results. Though, we decided to choose CFM as the optimum extractant considering the practicality, and time issues, where in case of CFM, there was no need for freezing step as needed to solidify the organic solvent drop in case of using 1-UN and/or 1-DO. The CMF was collected from the bottom of extraction tube and transferred directly to the CE injection vial for analysis.

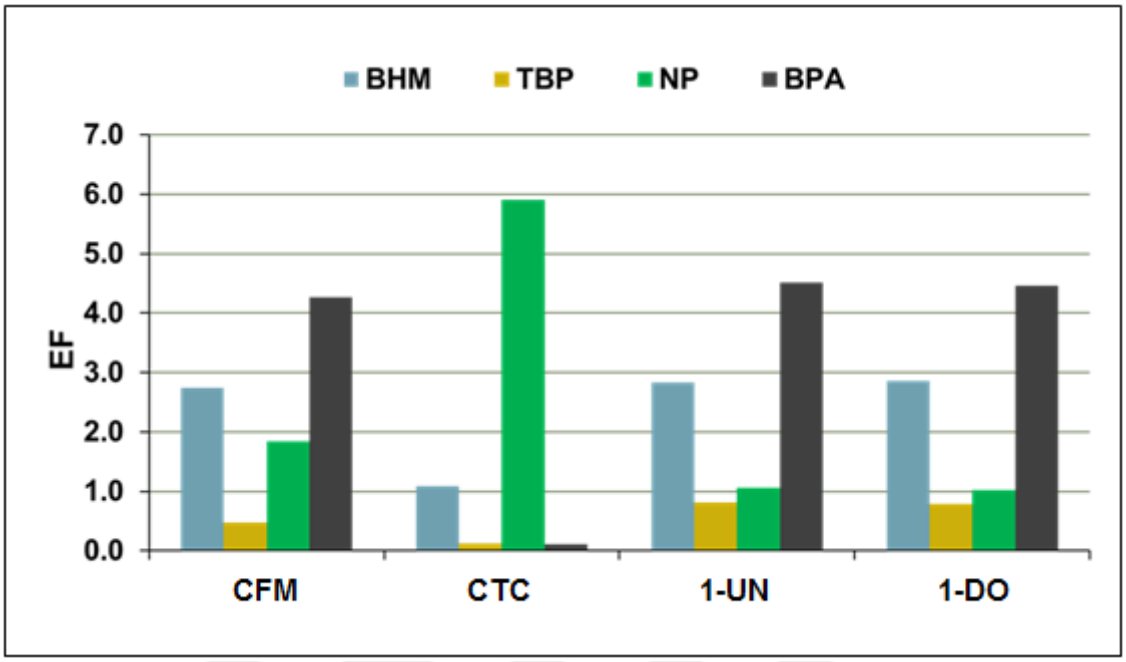


Figure 4.13. Type of organic extraction solvent

Volume of organic extractant

Generally, in LLME and particularly in SDME method, the volume of extraction solvent is a key parameter that affects extraction kinetics and therefore enrichment factors. Its effect on the extraction efficiency (recoveries) and analytical signal of the target analytes (Aps) was studied in the range of 100 –200 µL. As can be seen in Figure 4.14, the analytical signal (shown as peak areas) of the target analytes varied by changing the volume of extraction solvent showing the best results for most of target analytes. Based on these observations, the volume of 150 µL was chosen as the optimum value and used for further experiments.

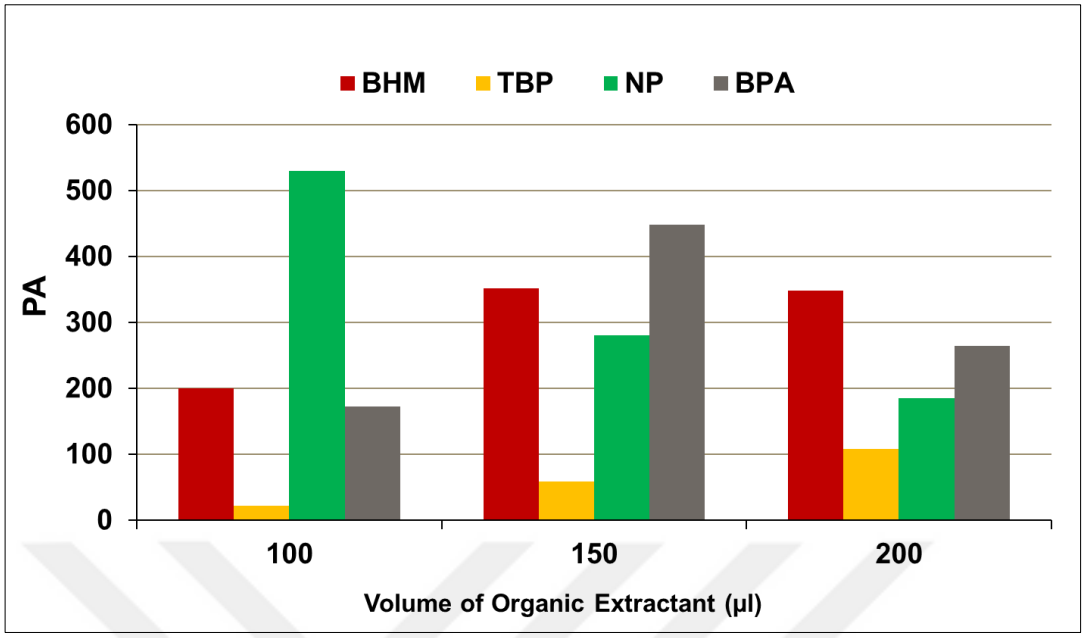


Figure 4.14. Volume of organic extraction solvent.

Volume of acetonitrile as dispersing agent

As the ACN was used in the milk sample pretreatment and in the same time it has the characteristics required for efficient DLLME, mainly its miscibility with both extraction solvent and sample solution, it was used as dispersing agent in this study. To investigate the optimum volume of dispersing agent of APs extraction from milk samples, different volumes of ACN in the range of 0.5 – 2.5 mL were studied and as shown in Figure 4.15, for most of target analytes (three analytes out of four) the volume 1.5 mL of ACN increased recoveries and thus the highest peak areas were obtained. The volume of 1.5 mL of ACN as dispersing agent was set as optimum value and used in the following experiments.

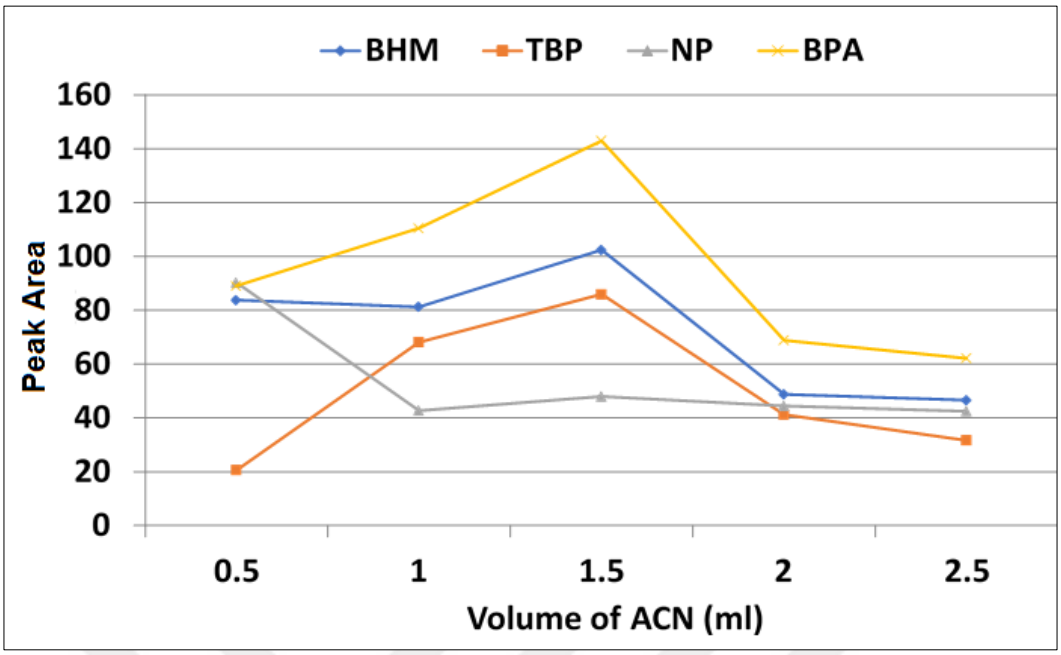


Figure 4.15. Volume of dispersive solvent (ACN).

Salting-out effect

The use of salting-out effect with water-miscible organic solvents, such as acetonitrile and acetone, for extraction is known for many years , especially its usage for the extraction of metal chelates into organic solvents prior to atomic absorption and high-performance liquid chromatography (HPLC); however, its exploitation as a preconcentration method for the trace analysis of neutrals as well as charged organic solutes have been very limited (So & Huie, 2001). An important but often unappreciated characteristic of the salting-out solvent extraction method is that even when two distinct layers appeared to be formed after the extraction, an appreciable amount of water can be found in the upper organic layer. Meaning that, a significant enhancement in detection sensitivity can be obtained by optimizing the salting-out conditions to cause favorable partitioning of the nonpolar as well as polar analytes into a relatively small volume of mostly organic layer. In this study, sodium chloride and ACN were used as the salting-out agent and water-soluble extraction solvent, respectively. It was clear that the homogeneous solution of water and ACN was broken by addition of the salt. Further sample treatment and extraction procedures were followed.

Type and volume of back extraction solution

The effect of volume of back-extraction solution on extraction recoveries was investigated over the range 60 to 110 µL. As can be seen in Figure 4.16, for BPA and BHM, the extraction efficiency gradually decreased with increasing the volume of BES while there was not significant change in the cases of NP and TBP. The decrease in extraction efficiency with increasing the BES volume in the case of BPA and BHM is mainly due to dilution effect. Lower volumes than 60 µL could have resulted in higher extraction efficiency but when lower volumes were used, it was practically difficult to handle the sample in the injection vial and to perform injection from the same vial.

