**T.R.N.C.**
**NEAR EAST UNIVERSITY**
**INSTITUTE OF HEALTH SCIENCES**

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**ANALYTICAL CHEMISTRY**

**MASTER OF SCIENCE THESIS**

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**SUPERVISOR**
**ASSIST. PROF. DR. USAMA ALSHANA**

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## DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

**Name, Last Name** **:** HÜSÜN TABUR

**Signature** **:**

**Date** **:** 28/05/2018

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To my lovely family.

## ABSTRACT

**Tabur, Hüsün. Dispersive liquid-liquid microextraction of caffeine from Turkish coffee prior to its determination by HPLC.**
**Near East University, Institute of Health Sciences, Analytical Chemistry Program, Master of Science Thesis, Nicosia, 2018.**

Dispersive liquid-liquid microextraction (DLLME) was used prior to high-performance liquid chromatography (HPLC) for the extraction and determination of caffeine in Turkish coffee samples. A reversed-phase column (Agilent Zorbax SB-Aq, 4.6 x 150 mm, 5 μm) was used for separating the analytes using a mobile phase consisting of 40% (v/v) methanol in water at room temperature, a flow rate of 0.8 mL $min^{-1}$, and an injection volume of 20 μL. The analytes were monitored using a diode array detector (DAD) at 273 nm. Optimum DLLME conditions were as follows: 100 μLof chloroform (as extraction solvent), 500 μL of ethanol (as disperser solvent) and 60 s extraction time. The analytes were back-extracted into 50.0 μL of 50/50% (v/v) methanol containing 50 mM NaOH solution within 60 s. All of the 24 different brands of Turkish coffee samples analyzed, contained caffeine at varying concentrations in the wide range of 0.89-15.40 μg $g^{-1}$. Theobromine and theophylline were not detected in any of the studied samples. The results proved that DLLME combined with a simple back-extraction step prior to HPLC could be of great interest in the determination of caffeine in foods and beverages in routine food analysis laboratories.



**Keywords:** Caffeine, determination, dispersive liquid-liquid microextraction, HPLC, Turkish coffee.

# ÖZET

**Tabur, Hüsün. Kafeinin Türk kahvesinde HPLC ile teyin öncesi dispersif sıvı-sıvı mikro ekstraksiyonu.**

**Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Analitik Kimya Programı, Yüksek Lisans Tezi, Lefkoşa, 2018.**

Türk kahvesi numunelerinde kafeinin yüksek performanslı sıvı kromatografisi (HPLC) ile tayin öncesi ekstraksiyonu için dispersif sıvı-sıvı mikro ekstraksiyon (DLLME) yöntemi kullanılmıştır. Analitleri ayrılmasında ters faz kolonu (Agilent Zorbax SB-Aq, 4.6 x 150 mm, 5 µm) su içinde %40 (h/h) metanolden oluşan hareketli faz, oda sıcaklığında, 0.8 mL $dk^{-1}$ ve 20 µL injeksiyon hacmi uygulanmıştır. Analitler diyot serili dedektör ile 274 nm dalga boyunda izlenmiştir. Optimum ekstraksiyon koşulları aşağıdaki gibidir: 100 µL kloroform (ekstraksiyon çözücüsü), 500 µL etanol (dispersiyon çözücüsü) ve ekstraksiyon süresi 60 saniyedir. Analitler 50.0 µL hacminde metanol: sodyum hidroksit (50 mM) 50:50 (h/h) karışımı ile 60s süreyle geri ekstrakte edilmiştir. Bütün 24 farklı çeşit Türk kahvesi örneğinin 0.89-15.40 µg $g^{-1}$ geniş aralığında değişik konsantrasyonlarda kafein içerdiği bulunmuştur. Teobromin ve teofilin analiz edilen hiçbir örnekte saptanmamıştır. Sonuçlar HPLC'den önce geri ekstraksiyon ile dispersif sıvı-sıvı mikro ekstraksiyonun birlikte kullanılmasının rutin gıda analiz labratuvarlarında kafeinin gıda ve içeceklerdeki tayinlerinde önemli olabileceği gösterilmiştir.

**Anahtar kelimeler:** Dispersif sıvı-sıvı mikro ekstraksiyonu, HPLC, kafein, tayin, Türk kahvesi.

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## LIST OF ABBREVIATIONS

| ABBREVIATION | DEFINITION |
|---|---|
| 1-DO | 1-Dodecanol |
| 1-UN | 1-Undecanol |
| AAS | Atomic Absorption Spectrometry |
| AC | Affinity Chromatography |
| ACN | Acetonitrile |
| ACT | Acetone |
| BE | Back-Extraction |
| BES | Back-Extraction Solvent |
| CAF | Caffeine |
| CE | Capillary Electrophoresis |
| CF | Chloroform |
| DI | Deionized Water |
| DLLME | Dispersive Liquid-Liquid Microextraction |
| DPE | Diphenylether |
| EF | Enrichment Factor |
| ER | Extraction Recovery |
| ETD | Evaporation to Dryness |
| GC | Gas Chromatography |
| HPLC | High-Performance Liquid Chromatography |
| IEC | Ion-Exchange Chromatography |
| k’ | Retention Factor |
| LLE | Liquid-Liquid Extraction |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| LPME | Liquid-Phase Microextraction |
| MAE | Microwave-Assisted Extraction |
| MP | Mobile Phase |
| N | Number of Theoretical Plates |

| ABBREVIATION | DEFINITION |
|---|---|
| NP | Normal-Phase |
| ODS | Octadecylsilyl ($C_{18}$) |
| RP | Reversed-Phase |
| RR | Relative Recovery |
| $R_s$ | Resolution |
| RTIL | Room-Temperature Ionic Liquid |
| SEC | Size-Exclusion Chromatography |
| SLE | Solid-Liquid Extraction |
| SPE | Solid-Phase Extraction |
| TB | Theobromine |
| THF | Tetrahydrofuran |
| $t_M$ | Retention Time of an Unretained Species |
| TP | Theophylline |
| $t_R$ | Retention Time of the Analyte |
| TRNC | Turkish Republic of North Cyprus |
| $\alpha$ | Selectivity Factor |

# CHAPTER 1

# INTRODUCTION

## 1.1 History of Coffee

Aborgines of Africa were the first to use coffee. They used leaves, fruits and seeds of the coffee plant for food. Coffee plant has ripe fruits, which are red with sweet pulp containing the caffeine. At first, they used to chew that ripe fruits, then they started to use coffee in solid mixtures.

### 1.1.1 The spread of coffee-drinking habit

The time when people started to drink coffee is still a mystery. However, it is known that it was first discovered in Arabia about the middle of the fifteenth century.

### 1.1.2 Types of coffee seeds

There are three types of coffee seeds:

**Coffee Arabica**

Coffee Arabica comprises about 80 per cent of the world's consumed coffee.

**Robusta Coffee**

It occurs wild in the equatorial forest from West Africa to Lake Victoria. It grows in West Africa, Zaine, Sudan, Uganda, North-Western Tanzania and Angola. The sizes and shapes of Coffee Arabica anad Robusta Coffee are similar to each other.

**Liberica Coffee**

It was originally found near Monrovia in Liberia.

### 1.1.3 Botany of coffee

Coffee belongs to the genus Coffea which in turn is a member of the family *Rubiaceae*. All species of Coffea have opposite leaves and branches.

## 1.2 Caffeine

Caffeine (CAF) is an alkaloid natural compound, which is found in many plants such as leaves and beans of the coffee plant, in tea, yerba mate, and guarana berries, and in small quantities in cocoa, kola nuts and the Yaupon Holly. It acts as a natural pesticide against certain insects and as a stimulant in humans. CAF belongs to the group of xanthines, which has effect over the nervous central system. Coffee, tea, and cocoa are the most commonly used CAF-containing drinks. Coffee beans are the world's primary source of CAF. Coffee is brewed from the seeds of the coffee plant [1]. The chemical structure of the CAF is given in **Figure 1.1**.

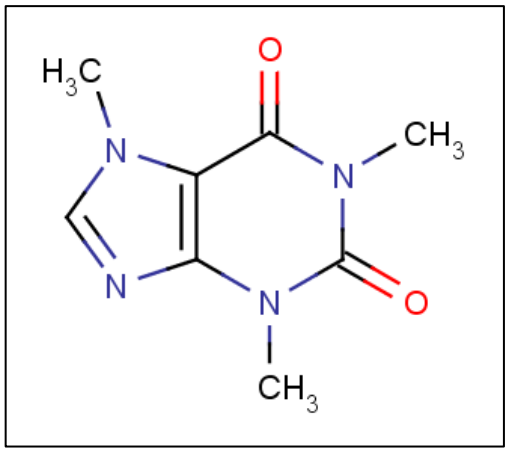


**Figure 1.1.** Structure of CAF.

## 1.3 Effect of CAF on Human Health

CAF has many effects such as cardiovascular effects, effects on bone and calcium balance, effects on human behavior, reproductive and developmental effects, as well as

effects on sleep [2]. Consumption of excessive amounts of CAF might cause "caffeinism" syndrome. Caffeinism is a condition where a person presents an intoxication due to abuse of CAF. This intoxication covers a variety of unpleasant physical and mental symptoms associated with the consumption of excessive amounts of CAF. The main symptoms of CAF overdose are summarized in **Figure 1.2** [3].

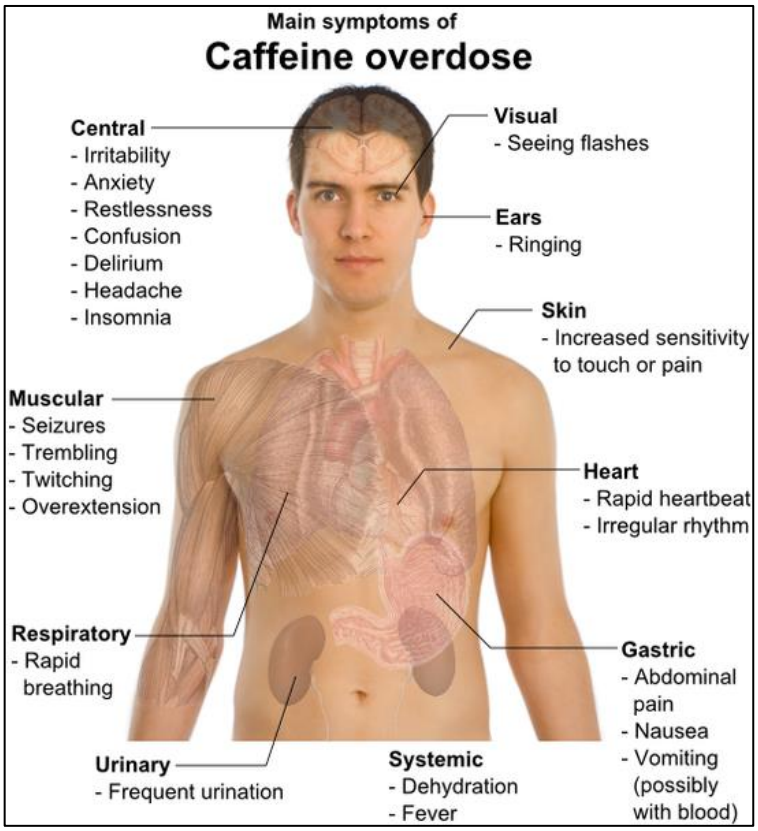


**Figure 1.2.** Main symptoms of CAF overdose.

### 1.3.1 Effect of CAF on cardiovascular system

There are several studies about the effects of CAF on heart rate, blood pressure serum cholesterol, and cardiacarrhythmia [4]. CAF is a stimulant that should be consumed within safe doses. The optimum dose of CAF is different for each person because of his or her sensitivity, weight, age, pregnancy and health history. Clinical studies show that

consuming ≤ 400 mg CAF per day or drinking four or fewer cups of coffee does not affect cardiovascular health negatively [5]. The recommended limits of CAF consumption are as shown below:

Healthy Adults: 300-400 mg per day
Children (13-18): 100 mg per day
Children (under twelve): None, but no more than 3 mg per kilogram of their body weight.
Pregnant Women: No more than 100-200 mg per day.

### 1.3.2 Effect of CAF on bone and calcium balance

Several studies proved that there is not a significant relation between CAF intake and bone density in adolescent woman, young woman between 20-30 years old, premenopausal and postmenopausal women [2]. Many clinical and epidemiological studies suggested that CAF intake is associated with significant increases in cholesterol levels in blood [6].

## 1.4 Theobromine

Theobromine (TB) is a methylxanthine, which is present in high concentrations in many natural products such as cacao beans, and it is the source of the typically bitter taste of chocolate. TB can occur as a product of CAF metabolism in bacteria and plants or can be synthesized [7]. It is highly fat-soluble, peaking in the plasma 1–2 h after ingestion. CAF has 5 times more stimulant effect than TB on human body. TB is known to lower blood pressure, it is also a smooth muscle relaxant and diuretic [8]. The structure of TB is shown in **Figure 1.3**.

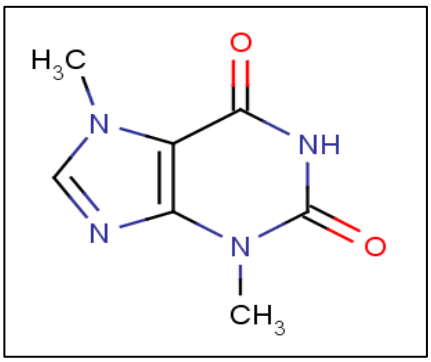


**Figure 1.3.** Structure of Theobromine.

## 1.5 Theophylline

Theophylline (TP) is a xanthine derivative that induces relaxation of smooth muscle in the bronchial tree causing bronchodilation. It is widely used in therapy of asthma and is not believed to cause liver injury. It is also used in human and veterinary medicine [9]. Overdoses of TP can cause emesis, tachyarrhythmias, hypotension, and seizure. Also metabolic derangements such as hypokalemia, hypomagnesemia, hypophosphatemia, lactic acidosis, hyperglycemia, and hyperthermia can be seen [10]. Chemical structure of TP is shown in **Figure 1.4**.

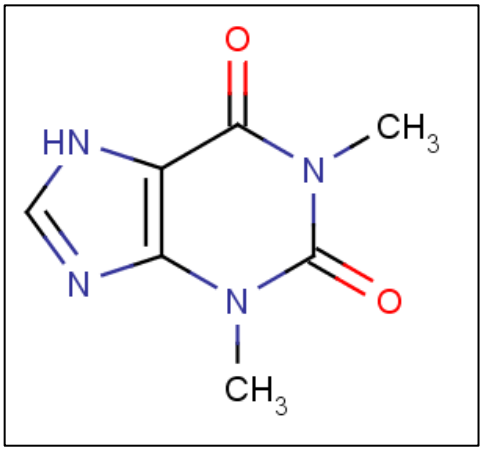


**Figure 1.4.** Structure of Theophylline.

## 1.6 Methods for Extraction of CAF

### 1.6.1 Supercritical $CO_2$

Studies show that extracting CAF from Robusta coffee husks by using $CO_2$ under supercritical conditions is possible. The amount of $CO_2$ and the extracted CAF are directly proportional to each other. CAF is extracted together with a mixture of pigments and fats. From this mixture, simple washing, followed by evaporation, can easily separate CAF. In supercritical $CO_2$, solubility of xanthines is low because of their high polarity. Therefore, addition of polar co-solvents, for instance, water or ethanol is necessary. These added co-solvents can improve physical interactions between the solutes and $CO_2$, making extraction easier. High pressure is generally required to obtain higher solubility. At 373 K and 300 bar a maximum yield 84% was obtained. Then, washing the mxture with water CAF was at least 94% pure. This method is environmentally friendly but it is slow and relatively expensive [1].