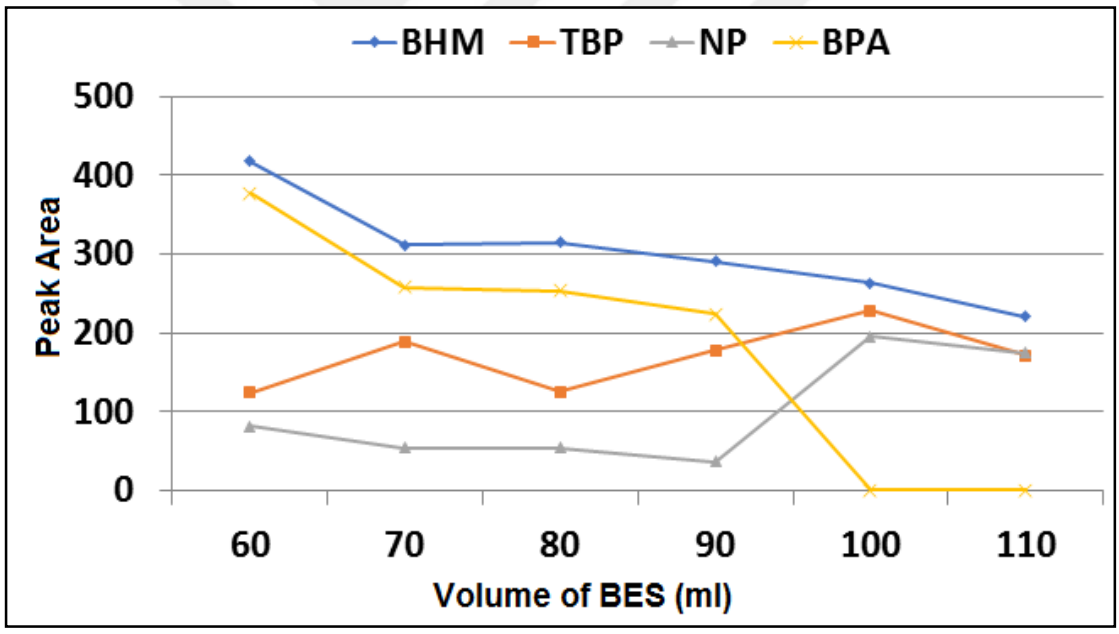


Figure 4.16. Volume of back extraction solution (BES)

pH of back extraction solution (BES)

the BES used in this study as a basic aqueous solution of sodium hydroxide. To investigate the optimum pH of the BES, the target analytes were back-extracted into an aqueous solution (BES) having varying pHs in the range of 10 – 12. Results shown in Figure 4.17 indicated that maximum extraction efficiency and thus peak

areas of all the target analyses were obtained using the BES having the pH of 11.5. so, this pH value was set as optimum and used in further experiments.

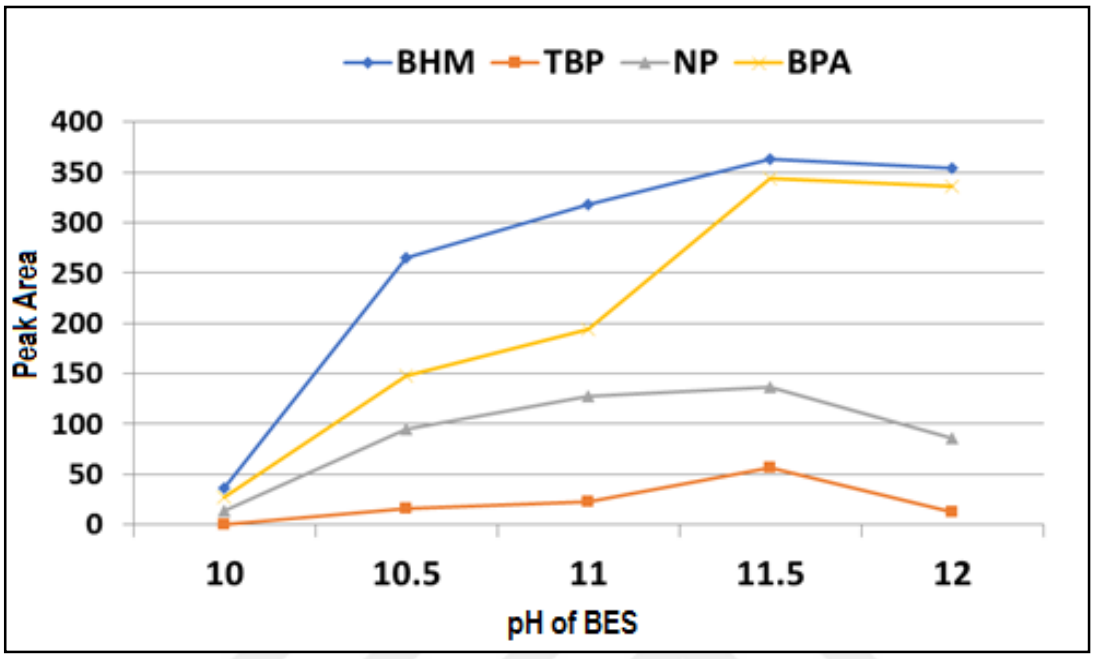


Figure 4.17. pH of back extraction solution

4.2.3. Optimization of parameters for CE analysis

Preparation and conditioning of the capillary

The capillary type and condition is can have impact on the efficiency and reproducibility of the CE analysis. In this part of the study, uncoated fused-silica capillary having the optimized dimensions of (total length: 48.5cm, effective length: 40 cm & internal diameters: 75 µm) was used for the analysis. To prepare the capillary for analysis, the capillary was cut to the appropriate length with a ceramic knife, to ensure a neat and flat edges. This is of particular importance with the inlet end of the capillary where sample is to be introduced. Closer to the outlet end (about 9 cm) a window (about 0.5cm) was created by removal of the capillary coating by burning-off the polyimide using a normal kitchen lighter and wiping the surface with an ethanol soaked smooth tissue papers. and the online detection took place through this window. Once the window is created, since the window area is very

fragile, we dealt with the capillary very carefully during handling and/or keeping on shelf after finishing the analysis.

Treatment of capillary before, during and after the electrophoretic separations is very crucial. So, before using the new capillary for analysis, the capillary was pre-conditioned by rinsing with water (10 minutes), then 1.0M NaOH (15 minutes), followed by DI water (20 minutes) before rinsing with the separation buffer (15 minutes). By doing this, the capillary was ready for further separations.

BGE and separation conditions

In general, to achieve analysis by CE, the BGE composition is the key parameter. First of all, the mobility of the ions of the BGE, must be closely matched with those of the analyte ions, for a better resolution and a symmetric peak shape. The components of BGE should not absorb UV radiation, as most of the inorganic anions do not absorb in the UV -range. In this study, for the separation of the four alkylphenols using CE analysis, the optimized composition of BGE was 7.0mM sodium borate, 20mM sodium dodecyl sulphate (SDS) and 5% methanol. This composition of the BGE was suitable for the separation and online enrichment using normal stacking - MEKC mode in CE analysis.

pH of the BGE was studied over the pH range (8 -11) taking into consideration the pKa values of the four analytes where 50% of these analytes are in the ionized form at the pKa value. The pH of 10.2 was found optimum in terms of separation, migration time and peak areas of the analytes. So, this pH was set as optimum and used in further experiments. Next, the effect of separation voltage on the separation of the four APs was investigated and it was found that higher voltages (e.g +25kV) offered faster separation and sharper peak shape. However, as the separation voltage increased, the baseline became less stable. Under the consideration of both separation time and baseline, +20 kV was chosen as the optimum separation voltage. The cassette temperature was also studied and the temperature of 22^{o}C was found optimum in terms of separation and migration time. Pressure sample

injection was used and the sample was introduced to the capillary at 50 mbar for 5 seconds. Signal collection was performed at 194 nm.

Normal stacking - micellar electrokinetic chromatography (MEKC)

In this part of study; stacking in the MEKC mode was used. Figure 4.18 shows schematic diagrams of the normal stacking-MEKC separation when SDS is used as surfactant. In normal stacking MEKC, the sample should be dissolved in a low-conductivity buffer or water, which was the case in our study as the analytes (alkylphenols) were prepared in water while the optimized BGE contained 7.0mM sodium borate, 5% MeOH and 20mM SDS to form the micelles. After the BGE and sample solutions were injected, respectively, a positive voltage was applied. Meanwhile, the SDS micelles from the inlet enter the sample zone (since the mobility of EOF is greater than that of the SDS micelles), and then carry the analytes to migrate. Once the SDS-analytes reach the boundaries between the sample zone and the BGE, sample focusing occurs. Following this, the SDS-analytes are separated by the MEKC mode.

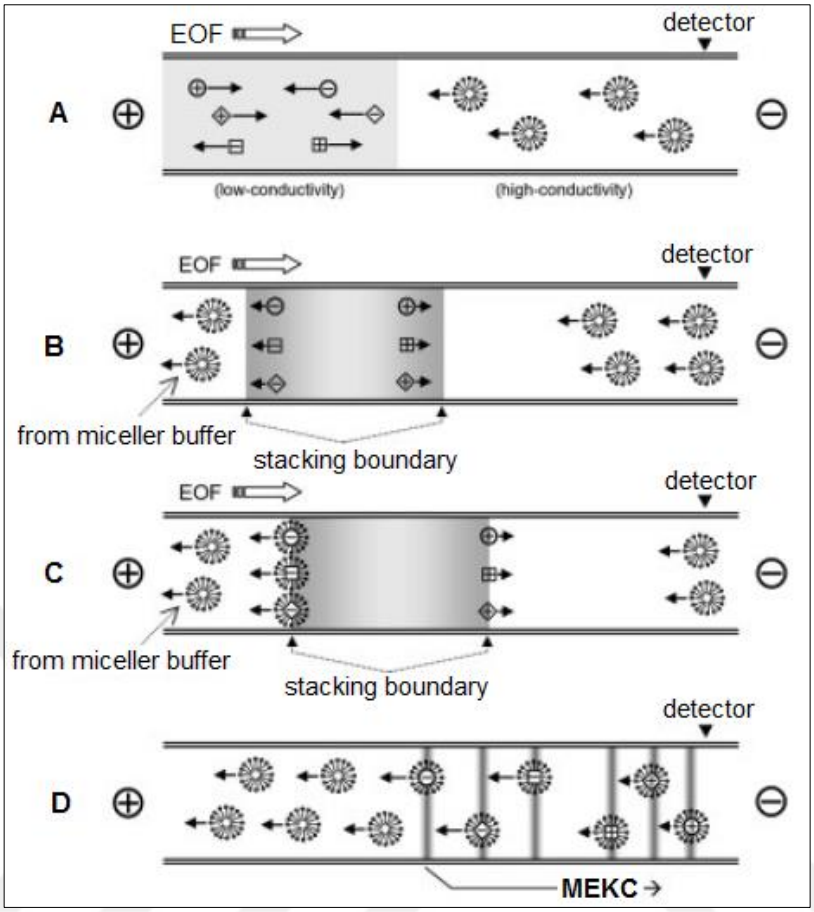


Figure 4.18. Schematic diagrams of a normal-stacking MEKC model.

Calibration curves, linearity and LODs

Calibration curves for the four analytes (BHM, BPA, TBP and NP) were constructed with concentrations ranging between 100 – 220 ng mL^{-1} and using the optimized pressure injection (5s). Each run was done in triplicates. The calibration curves based on peak areas and calibration equations, and coefficients of determination (R^2) for each of the four analytes are shown in Figure 4.19

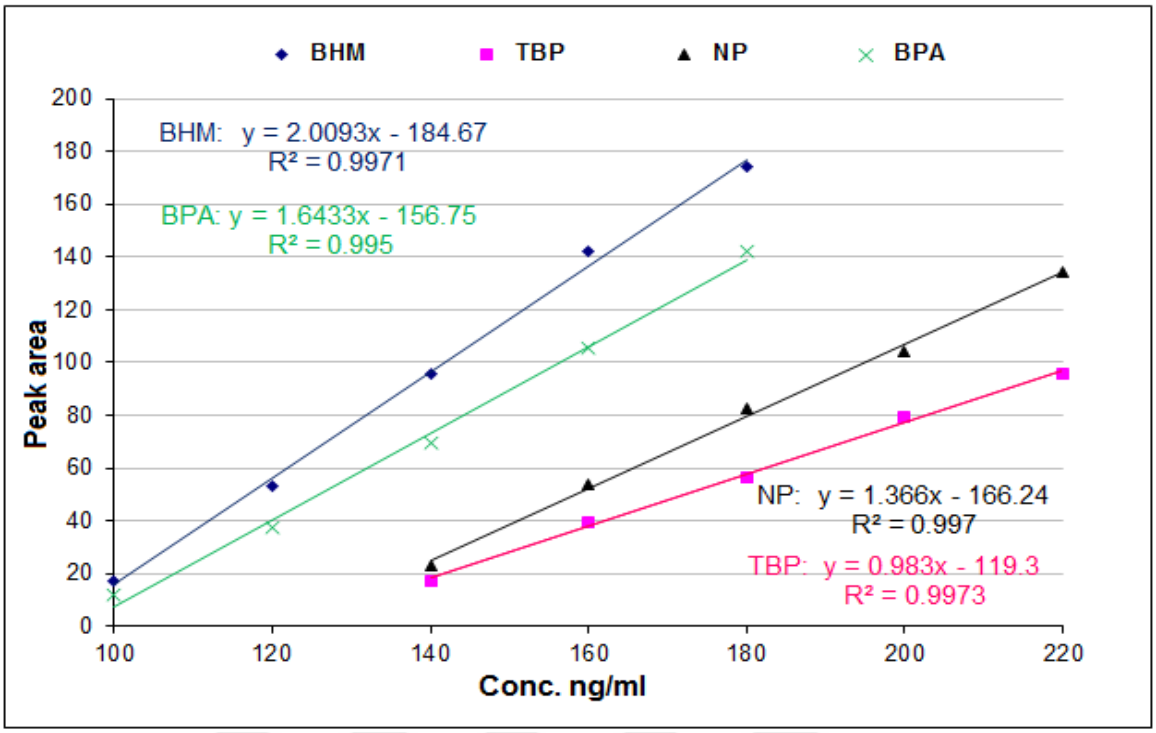


Figure 4.19. Calibration curves of the four APs analytes in milk samples.

Analytical figures of merit obtained for the developed method including LODs calculated at S/N = 3, LOQs and linear ranges of the four analytes are shown in Table 4.4.

Table 4.4: Analytical figures of merit of the developed method for APs.

Analyte	Linear Range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
BHM	100 - 180	25.6	85.3
BPA	100 – 180	29.7	99.0
TBP	140 – 220	48.1	160.3
NP	140 - 220	40.2	134.0

Figure 4.20 shows a comparison between two electropherograms obtained using the optimum extraction and analysis conditions and parameters. In a) a blank milk sample (not spiked) was passed through all the optimized extraction procedures and injected into the CE system. No peaks obtained in the corresponding electropherogram. In b) a spiked milk sample (5.0 μgL^{-1} BHM, BPA and 10 μgL^{-1} NP,

TBP) undergone all the developed pretreatment and extraction procedures and injected into the CE using the same analysis conditions and parameters used for the blank sample. The resultant electropherogram showed 4 peaks for the 4 target analytes. An investigation study for peak identification was performed and sequence and migration time of each peak was detected accordingly as can be seen in Figure 4.20.

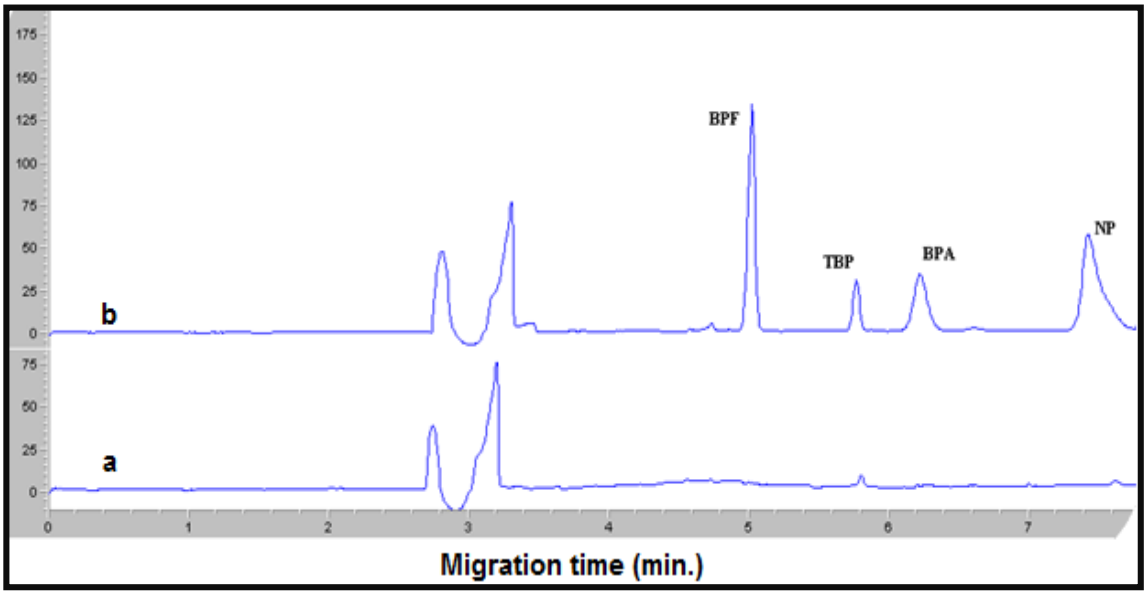


Figure 4.20. Electropherogram (a) Blank and (b) spiked milk sample (5 µg/mL BPF and BPA and 10 µg/mL NP and TBP)

4.2.4. Comparison with other preconcentration methods

To evaluate the DLLME-MEKC method developed in this study, it was compared with other preconcentration methods used for the determination of alkylphenols (BHM, BPA, TBP and NP or some of them) obtained in other published studies. Performing the comparison, different offline and online preconcentration methods combined with different analysis and detection techniques used for extraction and determination of alkylphenols were considered and the obtained results were compared in terms of LOD, linearity, RSD%, volume of extraction solvent and extraction time. As can be seen in Table 4.5., the present method is much simpler than the other extraction and analysis methods. Unfortunately, we couldn't find any

source of information about the analysis of alkylphenols in bottled milk samples using ME with CE. So, we compared the present method with some available information in literature that is close to our purpose of study.

This method had comparable RSD values with the compared methods and it provided acceptable LOD values and good linear ranges without the need to sophisticated and expensive extraction devices as most other methods use SPE kits or columns for packing and cleaning up samples and sometimes requiring derivatization reagents, which may complicate the extraction process and extend the extraction time. Also, while most of the other methods used more sensitive detectors such as FLD or MS which are expensive and are not affordable by many laboratories, we used the CE with UV detection.

Table 4.5. Comparison with other preconcentration methods.

Pre-concentration Method					
		SPE-MEKC	**VALLME[a]**	**LLE/SPE**	**DLLME- MEKC**
Sample type		Groundwater	Wastewater	Human milk	Bottled milk
Detection System		CE-UV	HPLC-FLD[b]	GC-MS	CE-UV
V_{ES}[c] (µL)		NA[d]	50	100	150
$T_{Extraction}$ (min)		NA	5	>40	21
LOD (ng/ml)	BHM	NA	NA	NA	25.6
	BPA	9.1	0.02	NA	29.7
	TBP	33.0	NA	NA	48.1
	NP	89.0	0.07	0.3 ng/g	40.2
Linear Range (ng/ml)	BHM	200–500	NA	NA	100-140
	BPA	200–500	0.05–100	NA	100-140
	TBP	200–500	NA	NA	140-220
	NP	200–500	0.50–100	1.7-1.6 ng/g	140-220
RSD (%)	BHM	NA	NA	NA	2.4
	BPA	2.02	2.0	NA	3.6
	TBP	1.11	NA	NA	5.0
	NP	3.26	7.0	7.0	4.7
Reference		(Li et al., 2005)	(Yiantzi, Psillakis, Tyrovola, & Kalogerakis, 2010)	(W. C. Lin, Wang, Cheng, & Ding, 2009)	This study

[a] Vortex assisted liquid-liquid microextraction
[b] Fluorescence detector
[c] Volume of extraction solvent
[d] Not available

4.3. Triphenylmethane Dyes

Triphenylmethane dyes, malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV) are antibacterial, antifungal and antiparasitic agents used for treatment of diseases in fish. There have been many reports of the inappropriate use of MG and CV as veterinary drugs. They are readily absorbed into fish tissue from water exposure, and are reduced metabolically by fish to leucomalachite green (LMG) and leucocrystal violet (LCV).