## 1.7 Solid-Phase Extraction (SPE)

Solid-phase extraction (SPE) has been reported for the extraction of CAF and other xanthines. This extraction technique is fast, powerful, reliable and cost-effective for extraction of compounds of interest from complex matrices. It generally needs less solvent than supercritical $CO_2$, which means that it can be more environmentally friendly. In a study, on-line SPE was performed using a $C_{18}$ minicolumn coupled to a flow injection system for the extraction and determination of CAF in green and roasted coffee beans [11]. Different roasted beans were studied and the results were compared with the methods, which use chloroform to carry out CAF extraction such as liquid-liquid extraction (LLE). An aqueous extraction step was carried out with hot water at 80 °C, which allowed a rapid and easy extraction of CAF from the coffee beans. Under final optimized conditions, the total analysis time (after the caffeine extraction process from coffee beans was carried out) was 6 min, including sample injection, on-line retention, on-line elution, regeneration of

the column and detection. The proposed method was a good option for the determination of CAF in coffee in routine analysis because it is a simple, rapid, precise and cheap.

### 1.7.1 Solid-liquid extraction (SLE)

Decaffeination of coffee is a solid-liquid extraction (SLE) process. In this method, CAF is transferred from the solid matrix into a suitable solvent. In a study performed on decaffeination of coffee bean waste by SLE [12], six different types of solvents (i.e., water, methanol, ethanol, acetonitrile, n-hexane, and dichloromethane) were used to find optimum conditions for the extraction of CAF. Ethanol/water (50/50, v/v) with a solid/solvent ratio of 1:20 (g $mL^{-1}$) and an extraction time of 60 min under a temperature of 80 °C were the optimum conditions for decaffeination of coffee bean waste.

### 1.7.2 Dispersive liquid-liquid microextraction (DLLME)

Even with the exponential growth in analytical techniques in the past few decades due to the design and application of sophisticated techniques such as chromatography, spectroscopy, electrochemistry and microscopy, the state of the current instrumentation is still not enough to get all information from a sample directly without some sample pre-treatment steps, known as sample preparation. In an analytical procedure, sample preparation involves an extraction process with the aim of isolation and enrichment of the analyte(s) from the sample matrix [13].

The drawbacks of conventional sample preparation methods are well documented in the literature. Some worth mentioning are the tedious and large consumption of toxic organic solvents involved in LLE, which are harmful to the researcher, living organisms and to the environment. SPE uses less volume of organic solvents but is still considered significant. In addition, SPE cartridges are expensive and disposable, generating waste which is harmful to the environment [14].

Recently, the focus is shifted towards the development of efficient, economic and miniaturized sample preparation techniques. Assadi and his team [13] developed a novel liquid-phase microextraction (LPME) technique in 2006, which was termed as dispersive liquid-liquid microextraction (DLLME). This novel technique has since then gained a wide acceptance, recognition and popularity among analytical chemists and in other fields due to its high rapidity of extraction, simplicity, environmental friendliness, high extraction efficiency and affordability [15].

DLLME consists of a ternary solvent system; namely, a disperser solvent, an extraction solvent and an aqueous sample. The extraction and the disperser solvents are rapidly injected into an aqueous sample in a conical test tube to form a cloudy solution containing micro droplets of the extraction solvent, which are dispersed fully in the aqueous solution. Equilibrium is achieved instantaneously due to the infinitely large surface area of contact between the acceptor and the donor phase making extraction time to be very fast which is one of the major advantages of this method. A centrifugation step is necessary to collect the extraction phase at the bottom of the conical tube. The choice of conical tube is for easy collection of the extraction phase.

The choice of the extraction solvent is based on the ability of the solvent to extract the analyte from the sample matrix and immiscibility with the aqueous phase, while the disperser solvent has to be miscible with both the extraction solvent and the aqueous solution [14]. The extraction solvent can be denser than water such as chlorinated solvents which include chloroform dichloromethane, and tetrachloromethane or less dense than water such as 1-undecanol, 1-dodecanol, 2-dodecanol, hexadecane, in which case solidification of the floating organic drop can be applied for those solvents which solidify at room temperature [16].

For lower density solvents that do not solidify at room temperature, special devices can be used for collecting the extraction solvent at the top of the aqueous sample, low density based solvent de-emulsification, adjustment of the solvent's mixture density and

sequential injection-DLLME [17]. Some of these methods also eliminate the need of the centrifugation step which is considered as the most time-consuming step of this method [18]. The disperser solvent is selected on the bases of miscibility with the extraction solvent and aqueous sample. Common disperser solvents used in DLLME include acetonitrile, acetone, methanol and ethanol [16].

Gas chromatography (GC) was the first instrument to be used for DLLME [13]. in which case the extract could be injected directly into the instrument due to the compatibility of the organic extraction solvent with the instrument. Other instruments such as capillary electrophoresis (CE) [19] and atomic absorption spectrometry (AAS) [20] were reported in the literature. HPLC is now the most widely used instrument with DLLME [21-26].

Recent advances in DLLME are geared towards the use of less toxic solvents due to the high toxicity of chlorinated solvents. Ionic liquids are considered as "green solvents" capable of replacing toxic organic solvents used in DLLME. They are a group of non-molecular organic salts with meting point below 100 ˚C which causes them to remain in the liquid form at room temperature, hence the name room-temperature ionic liquid (RTIL) [27]. A review by Trujillo-Rodríguez et al. [28] gives a detailed explanation of the various modes of ionic liquid dispersive liquid-liquid microextraction (IL-DLLME). The use of nanoparticles for enhancement of DLLME is a recent development in which the unique characteristics of nanoparticles such as increased surface area, optical, electrical, magnetic, catalytic properties and their ability to retain different functional groups to their surface have made them applicable in solid-liquid sorption processes applicable to DLLME [29].DLLME has many advantages which include the following:

- It is very rapid
- It is easy
- It can be automated
- It requires small volumes of solvent (few microliters)
- It is economical

- It has high enrichment factor and high extraction recoveries
- it reduces the chance of sample contamination because it is a closed system
- It can isolate and preconcentrate target analytes from the samples
- It can reduce the interferences by providing a high degree of sample clean-up
- It keeps the hazard analytes away from the analyst due to the use of a closed system.
- It has high extraction yield.

## 1.8 Calculations in DLLME

In DLLME, the enrichment factor (EF) and percentage extraction recovery should be taken into consideration to evaluate the efficiency of the method. Assadi et al. [13] defined EF as shown in **Equation 1.1.**

$$EF = \frac{C_i}{C_f} \qquad \textbf{Equation 1.1}$$

where, $C_i$ is the analyte concentration in the sample solution and $C_f$ is the analyte concentration in the final extract. Percentage extraction recovery (%ER) can be defined as the percentage ratio of the amount of analyte in the final extract to the initial concentration in the sample **Equation 1.2**.

$$\%ER = \frac{n_f}{n_i} \times 100 = \frac{C_f \times V_f}{C_i \times V_i} \times 100 \qquad \textbf{Equation 1.2}$$

where, $n_f$ and $n_i$ are the amounts (in mol) of the analyte in the final extract and the initial sample solution, respectively. $V_f$ and $V_i$ are the volumes of the final extract and the initial sample solution, respectively. %ER can also be calculated from the calibration of caffeine in the back-extraction solution (BES) **Equation 1.3**.

$$\%ER = \frac{V_f}{V_i} \times EF \times 100 \qquad \textbf{Equation 1.3}$$

where, EF is the enrichment factor.

The percentage relative recoveries (%RR) can be calculated from **Equation 1.4**.

$$\%RR = \frac{C_{found-C_{added}}}{C_{real}} \times 100 \qquad \textbf{Equation 1.4}$$

where, $C_{found}$ is the analyte concentration measured from the sample after it is spiked with the analyte, $C_{real}$ is the native analyte concentration in the sample and $C_{added}$ is the amount of the analyte that was spiked into the sample solution.

## 1.9 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a type of liquid chromatography technique, which is used to separate and distinguish between compounds in a mixture.

Based on working principle, liquid chromatography can fall into five different types:

1. Liquid-liquid chromatography (partition chromatography)
2. Liquid-solid chromatography (adsorption chromatography)
3. Ion-exchange chromatography
4. Size-exclusion chromatography
5. Affinity chromatography

In HPLC, the sample is dissolved in a suitable solvent and the liquid mobile phase is pumped through the column, which contains stationary phase, and the components are separated. Separation is determined by the interactions between the sample and the phases.

HPLC is an essential analytical technique for qualitative and quantitative determinations in many fields, for research, diagnostic, and manufacturing purposes, among many others.

### 1.9.1 Partition chromatography

Partition chromatography is the most widely used type of HPLC. The stationary phase and the mobile phase are both liquid, which are immiscible with each other. It can used for ionic and nonionic compounds. In this technique, the difference between the partition coefficients of the analytes in the two liquids help them to separate from one another. Partition chromatography has found a wide use in biochemical, food industry, forensic science, clinical chemistry, pharmaceuticals, pollutants and petrochemicals.

There are different types of partition chromatography depending on the polarity of the mobile phase being used. For polar mobile phases such as ACN, MeOH, and tetrahydrofuran (THF) eluted on a low-polarity stationary phase such as octadecyl ($C_{18}$) group-bonded silica gel (ODS), the mode of partition chromatography is known as reversed-phase (RP), while for non-polar mobile phase such as n-hexane (n-Hex) and chloroform eluted on a polar stationary phase like silica gel, the mode is known as normal-phase (NP) because it was the first principle that was applied for such chromatographic separations.

In normal-phase chromatography, the mobile phase is non-polar and the stationary phase is polar. In reversed-phase chromatography it is the opposite. Water can be used as the mobile phase in RP-HPLC. It is cheap, non-toxic, UV-transparent and compatible with biological analytes. In addition, mass transfer of polar mobile phases is faster. The mobile phases used in NP-HPLC are more toxic than those used in RP-HPLC. Because of these situations, RP-HPLC is the most widely used partition chromatography mode.

$$logP_{o/w} = log\frac{[analyte]_{octanol}}{[analyte]_{water}} \qquad \textbf{Equation 1.5}$$

Partition coefficient is one of the most important parameters that gives the concentration ratio of an analyte in biphasic system with two immiscible solvents . The logarithm of this ratio is known as log*P* which is defined in **Equation 1.5**.

### 1.9.2 Adsorption chromatography

Adsorption chromatography was first introduce by Tswett in the 19$^{th}$ century. There are solid and liquid phases in adsorption chromatography. The mobile phase is the one which repels the analyte while the stationary phase is the one to which the analyte is adsorbed. Separation occurs depending on the difference of adsorption of the analytes to stationary phase. Adsorption chromatography can distinguish between some compounds, which cannot be separated by other chromatography techniques.

### 1.9.3 Ion-exchange chromatography (IEC)

By using ion-exchange chromatography (IEC), anions and cations can be separated from each other depending on their affinity for an ion-exchange resin. Developing ion-exchange resins in the mid-1970s showed that cations and anions can be separated from each other on HPLC columns. This technique is widely used in separation of anions on an anion-exchange column and separation of cations on a cation-exchange column.

### 1.9.4 Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) is also known as gel chromatography. Molecules are separated by their size and molecular weight. It is the most powerful and convenient method for determining the molecular weight of a polymer [30]. Larger molecules reach the detector before the small molecules do because the small ones get trapped within the pores. SEC is commonly used in separation of organic polymers and biological molecules and for the analysis of organic polymers.

### 1.9.5 Affinity chromatography (AC)

Affinity chromatography (AC) helps to separate biochemicals from each other. The separation occurs depending on the interaction difference between antibodies, enzymes, inhibitors, ligands and proteins. While the sample passes through the column if there is an interaction between the molecule and the affinity ligand they bind to each other. If there is not the molecule leaves the column with the mobile phase. Affinity chromatography is widely used in purification of proteins, nucleic acids, proteins and in the study of biomolecular interactions [31].

## 1.10 Types of Elution in HPLC

### 1.10.1 Isocratic elution

In isocratic elution, the composition of the mobile phase is constant during analysis. The solvent must be pre-mixed. Isocratic elution is available in all HPLC instruments. Its low cost and easy instrumentation makes it to be preferred for use in many applications, but it has long analysis time and poor resolution in many cases.

### 1.10.2 Gradient elution

Gradient elution can deliver variable mobile phase compositions during analysis and it is possible to pump more than one solvent simultaneously. Good resolution can be achieved within short analysis time. Gradient elution can also be used to improve the resolution for difficult-to-separate analytes. Equations and a graphical illustration that are used to select the suitable mode of elution (i.e., isocratic or gradient) are shown in **Figure 1.5** and **Figure 1.6.**

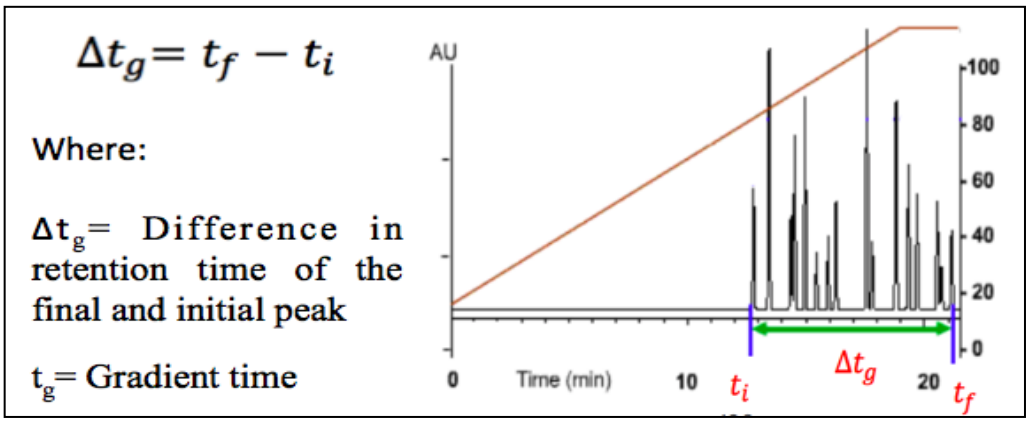


**Figure 1.5.** Equation and graphical illustration of determining the suitable mode of elution.

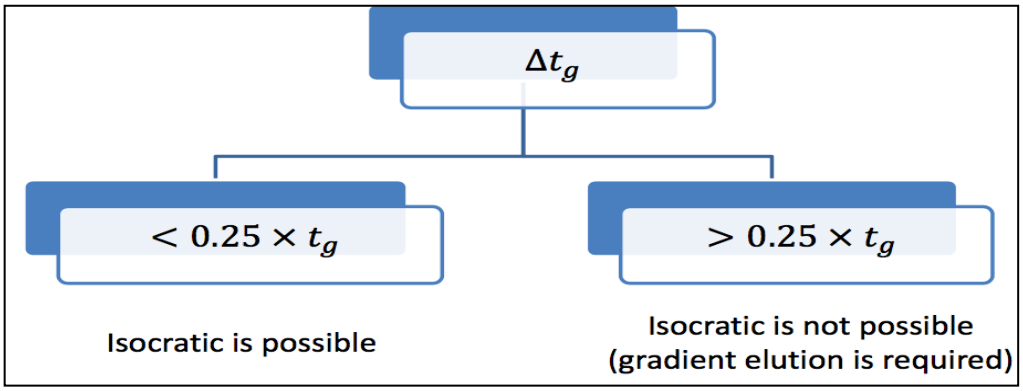


**Figure 1.6.** Deciding on the elution mode.

## 1.11 Optimization of HPLC Conditions

For optimization of the HPLC conditions, there are two common ways. One is the "Random Walk" approach, which is performed in 'random', uncoordinated experiments. Using the Random Walk, acceptable separations can be achieved sometimes but it would be done without understanding or clear insight into sensitivity of modifications necessary for the conditions. The other approach is the "Systematic Approach", which is always more recommended.

## 1.12 Equations Describing the Factors Affecting Resolution in HPLC

Retention factor ($k'$), selectivity factor ($\propto$) and number of theoretical plate (N) can all affect the resolution ($R_s$). Mathematical equations describing these terms are given in **Table 1.1**.

**Table 1.1.**Equations describing separation in chromatography.

| Factor | Term | Meaning | Equation |
|---|---|---|---|
| $k' = \frac{t_R - t_M}{t_M}$ | $k'$<br>$t_R$<br>$t_M$ | Retention (capacity) factor<br>Retention time<br>Dead time | **Equation 1.6** |
| $\propto = \frac{k'_B}{k'_A} = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$ | $\propto$ | Selectivity factor | **Equation 1.7** |
| $N = 16\left(\frac{t_R}{W}\right)^2$ | $N$<br>$W$ | Number of theoretical plate<br>Peak width | **Equation 1.8** |
| $R_s = \frac{\sqrt{N_{av}}}{4} \times \frac{k'_{av}}{k'_{av}+1} \times \frac{\propto -1}{\propto}$ | $R_s$<br>$N_{av}$<br>$k'_{av}$ | Resolution<br>Average $N$ of two adjacent peaks<br>Average $k'$of two adjacent peaks | **Equation 1.9** |

### 1.12.1 Changing $k'$

To obtain an efficient separation, the column should be able to retain the analyte and separate the other components of the sample. $k'$ can be defined as in **Equation 1.6,** $t_R$ is the retention time of the analyte (i.e., the time consumed by the sample to reach the detector) and $t_M$ is the retention time of an unretained species.

The larger the retention factor, the higher the ability of the column to retain analytes, which will improve the resolution. An ideal value for $k'$ would fall between 5 and 7, illustrating a good balance between analysis time and resolution. Changing the

composition of the mobile phase, pH, buffer concentrations and column temperature can affect $k'$.

### 1.12.2 Changing α

The selectivity factor ($\propto$) of a column is defined as the degree of separation between successive peaks (generally called as critical pair). For the two species A and B, α can be defined as in **Equation 1.7**. $k'_A$ and $k'_B$ are the retention factors of A and B, respectively. When $\alpha = 1$, the retention time of the two compounds, A and B, are equal [i.e., $(t_R)_A = (t_R)_B$], resulting in a complete overlap of the critical pair. $\propto$ can be controlled by changing the mobile phase identity or changing the column.

### 1.12.3 Changing *N*

Theoretical plate number ($N$) is a measure of column efficiency. It describes the number of plates as defined according to plate theory, and can be used to determine column efficiency. The higher the value for $N$, the sharper the peaks, the greater the peak efficiency. N can be calculated for any peak in the chromatogram using **Equation 1.8**. Column length, diameter and particle size as well as the flow rate can affect $N$.

### 1.12.4 Effect of $k'$, α and N on resolution

As can be seen in **Figure 1.7,** increasing N and αimprove$R_s$; they are directly proportional to each other. On the other hand, increasing $k'$ until 10 will improve $R_s$ rapidly but after 10, it will not affect significantly.

The relationship between the resolution and these separation factors can be described by **Equation 1.9**.

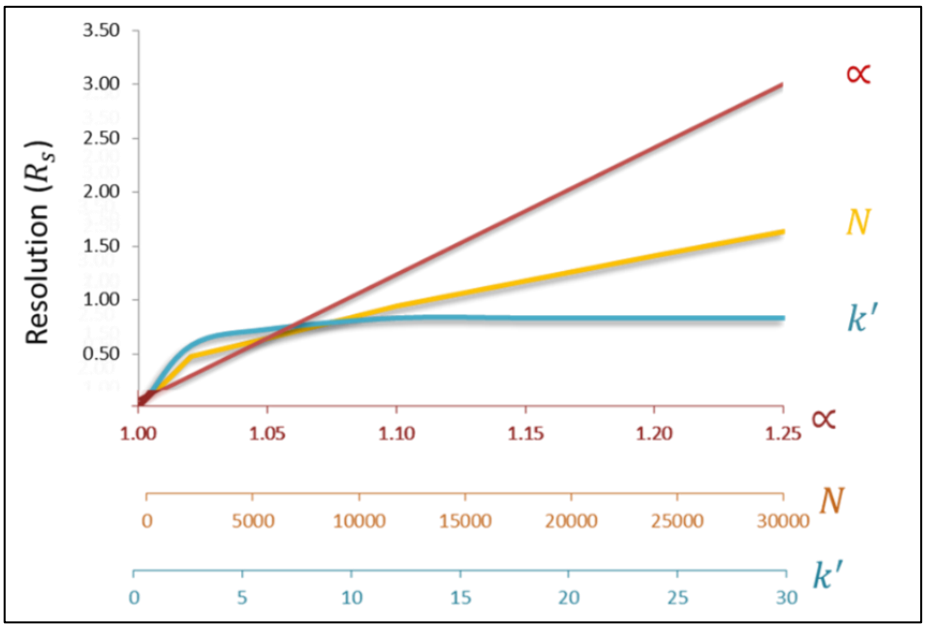


**Figure 1.7.** Effect of $k'$, $\alpha$ and $N$ on resolution

Optimum resolution can be obtained within the shortest time using a systematic approach such as the one summarized in **Figure 1.8**. Factors that can affect the separation of target analytes in an HPLC system include the following: Type of the column packing, column dimensions, particle size, composition of the mobile phase, flow rate of the mobile phase, identity of the mobile phase, pH of the column, temperature of the column, concentration of buffer used for adjusting the pH, concentration and type of acid modifier.

An approach to the design of this HPLC assay can be thought of according to the following six steps:

1. Selecting an HPLC methodology,
2. Selecting an HPLC column,
3. Selecting initial experimental conditions,
4. Carrying out an initial separation,
5. Evaluating the initial chromatogram and determining what change in resolution is required,
6. Establishing conditions required for the necessary final resolution.

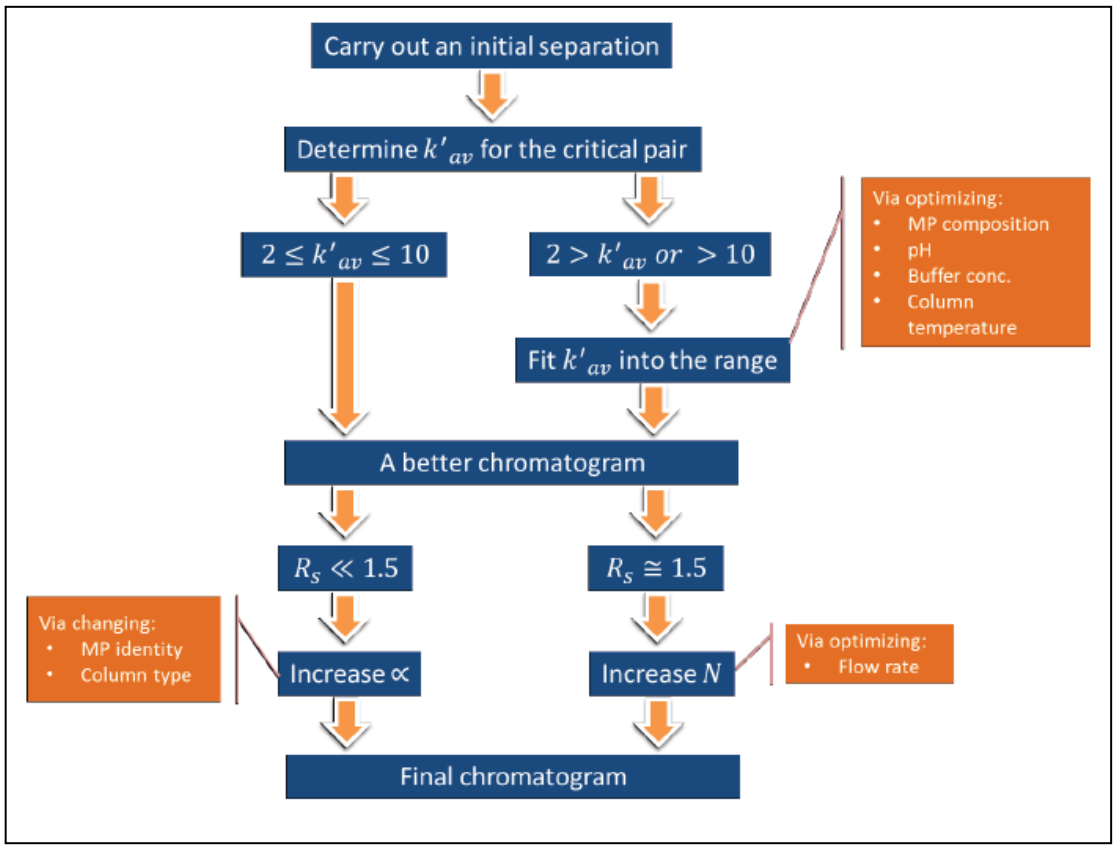


**Figure 1.8.** Systematic approach to HPLC optimization.

After selecting an HPLC methodology and a suitable HPLC column, initial experimental conditions are decided on and an initial injection is done. Evaluating the initial chromatogram helps to understand the conditions that need to be changed. If $k'$ is within the optimum range of 5 and 7, and resolution between two adjacent (critical) peaks is close to 1.5, increasing N would give the required resolution with minimum number of experiments. However, if $k'$ does not fall into its optimum range, the fastest resolution can be obtained by fitting it into this range first by changing one or more of the chromatographic parameters that can affect it.

If $k'$ is within the optimum range but resolution between the critical peaks is still poor, changing N would probably require a very long separation time and if α is 1 it would be almost impossible. In this case, increasing α can work very well.

### 1.12.5 Selection of the mobile phase (MP)

Selection of the MP for HPLC should be based on the following criteria:

- Viscosity: a low-viscosity solvent produces a lower back pressure than a solvent with higher viscosity for a specific flow-rate. It also allows faster chromatography as mass transfer takes place faster.
- UV transparency: if a UV detector is used, the mobile phase must be completely transparent at the required wavelength.
- Purity: HPLC-grade solvents or better should always be used.
- Inert with respect to sample compounds: The mobile phase must not react at all with the sample mixture.
- Toxicity: Here the onus is on each individual laboratory to avoid toxic products as far as possible.
- Price: Solvent consumption in HPLC is relatively high. Therefore, solvents with high purity but moderate prices are preferred.

## 1.13 Literature Review

Kana et al. [32] simultaneously determinate trigonelline, caffeine, chlorogenic acid and their related compounds in instant coffee samples by HPLC using an acidic mobile phase containing octanesulfonate, as an ion-pairing reagent. Optimum mobile phase conditions were obtained with 0.1% phosphoric acid, 4 mM octanesulfonate, and 15% methanol at 35 °C. The analytes were extracted from each coffee sample (0.50 g) using SLE assisted by ultrasonication for 5 min at 45 kHz into 10 mL of 15% MeOH at room temperature. The mixture was centrifuged at 1200 rpm for 5 min and the supernatant was filtered through a 0.45-µm filter before it was injected into the HPLC instrument. Trigonelline, nicotinic acid, caffeine, theophylline, chlorogenic acid, and caffeic acid were determined in ten instant coffee samples. These analytes were detected in all samples except theophylline. An increase in the caffeine content in instant coffee samples tended to

decrease in both trigonelline and chlorogenic acid contents, and the trigonelline content was found to be correlated well with the chlorogenic acid content.

Belguidoum et al. [33] developed an HPLC coupled to UV-Vis detection for quantitative determination of some phenolic compounds and caffeine in different brands of coffee in the Algerian market. Eight phenolic acids, three flavonoids, and caffeine were determined in sixteen coffee samples (roasted, green and instant) collected from the Algerian market. The following parameters were taken into account for coffee analyses: packaging, roasting degree, grain size, instantaneity, and decaffeination. The analytes were extracted from 0.5 g of each coffee sample into 15 mL of 50% methanol/water, followed by 15 mL of 75% methanol/water, and finally with15 mL of 100% methanol. Extractions were performed on a ultrasound bath, operating at 25 kHz for 20 min at 60 °C. After each extraction step, the sample was centrifuged for 10 min at 10 °C and 4000 rpm. The supernatant was collected and the solid residue was subjected to the next extraction step. After the final extraction, the supernatants were combined, and water was added to obtain a final volume of 100 mL. 20 µL of the obtained extract were filtered and injected into the HPLC.

The total polyphenols and caffeine concentration in the coffee extracts were found to vary from $12.37 \pm 0.55$ to $200.08 \pm 6.47$ mg $L^{-1}$, and $38.00 \pm 1.89$ to $136.00 \pm 6.45$ mg $L^{-1}$, respectively. Evaluation of the chromatographic performance showed excellent reproducibility, resolution, selectivity, and reasonable peak symmetry. The limit of detection (LOD) ranged from 0.75 to 14.79 µg $L^{-1}$, while the limit of quantification (LOQ) ranged from 2.26 to 44.44 µg $L^{-1}$. The separation of all compounds was achieved within 13 min.

Liu et al. [34] proposed a simple, rapid method for the simultaneous extraction of trigonelline, nicotinic acid, and caffeine from coffee, and separation by two chromatographic columns in series. Trigonelline, nicotinic acid, and caffeine were extracted using microwave-assisted extraction (MAE). The optimal conditions selected were 3 min, 200 psi, and 120 °C. The chromatographic separation was performed with

two columns in series, polyaromatic hydrocarbon $C_{18}$ (250 x 4.6 mm id, 5 pm particle size) and Bondapak $NH_2$ (300 x 3.9 mm id, 5 pm particle size). Isocratic elution was applied with 0.02 M phosphoric acid in methanol (70:30, v/v) mobile phase at a flow rate of 0.8 mL $min^{-1}$.

A 200 mg sample was accurately weighed into a Teflon pressure vessel, and 20 mL water were added. The vessel was tightly sealed and placed in the microwave system. After MAE, the sample was cooled to room temperature. The mixture was filtered through 18 cm quantified filter paper and the final volume was made up with water to 50 mL. The solution was filtered with a 0.45 µm membrane and 10 µL were injected into HPLC for analysis.

Good recoveries and RSD values were found for all analytes in the matrix. LOD of the three analytes was 0.02 $mgL^{-1}$. The concentrations of trigonelline, nicotinic acid, and caffeine in instant coffee, roasted coffee, and raw coffee (Yunnan Arabica coffee) were assessed by MAE and hot-water extraction. The correlation coefficients between concentrations of the three compounds obtained were close to 1.