MG and CV have been banned for use as fungicides and antiseptics in aquaculture and fisheries because of their carcinogenic and mutagenic properties. Thus, it is necessary to develop a sensitive, rapid, inexpensive, and reliable method for the determination of MG, CV, and their leucometabolites in aquatic products. Over the years, an increasing number of analytical techniques such as spectrophotometry, high performance liquid chromatography (HPLC), capillary electrophoresis Raman spectroscopy (CE-RS) (Tsai, Lin, & Lin, 2007), liquid chromatography tandem mass spectrometry (LC/MSn) and RNA-Aptamer–based assay has been adapted for the detection of MG in various matrices. Although the aforementioned techniques are considered as routine MG detection techniques, they are time-consuming and require complicated sample pretreatment in addition to extraction, pre-concentration, derivatization, etc.). In this regard, a rapid, reliable and sensitive technique for TPM dyes identification in fish samples would be more beneficial (Fang et al., 2016).

In this study, a novel method of determination of these analytes in fish was developed. In the developed method, fish sample pretreatment followed by freezing assisted microextraction procedures combined with pseudo-isotachophoresis (p-ITP) as a stacking technique in CE analysis. In the following sections, the method and optimization of different parameters is discussed in detail.

4.3.1. Sample pretreatment and microextraction procedures

Sample extraction is always a crucial step in residue analysis, because the matrix of fish samples is very complicated. Fish samples were cut into small pieces then homogenized using the household blender, in order to improve the extraction efficiency by increasing the sample-extractant solvents interaction. Acetonitrile – ammonium acetate solution is commonly used in extraction procedures of triphenyl methane dyes from fish samples. Therefore, acetonitrile–ammonium acetate buffer was selected as the extraction solvent of extracting MG, CV, LMG, and LCV from the homogenized fish samples. Addition of NaCl was used as a salting out agent, which led to the improvement of extraction recoveries. The freezing step had a

crucial role of getting off fat contents of the sample, as both aqueous phase and fat contents freezes in the freezer temperature (-20 ^{o}C), pushing out the analytes to ACN phase which has the freezing point (-45 ^{o}C) and thus in liquid phase during this step. The fat contents appeared as a dense freeze white colored in the interface between water and ACN. During the freezing step, only the ACN phase (upper liquid phase) became colored, meaning that the TPM dye analytes are present/concentrated in this phase. Although the volume of ACN (the upper blueish colored phase) resulted from the freezing step was ranging from 2.2- 2.8 mL, only 2 mL were collected and transferred into a plastic eppendorf to be used in further extraction/purification procedures and this was done to avoid any contamination during collecting the ACN phase from the extraction tube. To preconcentrate and purify the collected ACN-analytes (in plastic eppendorf), evaporation to dryness under argon gas and using sample concentrator and heating blocks was performed. To the dried residue at the bottom of the plastic eppendorf, 0.5 mL ACN and 0.5 mL saturated solution of NaCl (makes total of 1.0 mL) were. Again, the eppendorf was left in in freezer (-20 ^{o}C, 5 minutes) and two phases were formed. At this stage, it was very clear the analytes were concentrated in the upper phase (ACN) as this phase became dark blue. 250µL of the upper phase was transferred into a CE glass vial followed by addition of 250 µL ACN, 150 µL of 0.25M formic acid and 350 µL DI water (total volume of 1.0 mL). This was the sample injected in CE for analysis.

4.3.2. Optimization of CE conditions and analysis parameters

Wavelengths of CE determination

As the spectral characteristics of the analytes investigated in this part of the study were different and thus the wavelengths of maximum absorbance were in different ranges. Taking into consideration the wavelength range of detection of the CE UV/Vis detector (190 – 600nm) and the spectral profiles showing lambda max of the four analytes (Figure 4.21), three different wavelengths were examined in the CE analysis (214, 260 and 580nm). During analysis, electropherograms obtained using the wavelength 214nm showed the peaks of the four analytes with minimal noise and interferences. In the wavelength 260nm, there were many interference peaks

and bad resolution was obtained. In, the wavelength 580nm, only peaks of MG and CV were detected, while the peaks of leuco metabolites were not detected in this wavelength. Also, the peak areas of MG and CV obtained in the wavelength 580 were higher than those obtained using the wavelength 214nm. So, the two wavelengths 214nm and 580nm were used for determination in further experiments. Recording electropherograms instantly at different wavelengths could be obtained easily using the DAD detector.

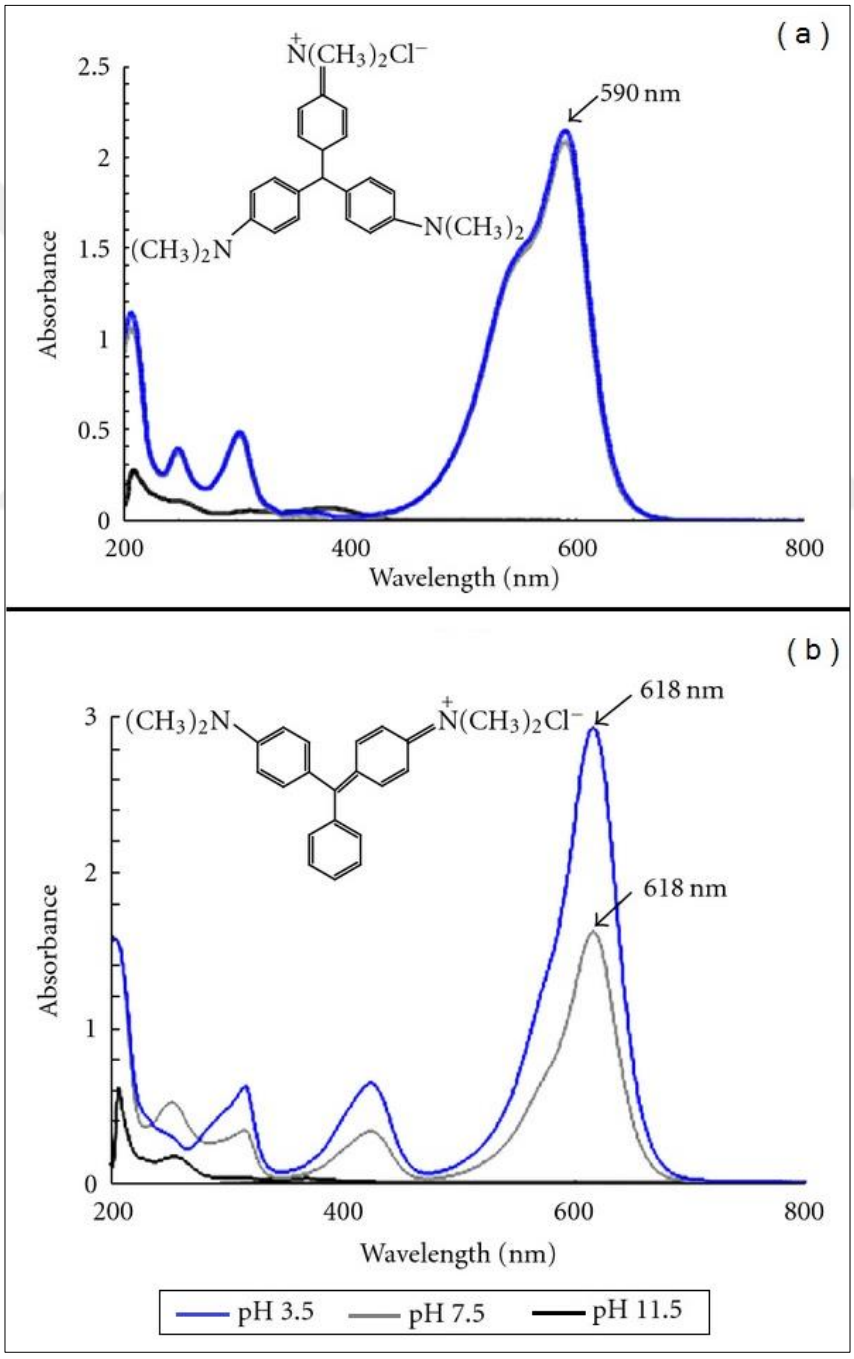


Figure 4.21. Spectra of (a) crystal violet and (b) malachite green.

BGE composition in FASI and FASS

The BGE used in TPM dyes CE analysis using FASI and FASS was composed of acetate buffer and acetonitrile as organic modifier. To obtain the best sensitivity and separation conditions in CE analysis, the optimum BGE composition was optimized. There are several factors affecting the efficiency of stacking. Among those the most crucial is the difference in conductivity between zones where analytes focusing occurs (Quirino & Terabe, 2000b). Taking this into account different concentrations of NaOAC in BGE in the range 20 – 80 mM were tested. As can be seen in Figure 4.22, by increasing NaOAC concentration, significant signal amplification was obtained up to 70mM. Higher concentrations than 70 mM were resulting in decrease in peak areas, baseline instability, current errors and analysis breakdowns. The value of 70 mM NaOAC was set as the optimum value and used in further experiments.

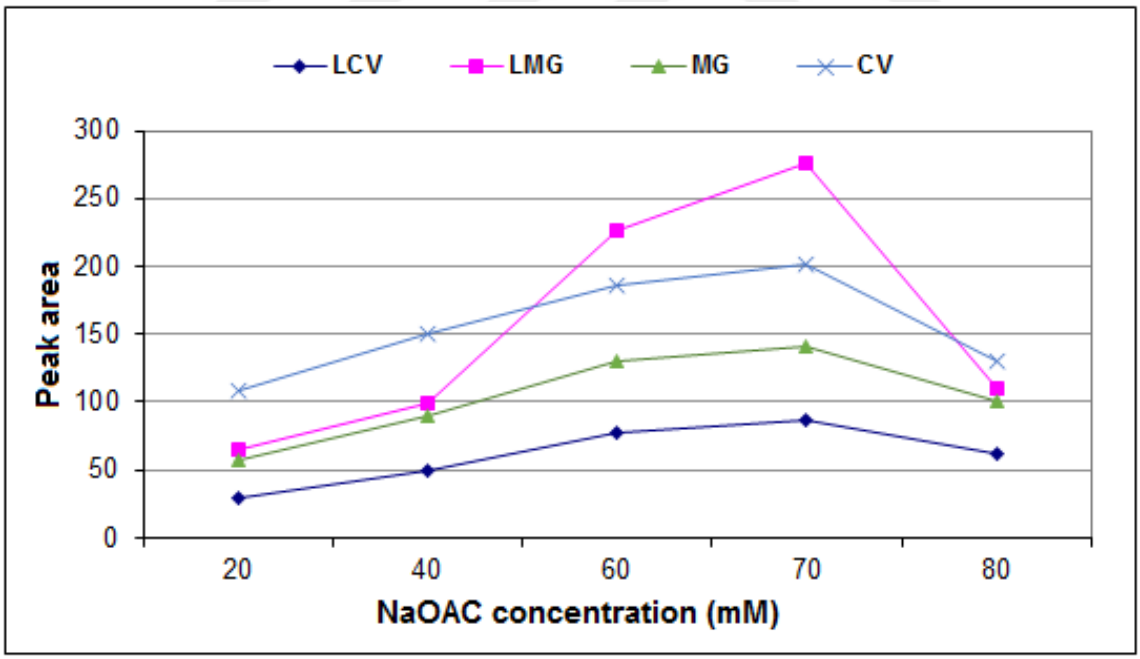


Figure 4.22: Effect of NaOAC concentration in the CE running buffer.