### 1.14 Aim of This Study

The aim of this study was to provide a simple, efficient, low cost and robust dispersive liquid-liquid microextraction method combined with reversed-phase high-performance liquid chromatography (DLLME-HPLC) for the determination of theobromine, theophylline and caffeine in Turkish coffee.

# CHAPTER 2
# EXPERIMENTAL

## 2.1 Instrumentation

Chromatographic separations were performed with an Agilent technologies 1200 series HPLC system (USA) equipped with a diode array detector, an autosampler, a degasser, a quaternary pump and a column oven. The instrument was controlled by Agilent ChemStation for LC systems software. A reversed-phase column (ZORBAX SB-Aq, 4.6 mm ID x 150 mm, 5 µm) was used.

## 2.2 Reagents and Solutions

HPLC-grade methanol, acetone and chloroform with purity higher than 99% were from Sigma-Aldrich, Germany. Ethanol was from EMSURE® (Darmstadt, Germany). 1-undecanol, 1-dodecanol, dichloromethane and diphenylether were obtained from Sigma-Aldrich (Steinheim, Germany). pH of the solutions was adjusted with 0.10 M NaOH. Caffeine, theobromine and theophylline standards (≥ 99.0%) was from Fluka (USA). Individual stock solutions of each analyte with a concentration of 1000 mg $L^{-1}$were prepared in methanol and stored in the refrigerator at -15 °C. Working standard solutions were used for optimization of the HPLC and DLLME methods and for drawing calibration graphs. All other reagents and solvents used were at least of analytical reagent grade.

## 2.3 Apparatus

Centrifugation was performed with HettichEba 20 centrifuge (Germany), while vortex was performed on a HeidolphReax top Vortex. Eppendorf micropipette (Sigma-Aldrich, USA) and tips were used for sample collection and transfer A Blomberg refrigerator was used for sample preservation, and Sinbo coffee grinder model SCM 2927 (P.R.C) was used for blending of the samples.

## 2.4 Sampling and Sample Pre-treatment

Turkish coffee (24 brands) were purchased from local markets in Nicosia, TRNC. A photograph of the samples analyzed is given in **Figure 2.1**, while the names and their abbreviations are given in **Table 2.1**.


**Figure 2.1.** Turkish coffee samples.

**Table 2.1**: Symbols of the Turkish coffee samples.

| # | **Sample Name** |
|---|---|
| 1. | A1 |
| 2. | A2 |
| 3. | A3 |
| 4. | AIE |
| 5. | C1 |
| 6. | C2 |
| 7. | C3 |
| 8. | C4 |
| 9. | E1 |
| 10. | E2 |
| 11. | H |
| 12. | M |
| 13. | O1 |
| 14. | O3 |
| 15. | OS1 |
| 16. | OS2 |
| 17. | OZ1 |
| 18. | OZ2 |
| 19. | OZ3 |
| 20. | S1 |
| 21. | S2 |
| 22. | S3 |
| 23. | T1 |
| 24. | T2 |

### 2.4.1 Blending of samples

Few grams (approx. 20 g) of each coffee sample were blended using the coffee grinder to a very fine powder and preserved in well-sealed glass bottles until analysis.

### 2.4.2 Solid-liquid extraction

0.5 g of coffee was taken into a 50-mL volumetric flask and boiled for 4-5 min into deionized water (DI). The mixture was filtered while hot through cotton wool and the volume was completed to the mark with DI water after cooling down to room temperature. A portion (10 mL) was collected into a falcon tube and centrifuged for 2 min at 6000 rpm. The supernatant was collected (hereafter referred to as sample solution).

### 2.4.3 DLLME

A portion (1.0 mL) of the sample solution was taken into a falcon tube and completed to 6.0 mL with DI water; 3 mL of 10% (w/v) NaCl was added to make the final percentage of NaCl 3 % (w/v) in the solution. 100 µL of $H_3PO_4$were added and the solution was vortexed for 1 min. 100 µL of chloroform (CF) were added as the extraction solvent and 500 µL of ethanol (EtOH) as the disperser solvent. The solution was vortexed for 1 min and centrifuged for 2 min at 6000 rpm. The chloroform layer sedimenting at the bottom of the flask was completely collected and transferred into a microtube for back-extraction.

### 2.4.4 Back-extraction

The chloroform layer was back-extracted with 50 Mm NaOH in the mobile phase (40% v/v MeOH) by vortexing for 1 min and centrifuging for 2 min at 6000 rpm in a microtube. 20 µL of the upper aqueous layer were collected and injected into HPLC for analysis. A schematic diagram of the DLLME-BE procedure is given in **Figure 2.2**.

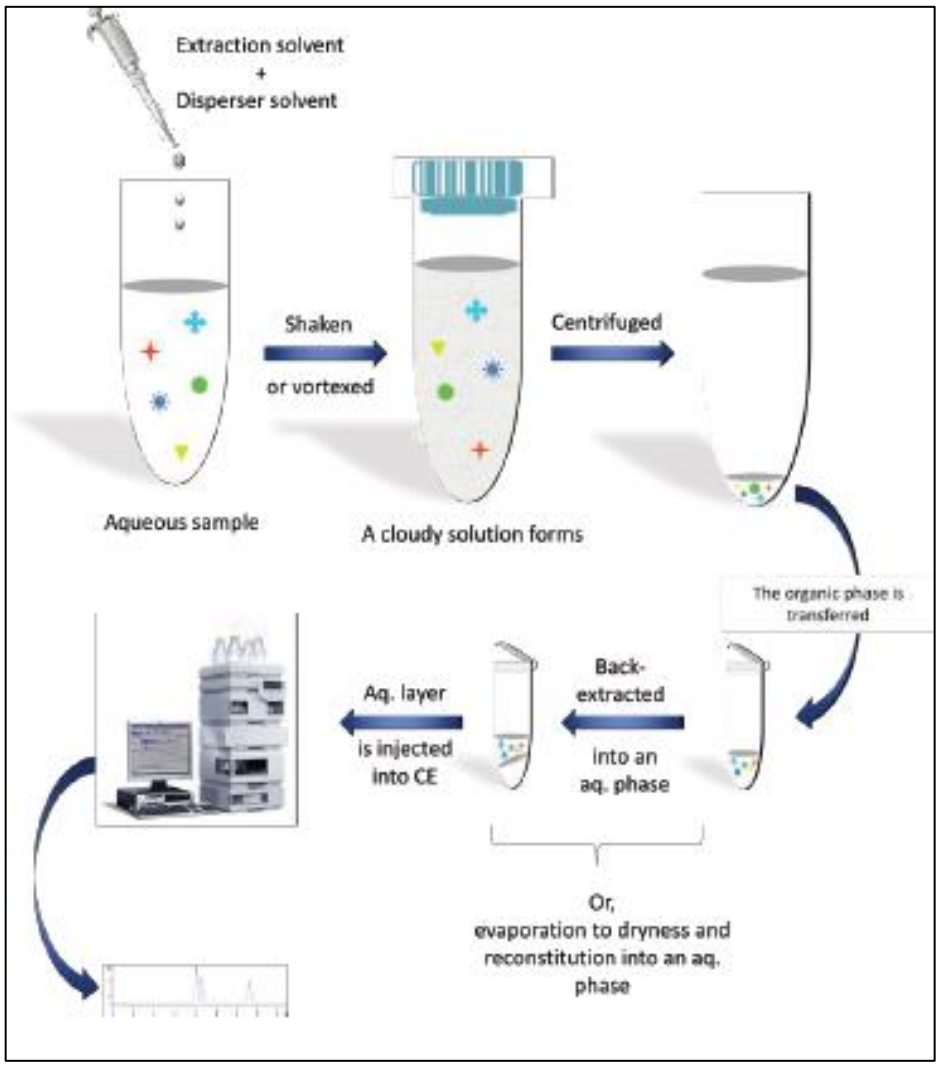


**Figure 2.2.** General DLLME procedure.

### 2.4.5 Standard addition method

In order to plot standard addition calibration graphs, 0.5 g of a pooled Turkish coffee sample (prepared by mixing equal masses of all samples) was weighed. To the solid, an appropriate volume from the 1000 mg $L^{-1}$ caffeine standard solution was added in a 50-mL volumetric flask to have spiked caffeine concentrations of 10, 20, 30, 40, 50 and 60 $mgL^{-1}$. The solutions were made up to the mark with DI water added and the mixture was boiled for 4-5 minutes. After boiling for 5 min, the mixture was filtered through a cotton

wool and the volume was made up again to 50 mL with DI water. 10 mL of this solutions, for each, were transferred into six test tubes and centrifuged for 2 minutes at 6000 rpm. To 1.0 mL from the supernatant obtained each taken tube after centrifugation, 6 mL of DI water, 3.0 mL of 10% NaCl and 100 µL $H_3PO_4$were added and the solution was vortexed for 1 minute. Then, 100µL of chloroform and 500µL of EtOH were added and the solution was vortexed again for 1 minute and centrifuged for 1 minute at 6000 rpm. In the back-extraction step, the sedimented phase (chloroform) was transferred completely into a 1.5 mL a microtube and 50 µL of the back extraction-solution (BES), composed of 50/50% (v/v) MeOH/50 mM NaOH, were added and the solution was vortexed for 60 s, followed by centrifugation at 6000 rpm for 2 min. This resulted into a two-phase system, the upper phase of which (containing the analyte) was collected for analysis with HPLC using optimized chromatographic conditions.

# CHAPTER 3
# RESULTS AND DISCUSSION

## 3.1 Selection of Maximum Absorption Wavelength ($\lambda_{max}$)

Selection of the wavelength of maximum absorption for TB, TP and CAF was necessary to have maximum sensitivity and robustness of the method. Literature shows that optimum wavelength for TB, TP and CAF is 280 nm [34].Injecting aqueous standards of TB, TP and CAF and monitoring their absorption in 3D plot (**Figure 3.1**)**,** UV absorption profiles (**Figure 3.2**) and isoabsorbance plot (**Figure 3.3**) showed that the three analytes had a maximum absorption wavelength ($\lambda_{max}$) of 273 nm. Hence, this wavelength was set optimum in subsequent experiments.

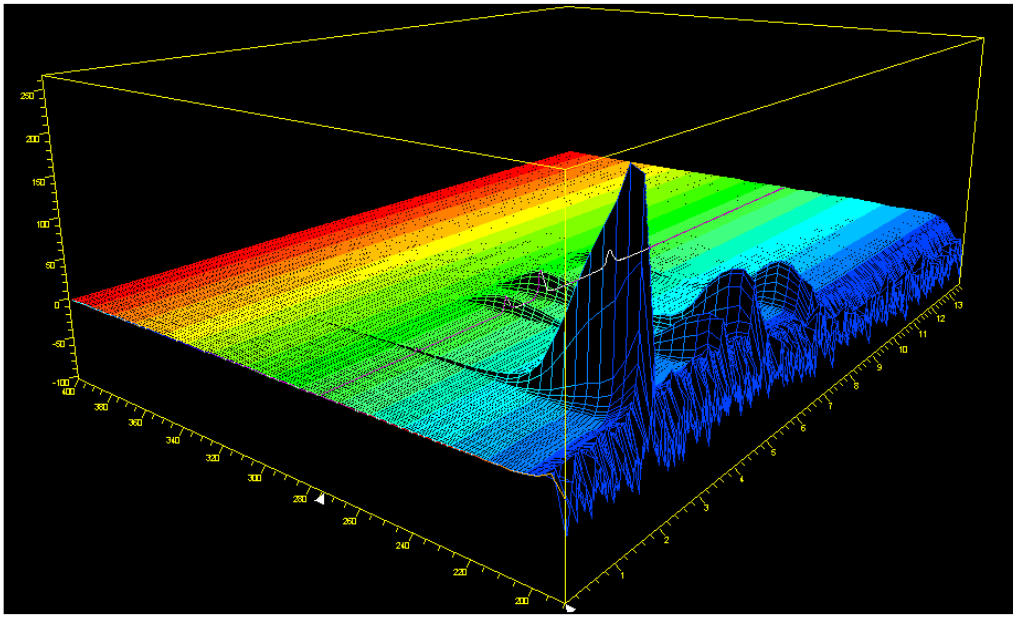

**Figure 3.1.** 3D plot of TB, TP and CF.

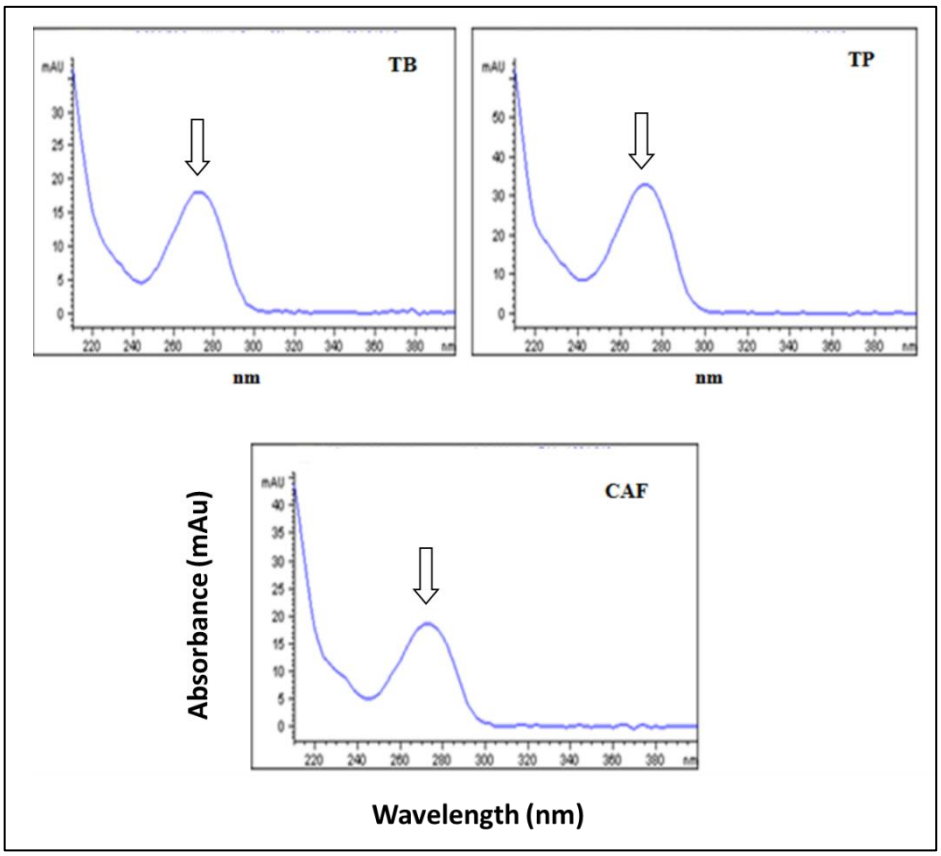


**Figure 3.2.** UV absorption profiles of TB, TP and CAF (at 50.0 mg $L^{-1}$ each prepared in the mobile phase).

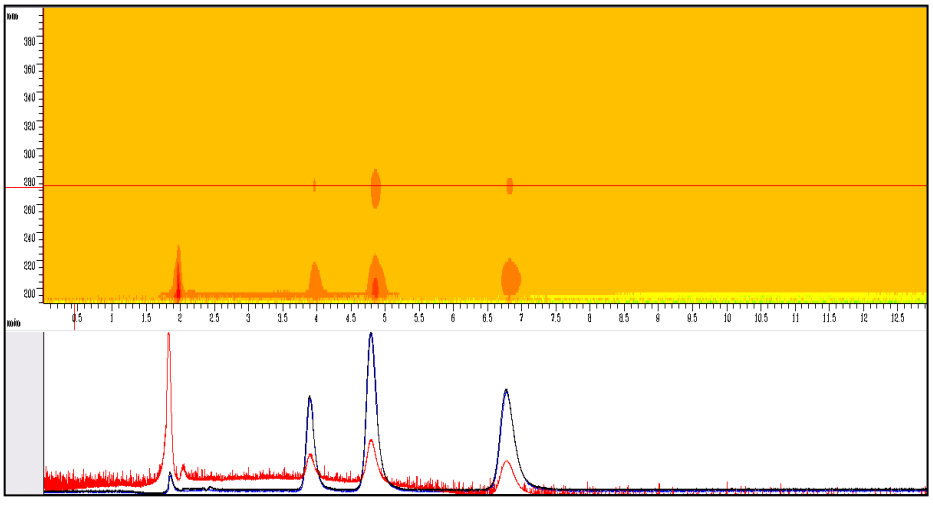

**Figure 3.3.** Isoabsorbance plot of a mixture of TB, CAF and TP (at 50.0 mg $L^{-1}$ each prepared in the mobile phase).

## 3.2 Optimization of HPLC Conditions

The systematic approach, described in **Section 1.12**, was applied in the optimization of HPLC conditions starting with the type of mobile phase. In this study, 40% of MeOH/$H_2O$ was used as the mobile phase composition.

### 3.2.1 Type of the column

With the use of Agilent Eclipse XDB-$C_{18}$ column [4.6 mm ID x 150 mm (5 µm)], the analytes could not be separated because of the closeness of their structures. The use of ZORBAX SB-Aq column [4.6 mm ID x 150 mm (5 µm)] drastically improved the resolution through changes of selectivity (α). It was stated by the manufacturer that Zorbax SB-Aq column are suitable for polar analytes. Since the analytes were polar, better separation was obtained with this column. Generally, it is not recommended to use more than 50% water in reversed-phase columns. However, the manufacturer also stated that Zorbax SB-Aq columns are compatible even with 100% water. A comparison between chromatograms obtained with both columns is shown in **Figure 3.4**.

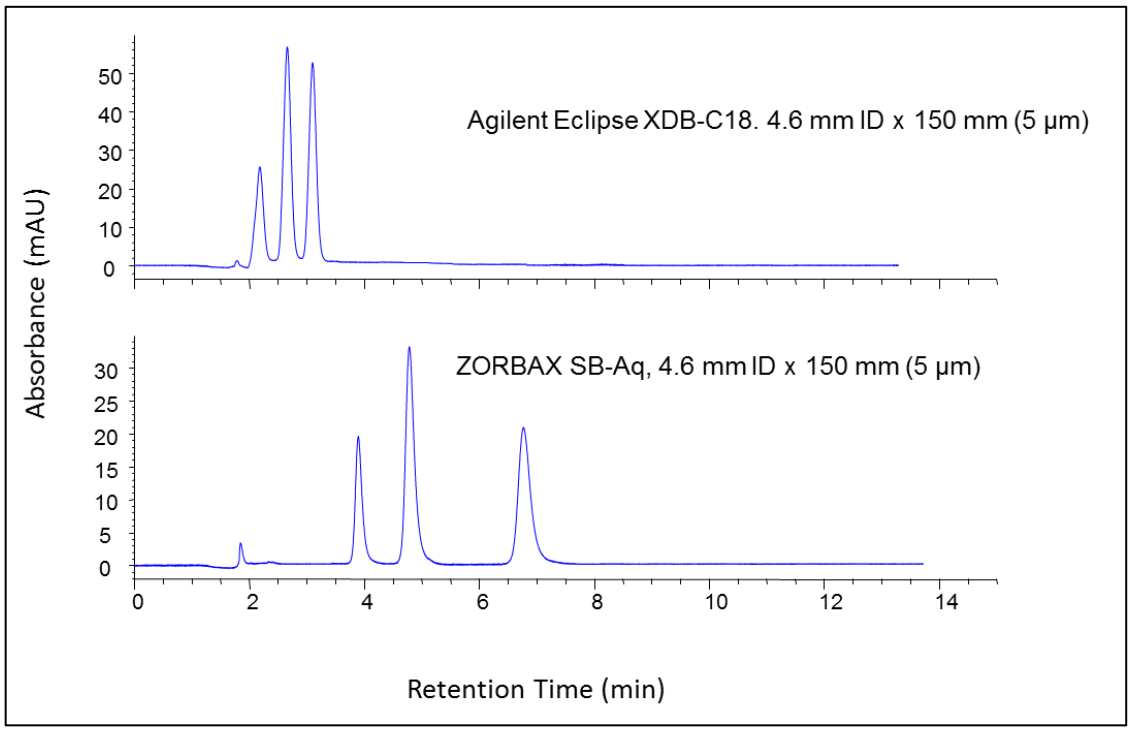


**Figure 3.4.** Effect of type of column on resolution.

### 3.2.2 Type and composition of the mobile phase (MP)

Acetonitrile (ACN) and MeOH were used for investigating the effect of type of the MP on the chromatographic behavior. MeOH is more polar than ACN and it was observed that the analytes were eluted faster from the column as the percentage of ACN in the MP was increased. In other words, $k'_{av}$ decreased upon increasing the percentage of ACN in the MP. Decreasing the percentage of ACN in the MP improved the chromatographic parameters but the chromatogram was not still acceptable because the peaks of TB and TP overlapped.

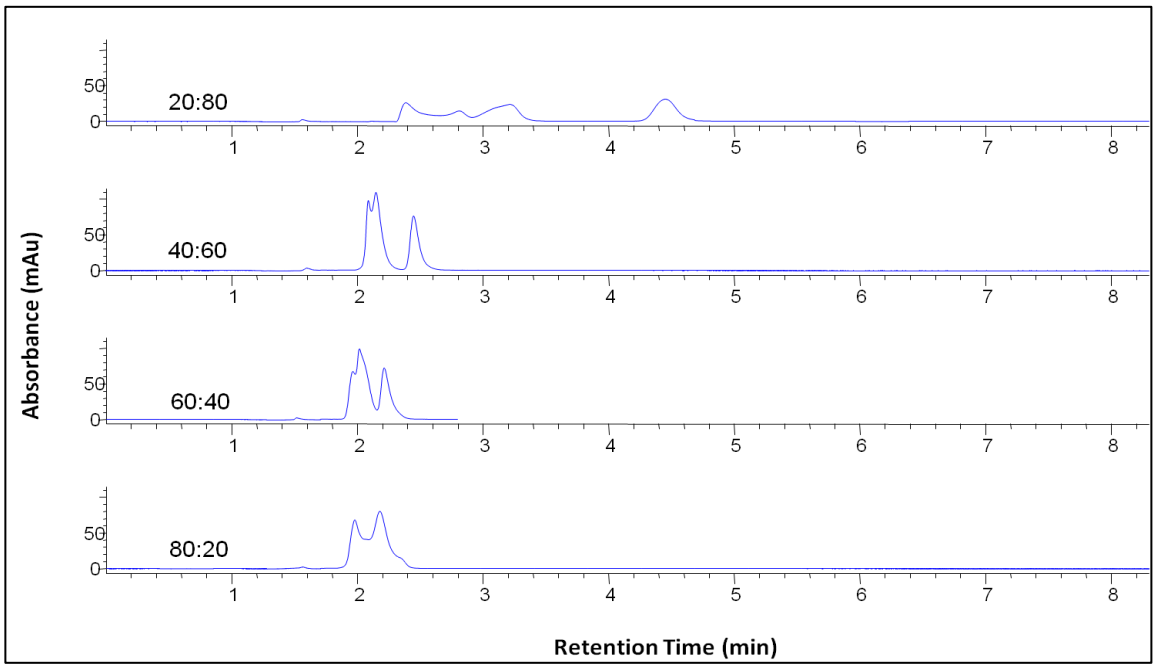


**Figure 3.5.** Chromatograms with decreasing ACN from 80% (down) to 20% (up).Conditions: Column, ZORBAX SB-Aq; Flow rate, 0.8 mL $min^{-1}$.

The effect of replacing ACN with MeOH in the mobile phase was investigated. Chromatographic behavior was studied under the following compositions of MeOH: 70, 60, 50, 40 and 30 and (v/v) in water. As shown in **Figure 3.6**, the use of MeOH, drastically improved selectivity (∝), resulting into a much higher resolution of all peaks.

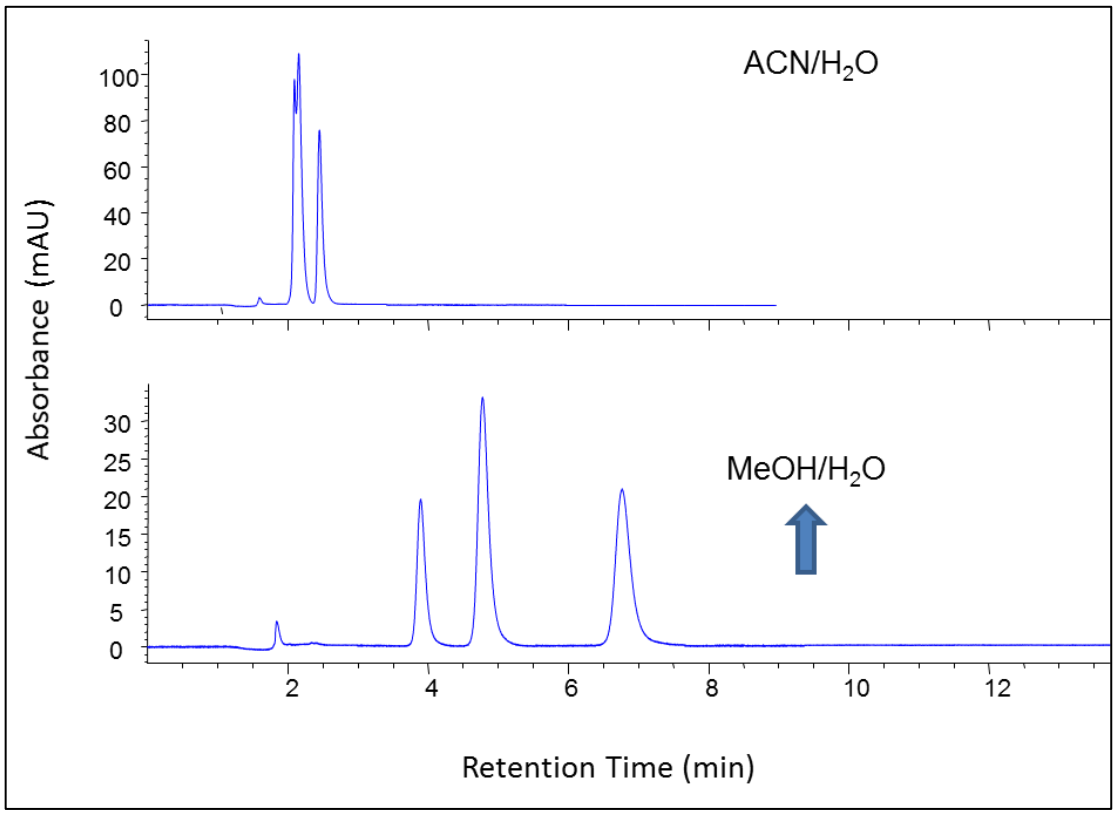


**Figure 3.6.** Effect of type of mobile phase. Conditions: Column, ZORBAX SB-Aq; Flow rate, 0.8 mL $min^{-1}$.

Increasing the percentage of MeOH in the MP, decreased its polarity. Since the analytes are polar, they were eluted faster at lower concentrations of MeOH. When the MeOH was 70% the retention time reduced and the analytes could not be separated. Since the polarity of $H_2O$ is higher than that of MeOH, when the percentage of MeOH is reduced in the MP, the polarity was increased. At 50% MeOH and less, the overlapping problem for TB and TP analytes was solved. In previous experiments with 70% MeOH, the peaks were overlapping. On the other hand, increasing the volume of $H_2O$ too much makes the MP too polar, elution was faster and a good chromatogram could not be achieved. Therefore, a better chromatogram could be obtained with 40% MeOH as shown in **Figure 3.6**. With this composition, $k'_{av}$ of the three analytes was fell in the ideal range of 5-7.

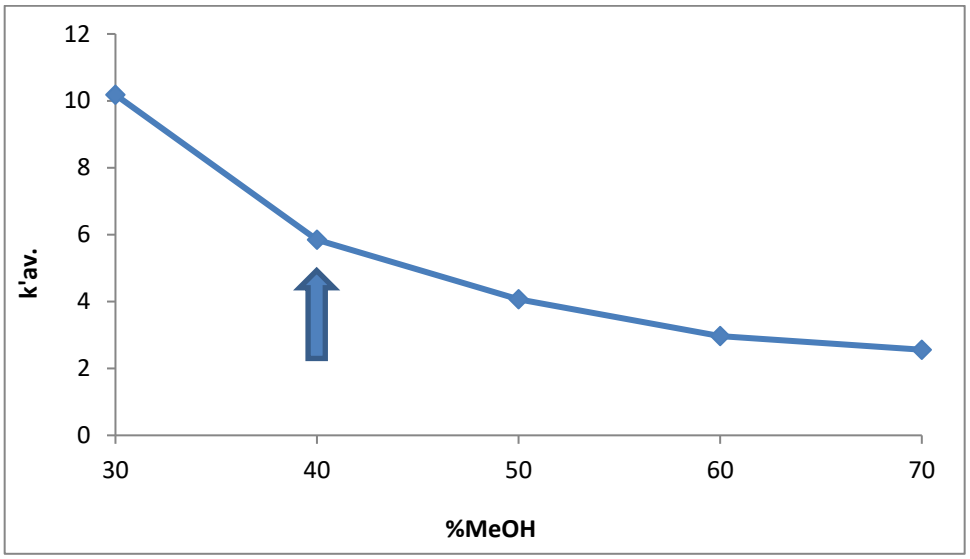


**Figure 3.7.** Optimum concentration of MeOH in the MP.

### 3.2.3 Effect of acid modifier in the MP

Acid modifiers are usually used to improve resolution or reduce retention time. The order of elution is due to polarity of the analytes. The more water, the faster the more polar analyte would be eluted. Since the analytes are basic, they would be present in their non-ionized form in acid medium. In other words, they would interact longer with the column, and retention times would slightly increase. The effect of adding an acid modifier to the MP (i.e., acetic acid) was investigated throughout varying the concentration in the range of 0.0 to 1.0% (v/v). It was observed that the addition of acetic acid to the MP had little effect (**Figure 3.8**). Hence, no acid was added in subsequent experiments.