Effect of pH of BGE

In CE, it is extremely important to properly control pH since it affects analyte charge, electroosmotic flow (Figure 4.23), and, by affecting current, heat production. Thus, small changes in pH tend to have greater impact in CE than do comparable pH variations in HPLC. In electrophoretic separations of ionizable compounds (e.g. TPM dyes investigated in this study), pH plays an important role as it determines the extent of ionization of each individual analyte. Failure to properly control pH is one of the major causes of poor reproducibility in CE.

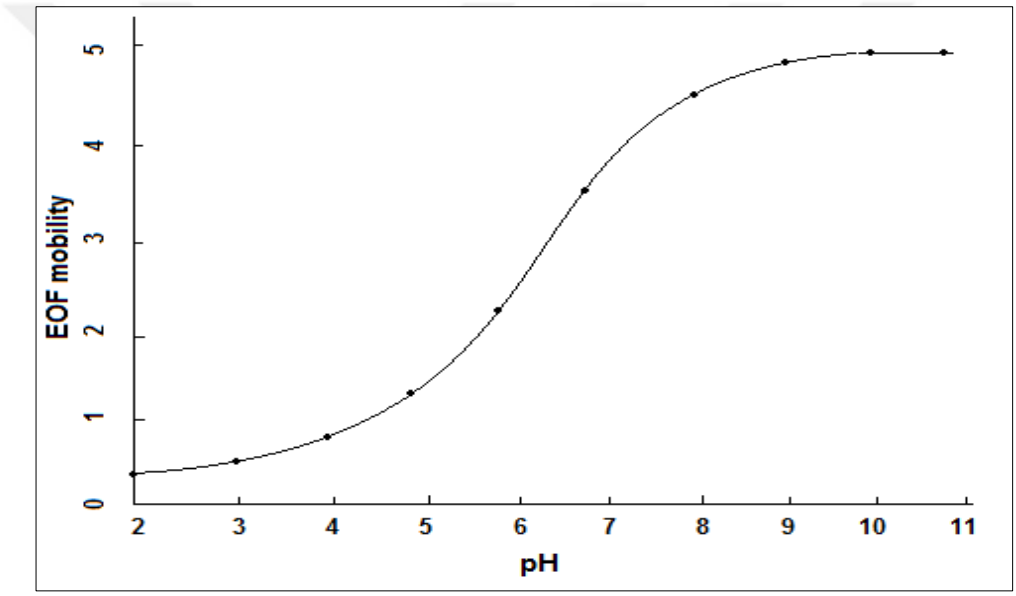


Figure 4.23. Variation of EOF mobility with changing pH for typical uncoated CE capillary (simulated data).

To study the effect of BGE pH on CE analysis of TPM dyes, BGE with different pHs (3.5, 4,0 and 4.5) were studied and the pH of 4.0 showed the highest peak areas for most of the analytes (Figure 4.24) and thus this pH value was set as the optimum value of BGE pH and used in further experiments. In choosing the pH range of BGE, pKa values of the studied analytes and BGE buffer range were considered. The pKa of MG and CV were 10.3 and 9.3, respectively. LMG and LCV are alkaline due to the presence of two basic amines, but there was no available information on their pKa values. Using aqueous electrolytes, the separation of these four analytes could not be achieved over a broad pH range. The pKa values for these compounds in

organic solvents can be significantly different from those in water. The pH value of the acetonitrile–buffer solution mainly influenced the resolution and the apparent mobility of the target analytes and the four analytes were protonated easily in acidic medium.

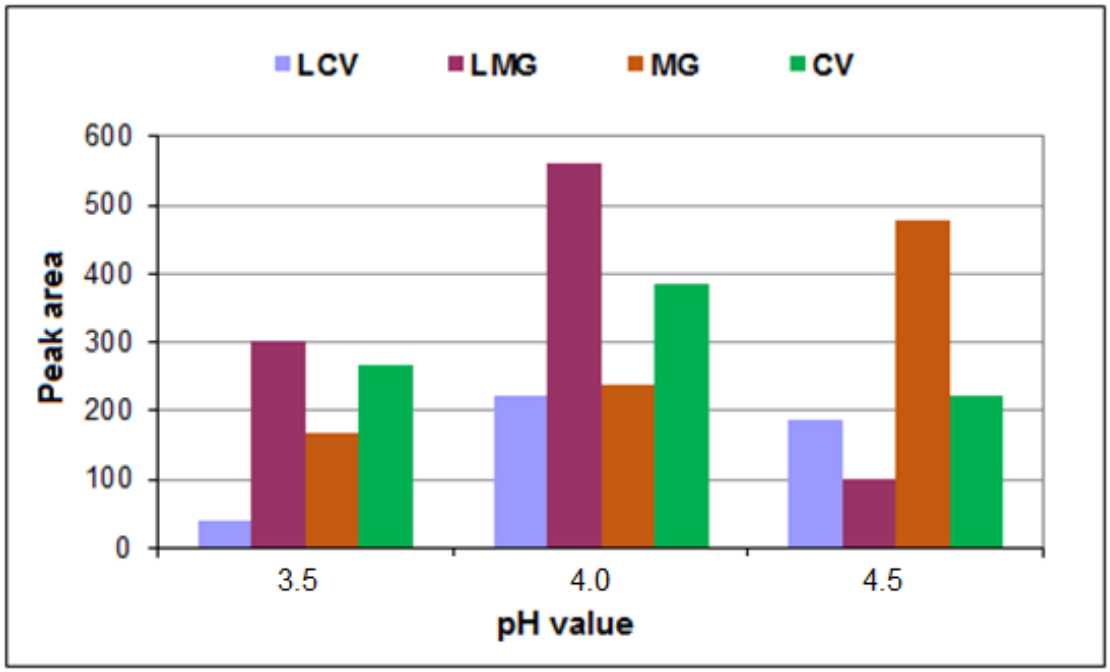


Figure 4.24. Effect of BGE pH on analysis sensitivity

Effect of acetonitrile content in BGE

Actually, the addition of acetonitrile to the BGE used in this part of the study, played an important role and was crucial for increasing the solubility of the analytes inside the capillary during the CE analysis, as the TPM dyes have limited solubility in aqueous phase. Acetonitrile content was tested as a background electrolyte BGE additive and modifier at concentrations ranging between 15-35%. As can be noted in Figure 4.25, peak areas of the four analytes increased gradually by increasing the ACN concentration and reached the highest level at the value of 25%, then started to decrease again after this value. This value was set as optimum and used in further experiments. The decrease in peak areas of the analytes after the optimum value was due to dilution effect.

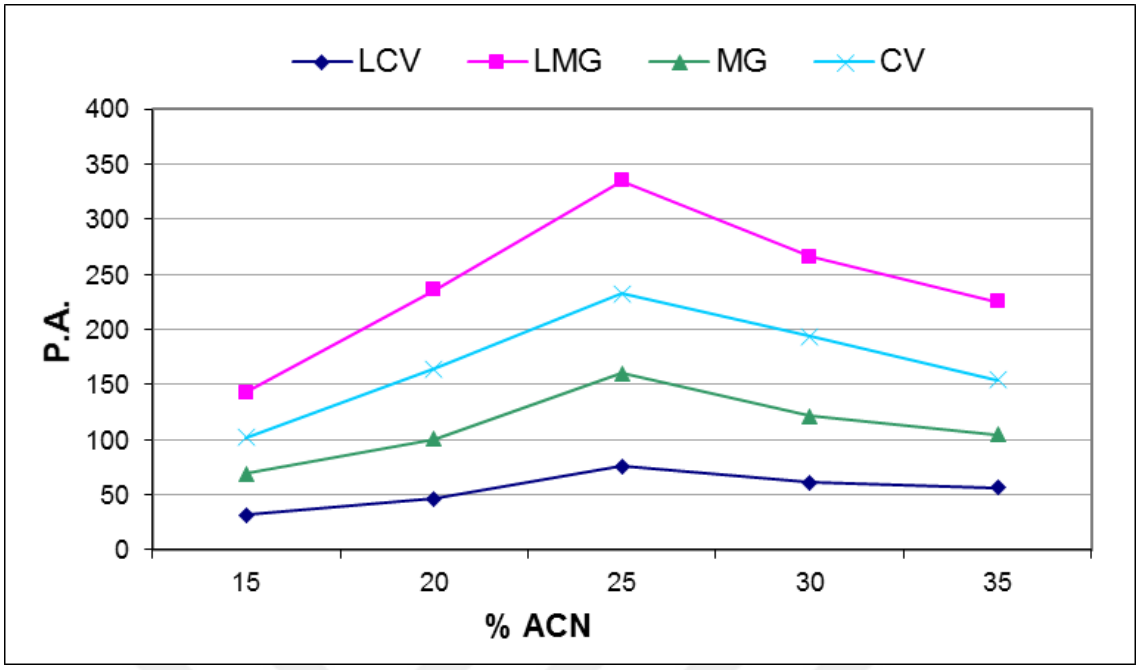


Figure 4.25. Optimization of ACN content in BGE.

FASI and pseudo-isotachophoresis (pITP)

Studies conducted in recent years have shown that application of on-line preconcentration techniques can make CE even more sensitive than LC. It reveals the potential of in-capillary preconcentration techniques. Standard stacking technique is based on focusing of analytes on the boundary between zone of different electric field strength. Both hydrodynamic and electrokinetic injection modes have been developed for stacking of analytes. FASI was found to be more sensitive than FASS technique. It was pointed out by Chien et al. that the number of sample ions injected under FASI conditions is not limited by the sample volume. Combination of FASI technique with other on-line preconcentration mechanisms like sweeping and/or ITP provided the most powerful in-capillary enrichment techniques (Dziomba, Kowalski, Slominska, & Baczek, 2014). The most crucial element of ITP optimization process is a proper choose of leading and terminating electrolyte. Shihabi reported a mode of ITP in which organic solvent acts as a terminating electrolyte (p-ITP) (Shihabi, 2006). It provides some advantages over traditional ITP like possibility of shortening of sample preparation step and lack of typical terminating ion. The p-ITP was reported to be useful in a number of analyses. In all reported papers, about 100-fold enhancement sensitivity was obtained, when

hydrodynamic injection was employed. The application of electrokinetic injection can successfully provide about 1100-fold sensitivity improvement. Zhang et al. found a synergistic effect of FASI and acid-induced p-ITP resulting in three orders of magnitude signal enhancement (Zhang, Zhang, Wang, & Zhang, 2008).

In this part of the study, a synergistic effect between FASI and p-ITP in capillary zone electrophoresis (CZE) has been evaluated. The method was developed and applied for the determination of TPM dyes (MG, CV, LMG and LCV in aquaculture fish samples after the novel DLLME method developed in this study which shortened sample preparation procedure and improved the sensitivity. In the present study, we have proposed the new combination of offline and online preconcentration methods to solve the problems of sample pretreatment, extraction, peconcentration and signal amplification when simple CZE-UV technique with classical extraction and stacking techniques are employed for quantification of TPM dyes in fish samples.

Concentration of formic acid in the sample

Presence of acid in analytical matrix in FASI technique can strongly enhance stacking of organic cations (Shihabi, 2005). Formic acid (HCOOH) was used to play this role in the present study as the target analytes (TPMs) become protonated at low pH values (acidic medium). Influence of different concentrations of HCOOH in the range of (10 – 30 mM) in the analytical matrix was tested and results are shown in Figure 4.26. As it can be noted even low concentrations of formic acid (10mM) improved preconcentration process. Further addition of the acid caused peaks deterioration which can be explained by increased the ionic strength of the analytical matrix. The greatest signal amplification effect and thus highest peak areas were observed at 20mM concentration of HCOOH. Generally, acid addition improved precision of injections. This can be explained by the stabilization of current during electrokinetic injection (Anres, Delaunay, Vial, Thormann, & Gareil, 2013).

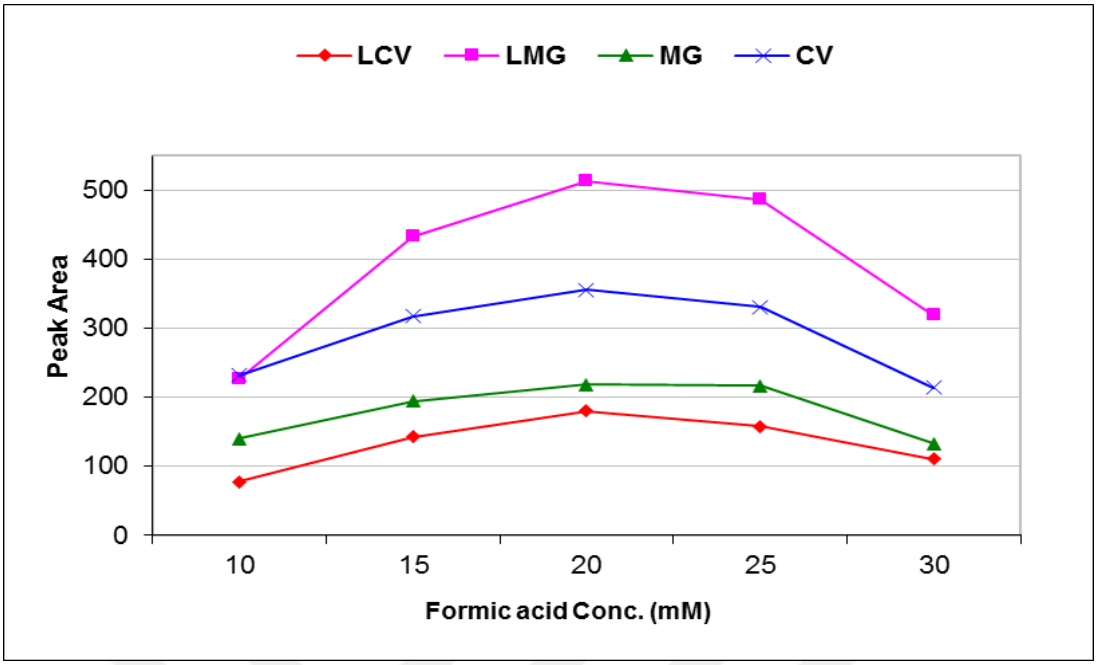
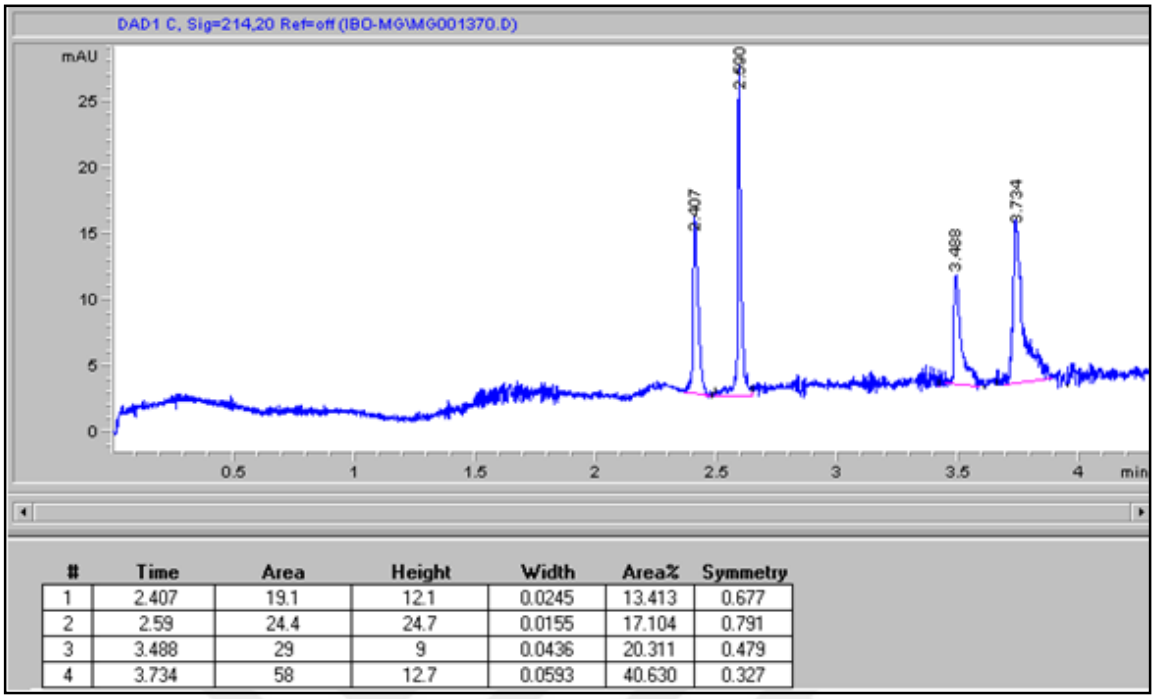


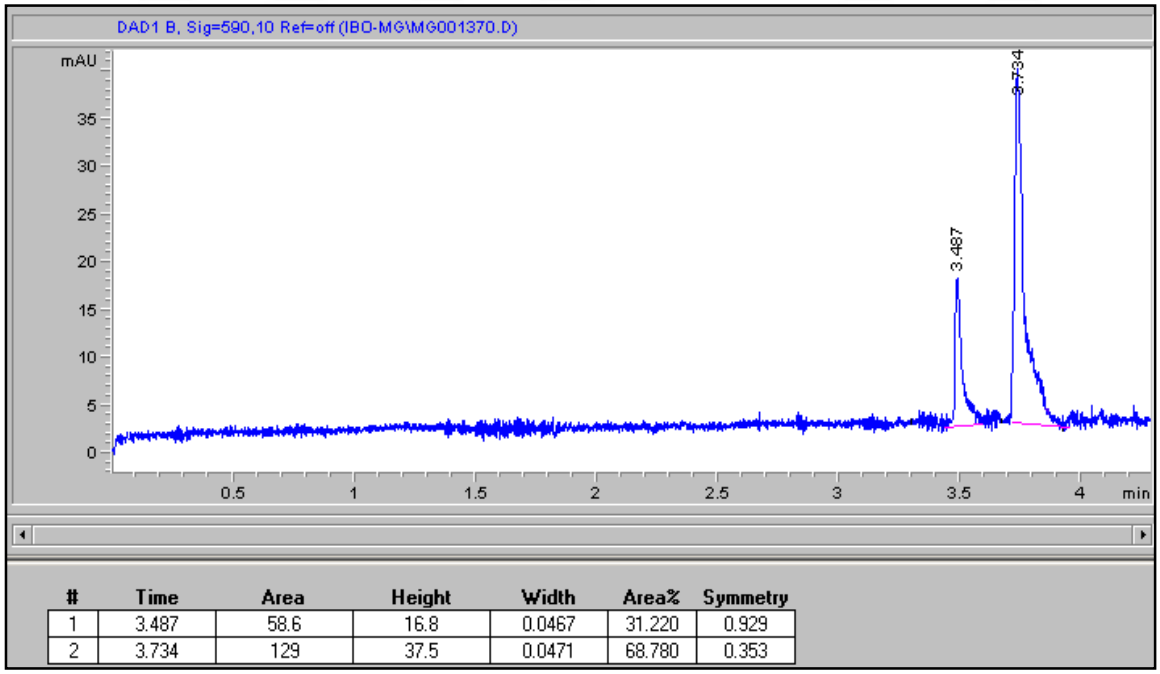
Figure 4.26. Effect of formic acid concentration in sample on analysis sensitivity

In Figure 4.27, there is a typical electropherogram of the four TPM dye analytes collected at 214 nm. The measurement was done following to the developed extraction procedures and the fish samples were spiked at 10 ng g^{-1}. Collecting the peaks at this wavelength enabled us to have the four peaks of the target analytes, but with lower peak areas for MG and CV originated from the low absorptivity of the two analytes at this wavelength. The electropherogram collected for the same sample at the wavelength 580 nm (Figure 4.28) under the same extraction and analysis conditions, only MG and CV were detected at this wavelength and appeared in the electropherogram but this time with larger peak areas for both, so for calculations and calibrations peak areas obtained at 580 nm were considered for MG and CV.