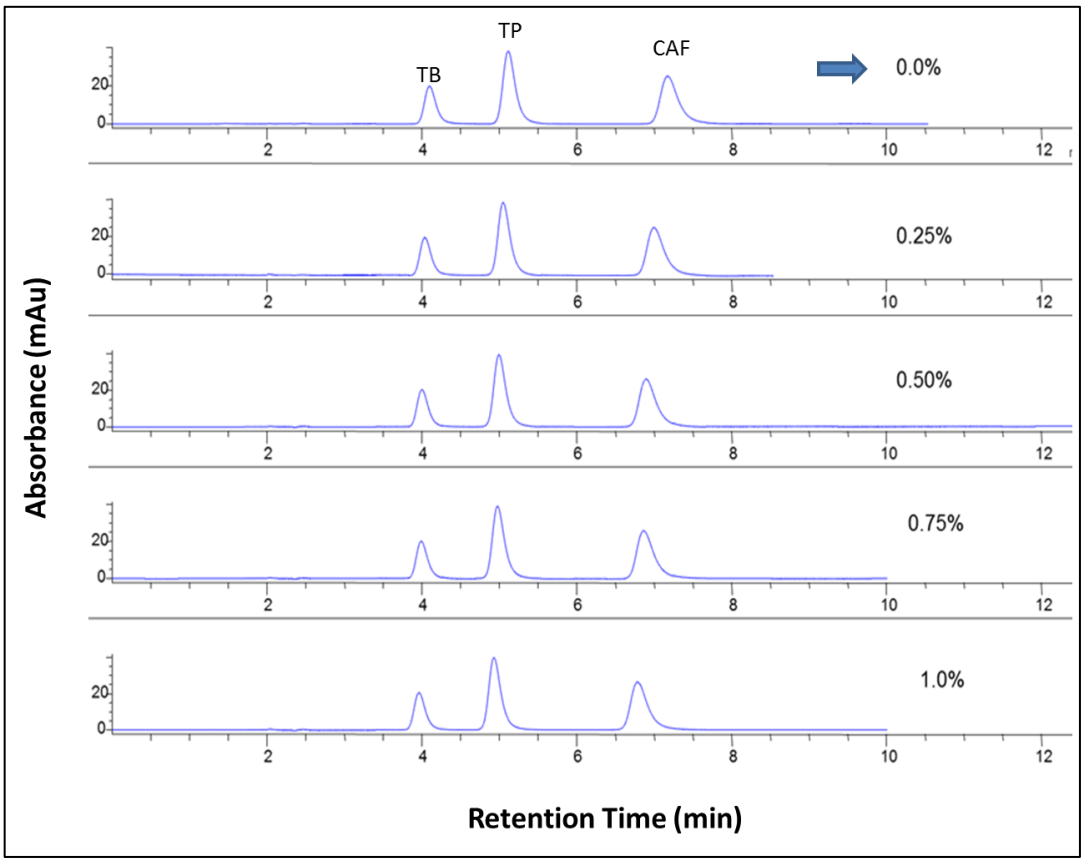


**Figure 3.8.** Effect of acid modifier

### 3.2.4 Effect of flow rate

The aim of determining the optimum flow rate was to reduce the retention time without affecting resolution. Increasing flow rate, increases the back-pressure in the column and can reduce the column lifetime. On the other hand, if the flow rate is low, the analysis time will increase. Peak area or retention time alone cannot be used as the basis for the selection of optimum condition for flow rate. This is due to the fact that increase in peak area and reduction in retention time can both reduce the resolution of the peaks. It is, therefore, necessary to use the corrected peak area to better account for the effect on resolution. This the factor is calculated by dividing the peak area by the retention time and plotting the ratio against flow rate. The optimum flow rate is then selected as the point

where the trend is constant. As can be seen from **Figure 3.9**, flow rate did not have a significant effect on corrected peak area throughout the studied range of 0.6 to 1.0 $mLmin^{-1}$. Hence, 0.8 $mLmin^{-1}$was considered optimum for the flow rate as a compromise between retention time, peak area and back-pressure.

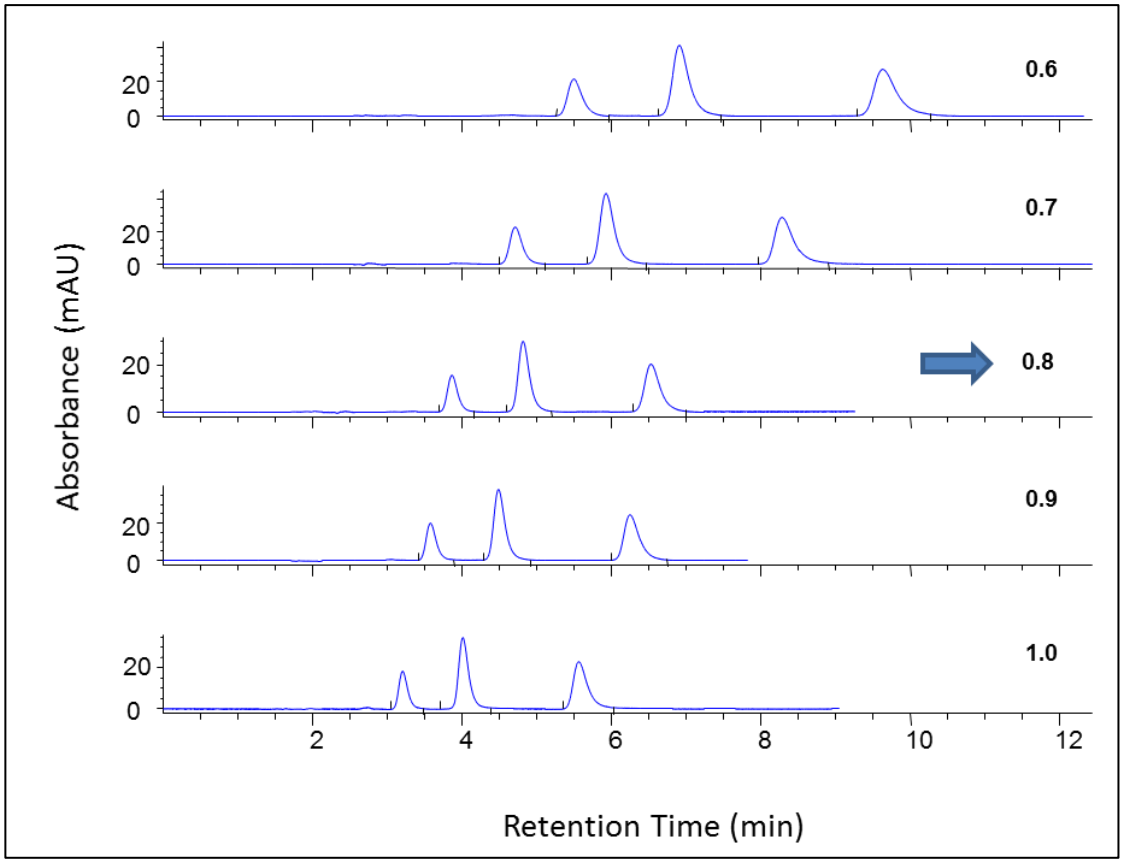


**Figure 3.9.** Effect of flow rate.

### 3.2.5 Optimum HPLC conditions

The optimum HPLC conditions in this study are summarized in **Table 3.1**.

**Table 3.1.** Optimum HPLC Conditions.

| | | |
|---|---|---|
| Physical parameters | Column | ZORBAX SB-Aq, 4.6 mm ID x 150 mm (5 µm) |
| | Flow Rate | 0.80 $mLmin^{-1}$ |
| | Temperature | Room temp. |
| | Detector/wavelength | UV. 273 nm (BW 4). Reference none |
| | Injection volume | 20 µL |
| Chemical parameters | Mobile phase | MeOH:$H_2O$ 40:60 (% v/v) |
| | pH | Not adjusted (no HAc added) |

## 3.3 Optimization of the Extraction Methods

Two extraction methods were considered, DLLME with back-extraction (DLLME-BE) and DLLME with evaporation-to dryness under a stream of nitrogen (DLLME-ETD).

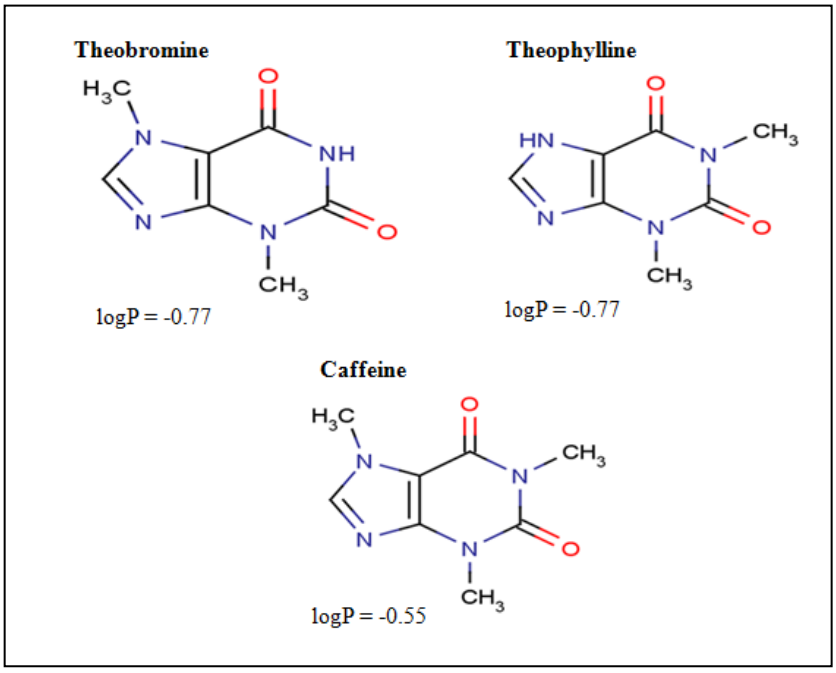


**Figure 3.10.** Structures and **log*P*** values of TB, TP and CF.

In order to determine the extraction parameters, logP values and microspecies distribution of the analytes at different pH provide valuable information for extractions and separation with RP-HPLC are not sufficient. Having low log*P* values means that the analytes are polar. Hence, RP-HPLC is the most suitable mode HPLC for their separation. However, the polar mobile phases used in this mode would not be miscible with the final organic extract having the enriched analytes. Thus, this extract needs to be replaced with another that is compatible with the MP. In order to do so, two possibilities are available, evaporation-to-dryness and reconstitution of into the MP, or the analytes can be back-extracted into an aqueous solution. In order to understand whether the analytes can be back-extracted, one needs to consider their microspecies distribution and polarities. Microspecies distribution curves of CAF, TB, and TP are given in **Figure 3.11**.

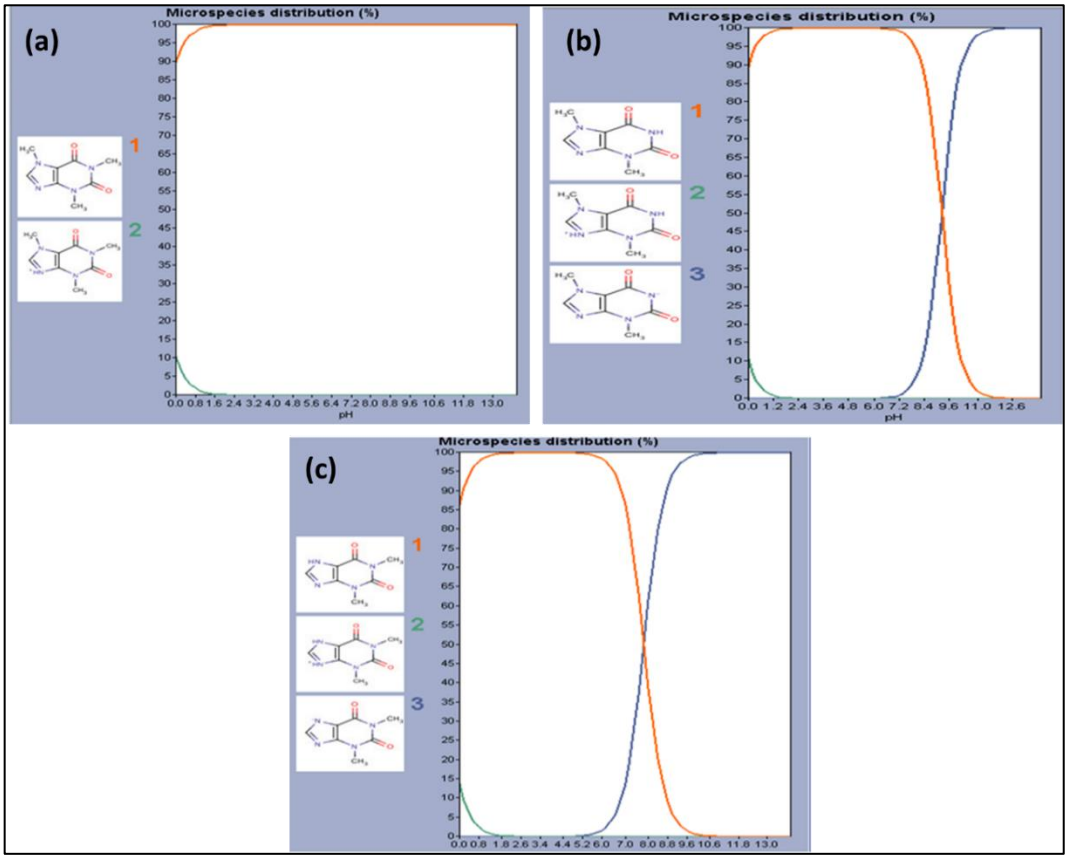


**Figure 3.11.** Microspecies distribution curves of (a) CAF, (b) TB, and (c) TP.

It is clear from **Figure 3.11** that CAF is almost present non-ionizable throughout the whole pH scale. TB and TP are ionizable at basic (above pH 9.0) and non-ionizable in acidic media. Therefore, in order to be able to extract the analytes into an organic solvent (acceptor phase), the sample solution (donor phase) needs to be acidified. It can also be possible, especially for TB and TP, to back-extract the analytes into a basic aqueous acceptor phase, which can be compatible with the MP.

## 3.4 DLLME

DLLME is a powerful miniaturized extraction technique, which provides high extraction efficiency due to the dispersion of the water-immiscible extraction solvent into fine droplets with very large surface area through the use of a disperser solvent. The infinitely large surface area of contact shortens the extraction time significantly. In some cases, salt addition to the aqueous sample solution can improve the recovery of extractable analytes. The most influential extraction parameters in DLLME were studied in details and optimized.

### 3.4.1 Optimization of the type of extraction solvent in DLLME

The selection of a suitable extraction solvent is a very important step in the optimization of DLLME conditions. There are four requirements for appropriate extraction solvent. It should have [35].

- higher density than water for easy collection, however, low-density solvents can also be used
- good chromatographic behavior
- high extraction capability of analytes
- low water solubility
- low toxicity
- high purity
- low price

In this experiment, high-density [chloroform(CF), diphenylether (DPE)],and two low-density [1-dodecanol (1-DO) and 1-undecanol (1-UN)]solvents were examined as the extraction solvents.CF gave the highest extraction efficiency as indicated by highest peak area of caffeine. It should be noted here that DLLME was optimized for caffeine only because preliminary experiments with the coffee samples revealed that none of them contained TB or TP at detectable concentrations.