#	Time	Area	Height	Width	Area%	Symmetry
1	2.407	19.1	12.1	0.0245	13.413	0.677
2	2.59	24.4	24.7	0.0155	17.104	0.791
3	3.488	29	9	0.0436	20.311	0.479
4	3.734	58	12.7	0.0593	40.630	0.327

Figure 4.27. Typical electropherogram of spiked culture fish measured at 214nm, from left to right: LCV, LMG, MG and CV. Final concentration of 10ng/g



#	Time	Area	Height	Width	Area%	Symmetry
1	3.487	58.6	16.8	0.0467	31.220	0.929
2	3.734	129	37.5	0.0471	68.780	0.353

Figure 4.28. Typical electropherogram of spiked culture fish measured at 590nm, from left to right: MG and CV. Final concentration of 10ng/g

Calibration curves, linearity and LODs

Calibration curves for the four analytes (MG, CV, LMG and LCV) were constructed with concentrations ranging between 10 - 100 ng/g and using the optimized electrokinetic injection (12kV, 70s). Each run was done in triplicates. The calibration curves based on peak areas, calibration equations, and coefficients of determination (R^2) of the four analytes are shown in Figure 4.29

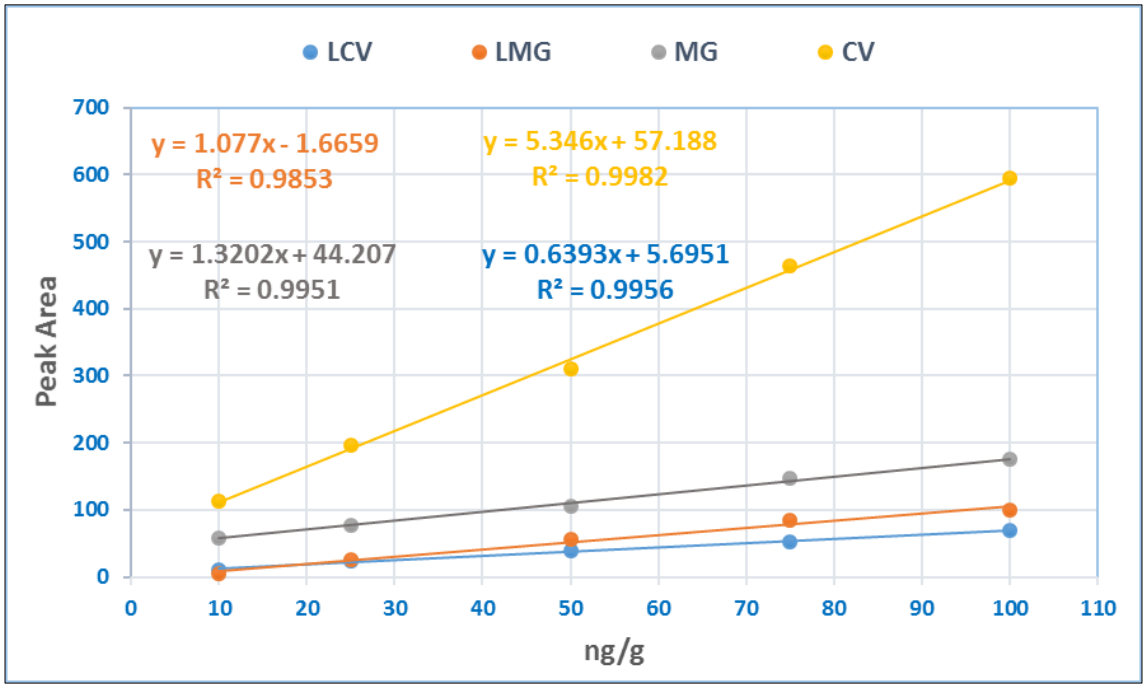


Figure 4.29. Calibration curves of the four analytes in real fish samples.

In addition, the analytical figures of merit of the developed method including LODs at S/N = 3, LOQs and linear ranges of the four analytes are shown in Table 4.6.

Table 4.6. Analytical figures of merit for TPM method.

Analyte	Linear Range (ng/g)	LOD (ng/g)	LOQ (ng/g)
MG	10 – 100	2.3	7.6
CV	10 – 100	1.0	3.3
LMG	10 – 100	1.6	5.3
LCV	10 – 100	3.2	10.0

To evaluate the efficiency of the developed method, recoveries of the four TPM dye analytes are calculated after the addition of a known amount of analyte to fish samples (sample fortification) at different concentration levels (20, 60 and 90 ng/g) and then the percent recoveries of each of the analytes added was detected at the above mention concentration levels. The results are shown in Table 4.7 and the mean recoveries were ranging between 81 – 98 %.

Table 4.7. Relative recoveries of the MG, CV, LMG and LCV in fish samples spiked at three concentration levels for each analyte.

Sample	**Analyte**	**Recovery (%)**			**Mean Recovery (%)**
		20 ng/g	60 ng/g	90 ng/g	
Çipura	MG	86	84	88	86
	CV	80	81	83	81
	LMG	103	95	97	98
	LCV	96	88	91	91
Somon	MG	94	91	93	93
	CV	96	93	95	95
	LMG	99	96	100	98
	LCV	101	98	98	99
Alabalık	MG	83	81	81	82
	CV	80	84	83	82
	LMG	88	90	92	90
	LCV	95	93	92	93

To estimate the precision of the recovery measurements, RSD values were calculated on inter-day and intraday basis and the results indicated that RSD values in the range of 1.1 – 3.8 for intraday and 3.2 – 8.7 for inter-day were obtained for the four investigated analytes (Table 4.8).

Table 4.8. Intra- and inter-day precision as relative standard deviation (RSD).

Analyte	RSD %					
	Inter-day (n=3)			Intraday (n=3)		
	20 ng/g	60 ng/g	90 ng/g	20 ng/g	60 ng/g	90 ng/g
MG	4.0	3.8	3.2	2.5	0.8	1.3
CV	4.1	3.7	3.9	2.8	1.0	1.1
LMG	5.1	5.2	4.2	3.4	2.6	2.0
LCV	8.7	6.8	4.1	3.8	3.4	2.7

4.3.3. Comparison with other preconcentration methods

To evaluate the LLME-FASI-pITP method developed in this study, it was compared with other preconcentration methods used for the determination of Triphenyl methane dyes (MG, LMG, CV and LCV) obtained in other studies using different offline and online preconcentration methods in terms of LOD, linearity, RSD%, volume of extraction solvent and extraction time. As can be seen in Table 4.9., the present method is most importantly much faster and simpler than the other extraction and analysis methods and can be considered more robust and handy.

This method had comparable RSD values with the other methods and it provided acceptable LOD values and good linear ranges without the need to sophisticated and expensive extraction devices or derivatization reagents, which may complicate the extraction process and extend the extraction time. Also, there was no need for applying more sensitive detectors such as MS which are expensive and are not affordable by many laboratories. One more important advantage of this method over the other compared methods is the relatively minimal usage of organic solvents as the other methods used relatively large and in some cases even incomparable amounts of organic solvents for cleaning up and/or extraction procedures, showing the environmental friendly character of our method between the other available methods. In short, the developed method proved to be simple, rapid, inexpensive and easy to apply.

Table 4.9. Comparison between the developed method and other preconcentration methods

Pre-concentration Method					
		SLE[a]	**FASI-MEKC**	**ASE[b]**	**LLME-FASI-pITP**
Sample type		Fish	Fish	Fish	Fish
Detection System		LC-MS/MS	CE-UV	CE-UV	CE-UV
V_{ES}[d] (µL)		1000	2000	NA[c]	250
$T_{Extraction}$ (min)		30	>60	>60	20
LOD (ng/g)	MG	0.43	70 µg/ml	0.39 µg/ml	2.3
	LMG	0.24	50	0.08	1,6
	CV	0.33	40	0.29	1.0
	LCV	0.28	50	0.10	3.2
Linear Range (ng/g)	MG	NA	0.14 – 44 µg/ml	1.32–300 µg/ml	10 – 100
	LMG	NA	0.14 - 44	0.27–100	10 – 100
	CV	NA	0.1 - 32	0.98–100	10 – 100
	LCV	NA	0.21 - 68	0.34–100	10 – 100
RSD (%)	MG	12	< 6.05	3.4	4.0
	LMG	15		1.9	5.1
	CV	6		1.6	4.1
	LCV	12		1.7	8.7
Reference		(Kaplan, Olgun, & Karaoglu, 2014)	(Ting-Fu Jiang et al., 2011)	(H. W. Sun & Qi, 2013)	This study

[a] Solid-liquid extraction
[b] Accelerated solvent extraction
[c] Not available
[d] Volume of extraction solvent

5. CONCLUSIONS

In the first part of this study, a novel combination of dispersive liquid-liquid microextraction based on solidification of organic drop (DLLME-SFOD) and Field amplified sample stacking (FASS) in capillary electrophoresis was successfully carried out for preconcentration and determination of bisphenol A in different water and human urine samples. Factors affecting the microextraction efficiency and CE analysis were systematically investigated and optimized. Under optimum conditions, this method gave an LOD at the μgL^{-1} level due to the high improvement factor obtained. Compared to conventional capillary zone electrophoresis (CZE), the proposed method provided high sensitivity, with a lower LOD by 1,200 times. Highly reproducible and interference-free electropherograms were obtained in the analysis of water and urine samples, indicating that the developed method has potential applicability in the determination of this target analyte in genuine samples. Although the absolute recoveries were not very high in urine samples, good recoveries (> 98%) were achieved with matrix-matched standards. Due to its simplicity, low cost, low volume of organic solvent requirement, high improvement factors and compatibility with CE, the proposed method can be used for preconcentration and determination of a variety of organic compounds in these matrices. The BPA peak appeared generally before 9 minutes with a relatively good retention time precision calculated for three repetitions (%RSD of retention time = 0.1). The developed CE analysis procedures for BPA gave a high performance in terms of separation efficiencie (N ≈ 468 000).