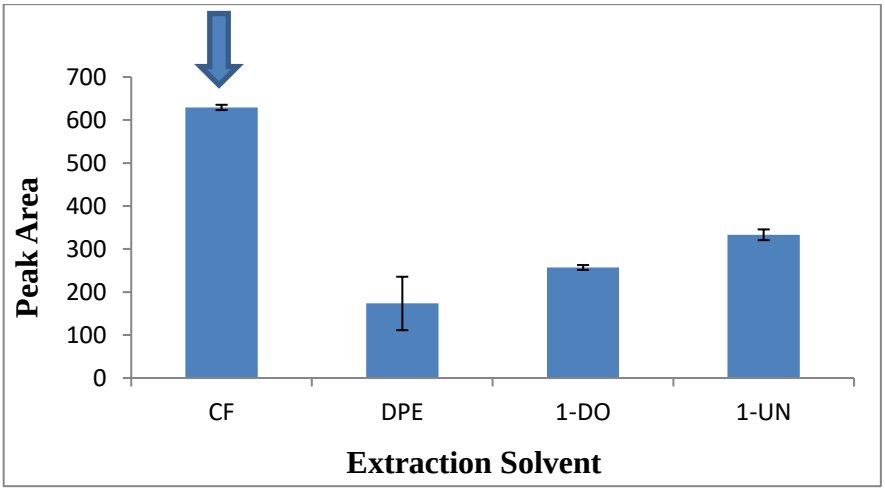


**Figure 3.12.** Effect of extraction solvent type on extraction efficiency.

### 3.4.2 Optimizing the volume of the extraction solvent

The volume of the extraction solvent may significantly affect the extraction efficiency. To evaluate the effect of extraction solvent volume, different volumes of CF (50-300 µL) used with a constant volume of ACN (2.0 mL) were subjected to the same DLLME procedure. As shown in **Figure 3.13**, by increasing the CF volume from 50 to100µL, peak area increased and then decreased afterwards. This trend can be explained by the fact that increasing the volume of CF increases its ability to extract more of the analyte due. However, higher volumes of the extraction solvent (i.e., 100 µL and above in this case) resulted in dilution of the analyte in the organic solvent. In addition, since the volume of the BES was kept constant, increasing the CF phase, decreased the ability of BES to back-extract the analyte from CF. As a result, the final trend is actually a resultant of both

extraction and back-extraction methods. Therefore, 100 µL of CF was selected as the optimal solvent extraction volume. It is worthy to note that the collected volume of CF after extraction was 110 ± 10 µL (n = 10).

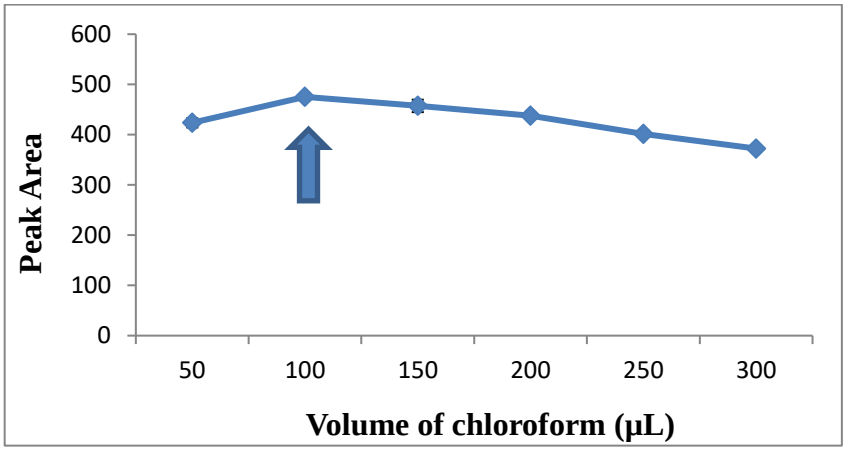


**Figure 3.13.** Effect of volume of the extraction solvent.

### 3.4.3 Optimization of the extraction time

In DLLME, extraction time can be considered as the time interval between the injection of the extraction solvent into the sample solution and the centrifugation time, which corresponds to the vortex time in this experiment. The vortex time was varied from 0 to 120 s within 30 s intervals. The maximum extraction was achieved at 60 s, after which the extraction efficiency remained constant (**Figure 3.11)**. Hence, 60 s was set optimum for further experiments.

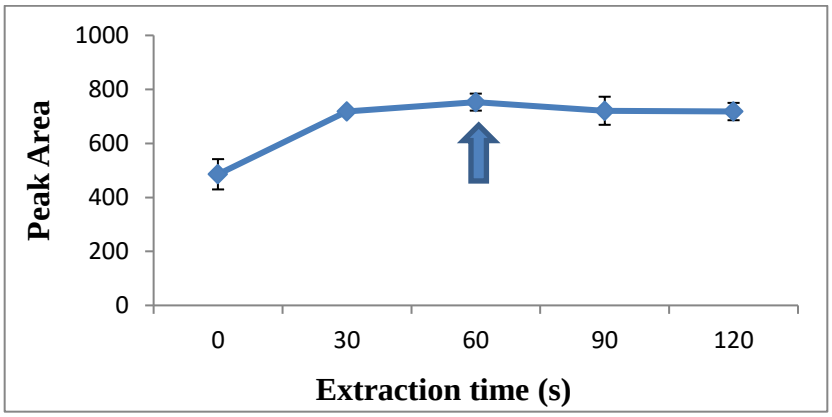


**Figure 3.14.** Effect of extraction time.

### 3.4.4 Effect of disperser solvent

The disperser solvents must be miscible with both the sample solution and extraction solvent. Choosing a suitable disperser solvent is very important to achieve high extraction efficiency. The use of a disperser solvent to disperse the water-immiscible organic solvent, directly affects the formation of a cloudy solution which decreases the interfacial tension between water and extracting solvent and increases the extraction efficiency [36]. In this experiment, EtOH provided the highest extraction efficiency as compared to the other solvents [i.e., MeOH, ACN and acetone (ACT)] as shown in **Figure 3.15**.

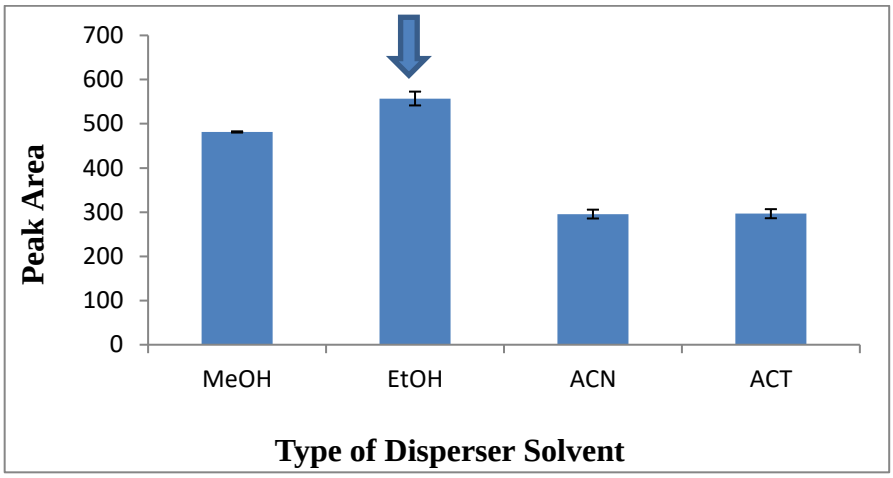


**Figure 3.15.** Effect of type of disperser solvent on extraction efficiency

### 3.4.5 Effect of the volume of the disperser solvent (EtOH)

In order to find the optimum volume of disperser solvent, the volume was varied from 250 mL to 1750 mL of EtOH with 250 mL intervals. The optimum volume was 500 mL, as shown in **Figure 3.16**. Peak area increased upon increasing the volume from 200 to 500 mL, after which it started to decrease gradually. This trend can be explained as follows. At low volumes of the disperser solvent, dispersing ability of the extraction solvent would be low. On the other hand, at higher-than-necessary volumes, the solubility of the analytes in the aqueous solution increases due to the presence of the disperser solvent. In addition, the solubility of extraction solvent would also increase, which decreases its recovery from the sample solution. Based on the above mentioned results, 500 mL of ethanol were used as the volume of disperser solvent in subsequent experiments.

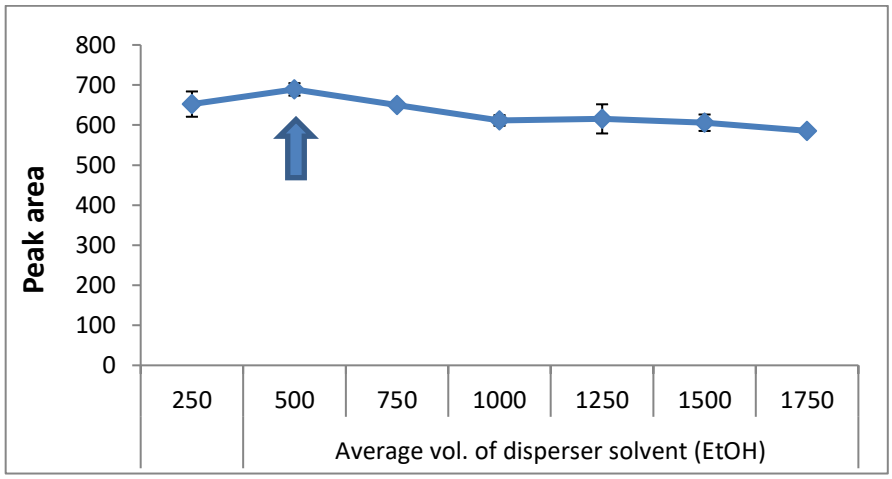


**Figure 3.16.** Effect of volume of the disperser solvent on extraction efficiency.

### 3.4.6 Effect of salt addition (NaCl)

Salt addition to sample solutions is a usually used technique in LLE to enhance phase separation and extraction of hydrophobic analytes by increasing the polarity of the donor phase. Salt addition can also increase the extraction efficiency by decreasing the solubility of analytes in the aqueous phase and salting them out into the organic phase. This experiment was performed by adding NaCl to the sample solution at increasing concentrations from no addition to 7% w/v. The addition of NaCl gave a positive effect on the extraction efficiency until 3% (w/v), but beyond this point, it gave a negative effect as can be seen in **Figure 3.17**. Thus, 3% (w/v) of NaCl was considered as an optimum value for subsequent experiments.

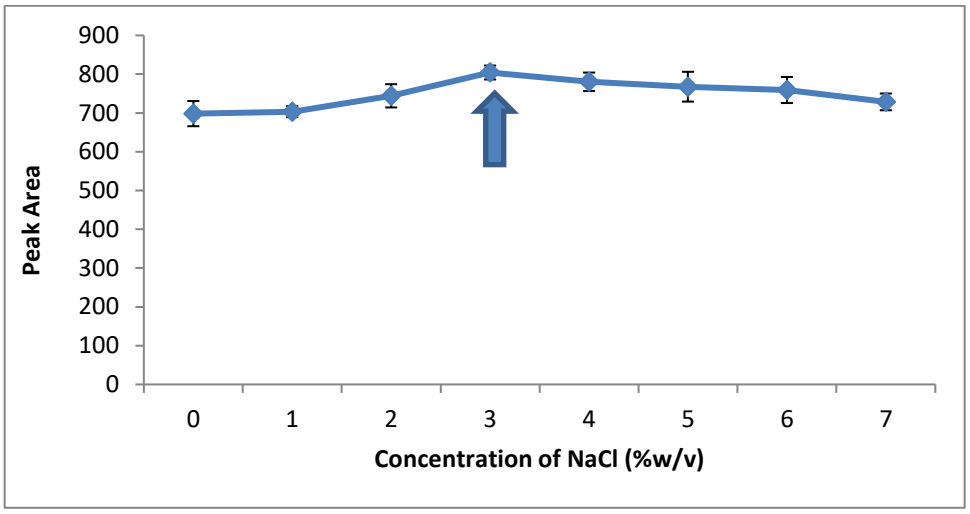


**Figure 3.17.** Effect of salt addition on extraction efficiency.

## 3.5 Optimization of Back-Extraction Conditions

After the analyte was extracted into CF, it was necessary to back-extract it before injection into the instrument. A basic solution (i.e., 50 Mm NaOH) was used as the back-extraction solution (BES). Evaporation-to-dryness and reconstitution into the MP was not preferred due to drawbacks associated with this method such as loss of analyte, long time, exposure to organic solvent vapor, etc.

### 3.5.1 Effect of back-extraction volume

The effect of BES volume on extraction efficiency was evaluated from 50 µL to 300 µL extraction efficiency was the highest at 50 µL, increasing BES volume more than 50 µL decreased the extraction efficiency (**Figure 3.18**). Lower volumes than 50 µL could not be used due to difficulty in collecting the extraction solvent and/or loss of phase separation in some cases.

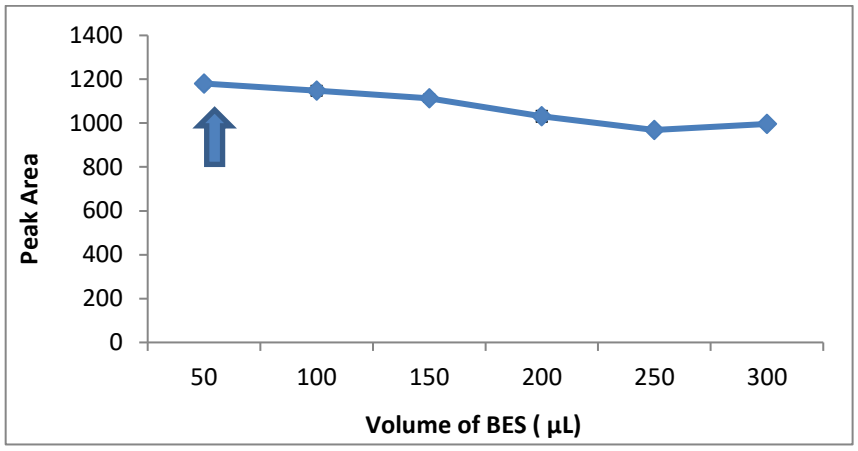


**Figure 3.18.** Effect of back-extraction volume.

### 3.5.2 Effect of back-extraction time

To study the effect of back-extraction time on the extraction efficiency, i.e., vortex time during back-extraction, was evaluated starting from 0 to 120 s with 30 s intervals. At 60 s, the peak area reached the maximum point as shown in **Figure 3.19**. This value (i.e., 60 s) was taken as the optimum back-extraction time.