In the second part of the study, new procedures to determine alkylphenols in water and urine samples were developed. In the new developed method, sample pre-treatment (clean-up) before microextraction procedures for milk samples proved to be very effective, since milk is a complex matrix. Milk samples were treated with phosphoric acid and centrifuged in order to precipitate the proteins. Sodium chloride also was added to enhance phase separation and salting out effect. The developed DLLME-CE method was applied to determine Alkylphenols in milk samples and the detection limits of 25.6, 29.7, 48.1 and 40.2 ng/mL for BPF, BPA, TBP and NP respectively were obtained with a precision (% RSD) lower than 5%. A separation

time of less than 9 minutes was typically achieved for the four APs with a good retention time precision as the %RSD of retention time for the APs mixtures was in the range of (0.09 – 0.44). The developed CE analysis procedures gave a high performance in terms of separation efficiencies (N ≈ 218 000, 83 500, 62 300, and 72 600 for BPF, BPA, TBP and NP respectively).

In the third and last part of this study, a sequential preconcentration technique of field amplified sample injection (FASI) induced by acetonitrile (ACN) and pseudo isotachophoresis (pITP) – acid stacking (AS) for separation and determination of Triphenylmethane dyes in fish sample solution was developed and optimized. Firstly, an excessive amount of sample solution containing 50% acetonitrile was injected electrokinetically and the analytes were concentrated by FASI. Then the injected sample zone was narrowed effectively by pseudo ITP–AS. Finally, the concentrated sample zone was separated by capillary zone electrophoresis (CZE). The proposed method for the determination of TPM dyes in culture fish could effectively separate and simultaneously determine MG, CV, LMG, and LCV, which have similar structures. The developed extraction procedures proved to be an effective tool for the extraction of the four compounds in culture fish without using special apparatus like accelerated solvent extraction or solid phase extraction. In addition, the freezing step in the extraction procedures proved to be very effective to get rid of fatty contents of the fish samples. This method can be very suitable for sensitive, rapid, inexpensive, and reliable determination of TPM dyes in culture fish. Capillary electrophoresis (CE) has the advantages of low solvent consumption and low cost. Capillary zone electrophoresis (CZE) has been primarily carried out using an aqueous electrolyte buffer. Using the p-ITP technique it was possible to separate and online concentrate the four analytes during the stacking step. A separation time of less than 4 minutes was typically achieved for the four TPMs with a relatively good retention time precision as the %RSD of retention time for the APs mixtures was in the range of (4.07 – 6.24). The developed CE analysis procedures provided a high performance in terms of separation efficiencies (N ≈ 69 000, 84 500, 75 000, and 55 000 for LCV, LMG, MG and CV respectively). In terms of resolution, for the four consequent peaks, resolution was calculated as higher than 4, meaning that the peaks are well separated.

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CURRICULUM VITAE

Personal Information

Date of Birth	: 23.01.1976
Nationality	: Palestinian
Place of Birth	: Deir Al Balah (Gaza Strip – Palestine)
Marital Status	: Married
Country of Residence	: Turkey
Telephone Number	: +90 553 232 61 09
e-mail Address	: ibolubbad@gmail.com
Mother Tongue	: Arabic
Other Languages	: 1) English (Fluent) 2) Turkish (Fluent) – Level C1 Diploma

Education

Degree	University	Date of Graduation
PhD in Analytical Chemistry	Gazi University (Ankara – Turkey) / Department of Analytical Chemistry – Faculty of Pharmacy	Continuing
Master of Science (MSc)	Middle East Technical University – METU (Ankara –Turkey) / Department of Chemistry – Faculty of Art and Science	2002
Bachelor of Science (BSc)	Al-Azhar University-Gaza, Palestine / Department of Pure Chemistry, Faculty of Science	1997
High School (Science Branch)	Al Manfalusti High School / Deir Albalah, Gaza Strip, Palestine	1993

Work Experience

Year	Work Place	Task
2016 – Present	UNICEF–Turkey, Ankara.	Non-formal Education Consultant
2011 – 2016	British Embassy School Ankara (BESA)	Science Teaching (Part time)
2012 – 2016	Freelance Translator	Freelance Translator
2010 – 2011	Faculty of Science, Islamic University of Gaza	Assistant of Vice Dean for Laboratory Affairs
2008 – 2011	Faculty of Science, Department of Chemistry, Islamic University of Gaza – Palestine	Instructor (Full Time)
2007 – 2008	Faculty of Science, Department of Chemistry, Islamic University of Gaza – Palestine	Teaching assistant
2006 – 2008	Palestinian Water Authority (PWA), Water Resources Directorate, Gaza – Palestine.	Hydrochemistry Expert
2006 – 2006	Water Quality Laboratory (JCP – UNRWA), Gaza – Palestine	Water Specialist
2004 – 2005	International Technical and Trading Co. (ITT), Abu Dhabi Fertilizers (ADFERT), Antalya-Turkey	Deputy General Manager
2002 – 2004	nternational Technical and Trading Co. (ITT), Abu Dhabi Fertilizers (ADFERT), Antalya-Turkey	Technical Manager
1997 – 1999	Palestinian Red Crescent – Middle Governorate Branch Deir Al Balah, Gaza Strip, Palestine.	Youth Director

Scientific Practical Skills

- Calibration and operation of analytical instruments:
  - ✓ Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES),
  - ✓ Flame and Graphite Furnace AAS,
  - ✓ High Performance Liquid Chromatography (HPLC),
  - ✓ Capillary Electrophoresis (CE),
  - ✓ Atomic Fluorescence Spectroscopy (AFS), and
  - ✓ Common Analytical Techniques.
- Collection/ pretreatment of different types of samples for Chemical Analysis.
- In environmental research: sampling (Water, Soil and Air), analysis, receptor modeling, statistical methods and data interpretation.

Conference Papers & Workshops

- Ibrahim Lubbad, UsamaAlshana, IsmetCok, Nilgun G. Goger, Ugur Tamer, NusretErtas, 2011. Solidification of organic drop microextraction combined with capillary electrophoresis for preconcentration and determination of bisphenolA in water samples, *The 7th International Conference on Instrumental Methods of Analysis, Modern Trends and Applications Chania Crete, GREECE*.

- Us,M.F., Alshana,U., Lubbad,I., Göğer,N.G., Ertaş,N., 2012. A Novel Combination of Dispersive Liquid-Liquid Microextraction Based on Solidification of Floating Organic Drop with Field-Amplified Sample Injection Capillary Electrophoresis for Preconcentration and Determination of Beta (2)-Agonists in Bovine Urine, *VI. UlusalAnalitikKimyaKongresi HATAY.*

- Alshana,U., Lubbad,I., Göğer,N.G., Çok,I., Tamer,U., Ertaş,N., 2012. Dispersive Liquid-Liquid Microextraction Based on Solidification of Floating Organic Drop Combined with Counter-Electroosmotic Flow Normal Stacking Mode in Capillary Electrophoresis for the Determination of Bisphenol A in Water and Urine Samples, *VI. Ulusal Analitik Kimya Kongresi HATAY.*

- Ibrahim LUBBAD, Usama Al SHANA, Nusret ERTAS, combining dispersive liquid-liquid microextraction with capillary electrophoresis to determine alkylphenols in bottled milk. *XIII UlusalSpektroskopiKongresi, 15-18 Mayis, 2013, Burdur, Turkiye.*
- LUBBAD Ibrahim, TUNCEL S., Al Agha O., Chemical Composition of Aerosols over Öludeniz Region. *Colloquium Spectropicum International XXXII.*

- Ibrahim Lubbad, Semra G. Tuncel, Chemical Composition and Source Determination of Aerosols in Öludeniz Region (Fethiye- Turkey). *"Aegean Analytical Chemistry Days"*, (09/2002), p.101. Kusadasi, Aydin, Turkey.

- LUBBAD Ibrahim, TUNCEL S., Composition of aerosols and Atmospheric Fluxes of Pollutants over Oludeniz Region. (2nd International Symposium on Air Quality Management, Istanbul, Turkey, September 2001.)

- Workshop (EUROTRAC TOR -2 Workshop Program, Ankara, Turkey, September 2001.
- Conference of (Better Environment – Aqua Abu Dhabi 2000)

JOURNAL PAPERS

Dispersive Liquid-Liquid microextraction based on Solidification of floating organic drop combined with counter-electroosmotic flow normal stacking mode in capillary electrophoresis for the determination of Bisphenol A in water and urine samples**.**
U Alshana, I Lubbad, NG Göğer, İ Çok, U Tamer, N Ertaş, Journal of Liquid Chromatography & Related Technologie, 12/2013; 36(20):2855-2870. DOI:10.1080/10826076.2012.725700.

Dispersive liquid–liquid microextraction based on solidification of floating organic drop combined with field-amplified sample injection in capillary electrophoresis for the determination of beta (2)-agonists in bovine urine.
MF Us, U Alshana, I Lubbad, NG Göğer, N Ertaş, Electrophoresis 34 (6), 854-861. DOI:10.1002/elps.201200348.

ATTENDED CERTIFIED COURSES

- Ethics and Integrity at UNICEF
- Prevention of Sexual Harassment and Abuse of Authority
- UN Human Rights and Responsibilities
- Results-Based Management (RBM) e-course
- Basic Security in the Field I and II (BSITF I&II)
- Harmonized Approach to Cash Transfers (HACT)
- Funding Authorization and Certificate of Expenditure (FACE) Form
- Instructional Design (30 hours)
- e – Learning (40 hours)
- Basic Child Protection Training Course.
- Child Protection Awareness in Education V2 (Level 2 Programme NSPCC).
- Paediatric First Aid (40 hours).
- Academy of International Students (Siyasallilar Vakfi & SETA Vakfi - Ankara).
  - ✓ Leadership.
  - ✓ International Relationships.
  - ✓ Islamic History and Ottoman Empire.
- Cultural and civil rehabilitation course (Summer Camp - 2 months).
- Basic Food Hygiene Training Course.
- Strategic Planning of Water and Wastewater Sectors.
- Wastewater Networks (40 hours).
- Geographic Information System (GIS)for operation and management of water and wastewater sectors.
- Statistical Package for Social Sciences (SPSS).
- Turkish Language Certificate (Level C1) (TÖMER – Ankara University).

AWARDS AND MEMBERSHIPS

- Republic of Turkey Prime Ministry – Turkey Scholarships, PhD Scholarship for International Students, 2011 – 2015.
- Associate Member of Royal Society of Chemistry (AMRSC)

GAZİLİ OLMAK AYRICALIKTIR