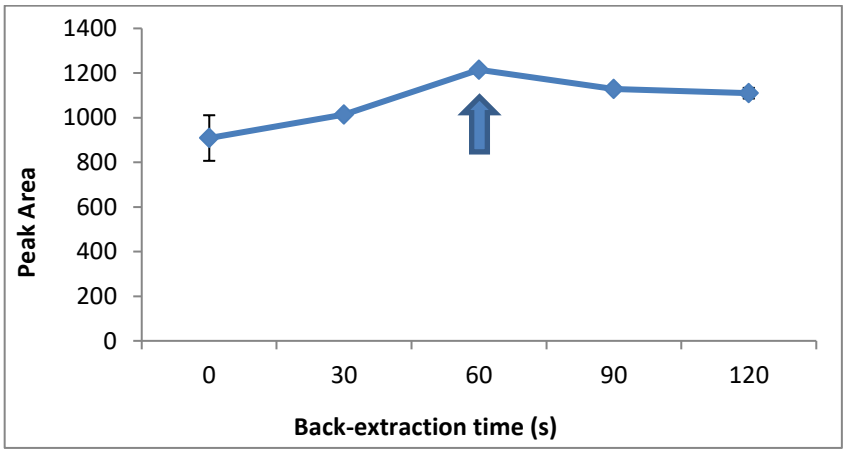


**Figure 3.19.** Effect of back-extraction time.

### 3.5.3 Optimum DLLME-BE conditions

**Table 3.2.** Optimum DLLME-BE Conditions.

| | | |
|---|---|---|
| DLLME | Extraction Solvent | Chloroform |
| | Volume of Extraction Solvent | 100 µL |
| | Disperser Solvent | EtOH |
| | Volume of Disperser Solvent | 500 µL |
| | Acidification | with 100 µL $H_3PO_4$ |
| | Extraction Time | 60s |
| BE | Back Extraction Solution | 50/50% (v/v) MeOH/50 mM NaOH |
| | Volume of Back Extraction Solution | 50 µL |
| | Back-Extraction Time | 60 s |

## 3.6 Peak Characterization

Peak characterization was done by injecting individual standards into HPLC and comparing their retention times with the mixed standard solution, each standard at a concentration of 5.0 $mgL^{-1}$. Since the identity of the standards is now known, each peak was assigned a name based on the retention time of the standards. The chromatograms obtained with the standards and the mixtures are given in **Figure 3.20**.

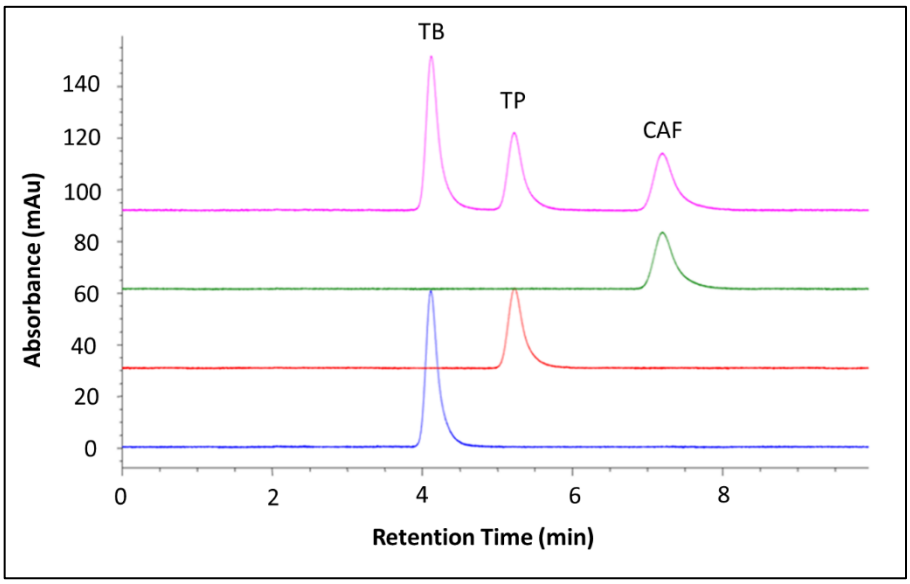


**Figure 3.20.** Peak characterization of TB, TP and CF.

## 3.7 Calibration, Quantitation and Figures of Merit

Under optimum DLLME-HPLC and conditions, the analytical figures of merit including linear dynamic range (LDR), limit of detection (LOD), limit of quantitation (LOQ), coefficient of determination ($R^2$) and percentage relative standard deviation (%RSD) were obtained to evaluate the method performance for the determination of caffeine in the Turkish coffee samples.

### 3.7.1 Standard addition calibration

Under optimized HPLC conditions, different caffeine concentrations ranging from 0 to 60 mg $L^{-1}$ prepared in the BES were injected (without extraction) and the results of the peak areas obtained were plotted (**Figure 3.21**). This graph was necessary to calculate the concentration of caffeine in the BES after applying DLLME, which helps to calculate EF, %ER, ER and %RR. In addition, the slope of this graph when compared with the slopes of standard addition calibration curves, can give an idea about matrix effect in the real samples.

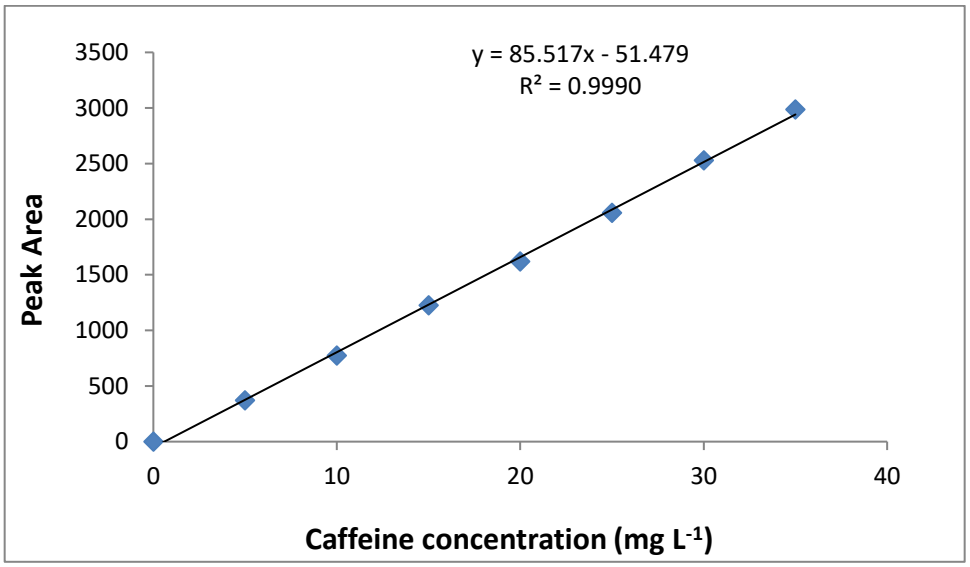


**Figure 3.21.** Standard external aqueous calibration graph for CF.

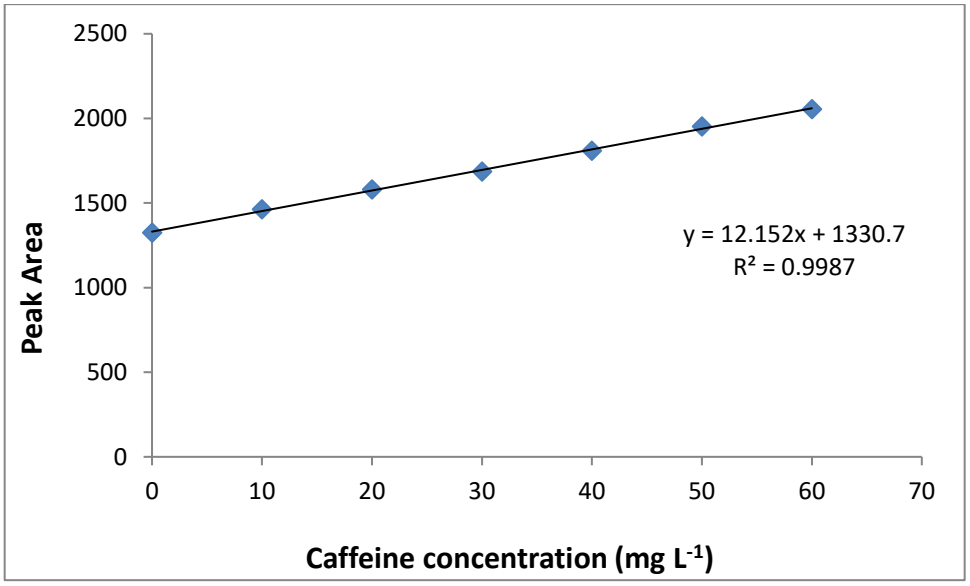


**Figure 3.22.** Standard addition calibration graph for CF in pooled coffee sample.

### 3.7.2 Representative chromatograms

Representative chromatograms obtained with unspiked coffee samples containing the lowest and highest concentrations of caffeine are shown in **Figure 3.23**.

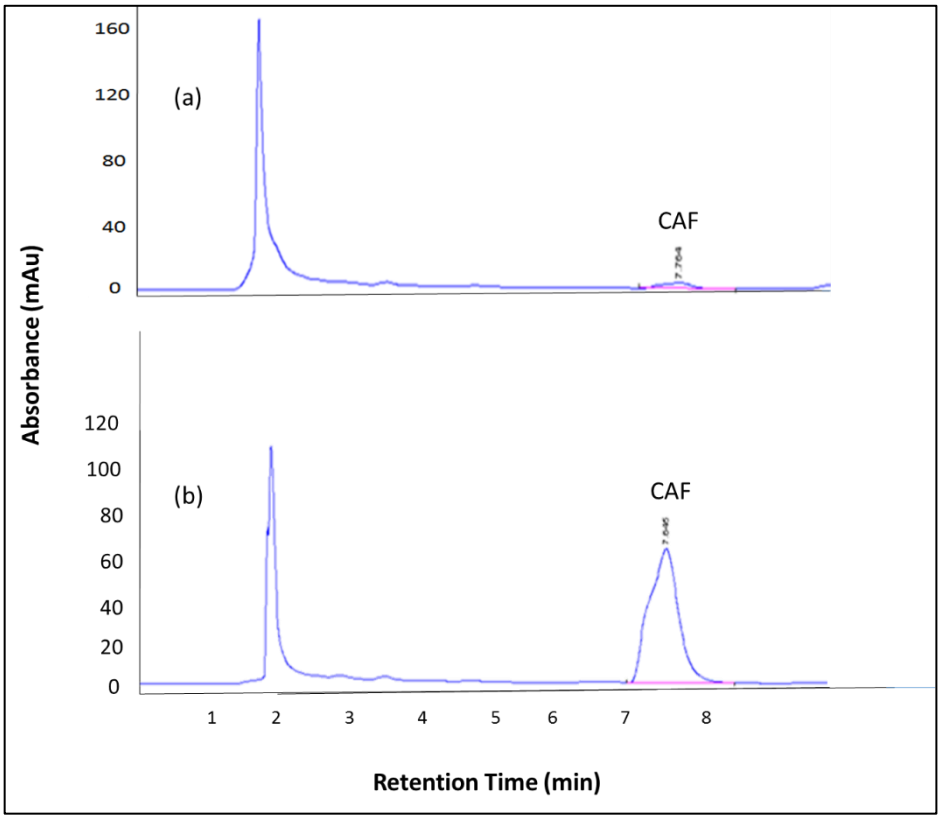


**Figure 3.23:** Representative chromatograms of unspiked coffee samples containing the lowest (a) and highest concentrations of caffeine (b).

**Table 3.2.** Analytical performance of DLLME-HPLC.

| Calibration | Regression equation[a] | $R^2$ | LOD[b] (mg $L^{-1}$) | LOQ[c] (mg $L^{-1}$) | LDR[d] (mg $L^{-1}$) | %RSD[e] | |
|---|---|---|---|---|---|---|---|
| | | | | | | Intraday | Interday |
| Aq. calibration | $y = 85.517(\pm 0.74)x - 51.479(\pm 15.48)$ | 0.9990 | 0.5 | 1.8 | 1.8-35.0 | 1.5 | 2.6 |
| Pooled | $\mathbf{y = 12.152(\pm 0.13)x + 1330.7(\pm 4.52)}$ | 0.9987 | 1.1 | 3.6 | 3.6-60.0 | 2.9 | 5.5 |

[a]$\text{Peak area} = \text{slope}(\pm\text{SD}) * \text{caffeine concentration}(\text{mgL}^{-1}) + \text{intercept}(\pm\text{SD})$

[b]Limit of detection

[c]Limit of quantitaion

[d]Linear dynamic range

[e]Percentage relative standard deviation $(n = 3)$

**Table 3.3.** Calculated concentrations of caffeine in Turkish coffee samples.

| **Sample** | **Concentration Found (mg $L^{-1}$)** | **Concentration Found (µg $L^{-1}$)** |
|---|---|---|
| C4 | 8.89 | 0.89 |
| A3 | 91.70 | 9.17 |
| T1 | 97.19 | 9.72 |
| OS1 | 101.66 | 10.17 |
| S2 | 102.64 | 10.17 |
| T2 | 104.53 | 10.45 |
| H | 105.75 | 10.58 |
| A2 | 107.13 | 10.71 |
| C2 | 110.86 | 11.09 |
| O3 | 111.56 | 11.16 |
| OZ1 | 113.55 | 11.36 |
| E2 | 114.62 | 11.46 |
| OZ3 | 115.19 | 11.52 |
| S1 | 116.64 | 11.66 |
| S3 | 120.51 | 12.05 |
| OS2 | 123.12 | 12.31 |
| A1 | 126.71 | 12.67 |
| OZ2 | 128.58 | 12.86 |
| C1 | 132.57 | 13.26 |
| E1 | 134.39 | 13.44 |
| C3 | 138.29 | 13.83 |
| M | 141.10 | 14.11 |
| AIE | 154.03 | 15.40 |
| O1 | 154.05 | 15.40 |

## 3.8 Comparison with Other Preconcentration Methods

Efficiency of the proposed DLLME–HPLC method for the extraction of caffeine was compared with other reported methods taking into account parameters such as extraction time, amount of sample used, total volume of organic solvents consumed per sample, LOD and LOQ. In comparison with other methods, the main advantages of this extraction method were rapidness, simplicity and cost effectiveness. As summarized in **Table 3.4**, the extraction time was only 2 min in this study, which was much shorter than the other extraction methods due to the large surface area of contact between the extraction solvent and the sample solution during emulsion formation. The other methods required a longer time for equilibrium to be established. In addition, this method required 0.5 mL of organic solvents for analysis, which is also much less compared with other methods. Acceptable LODs and LOQs were achieved considering the high concentration of caffeine generally found in coffee. MS and MS–MS detectors are inherently more sensitive than UV hence lower LODs or LOQs can be in the literature using these detectors. Yet, they are much more expensive and complicated than UV.

**Table 3.4:** Comparison of DLLME-HPLC with other methods for the extraction of caffeine.

| **Extraction method** | **Extraction Time (min)** | **$V_{org.}$[a] (mL)** | **LOD[b] (µg mL$^{-1}$)** | **LDR[c] (µg mL$^{-1}$)** | **Ref.** |
|---|---|---|---|---|---|
| LLE[d] | 240 | 50 | 0.30 | 0.4–8.2 | [37] |
| UAE[e] | 30 | 17.5 | 0.0028 | 1–500 | [38] |
| UAE[e] | 20 | ~33 | 0.004 | – | [39] |
| MAE[f] | 10 | 12.5 | 0.17 | 0.5–80 | [40] |
| DLLME | 2.0 | 0.5 | 1.1 | 3.6-60.0 | This study |

[a] Volume of organic solvents consumed per sample
[b] Limit of detection
[c] Linear dynamic range
[d] Liquid-liquid extraction
[e] Ultrasound-assisted extraction
[f] Microwave-assisted extraction

# CHAPTER 4
# CONCLUSION AND RECOMMENDATION

In this study, dispersive liquid-liquid microextraction (DLLME) was combined with a back-extraction step prior to high-performance liquid chromatography (HPLC) for the determination of caffeine in Turkish coffee. The developed DLLME method required minimum volume of organic solvent. Both extraction time in DLLME and retention time in HPLC had to be fast enough to be able to be applied for routine analysis. Therefore, DLLME and HPLC conditions were optimized in details to achieve this goal. In addition, the combination of DLLME with HPLC had to be simple enough requiring the use of basic HPLC instrument that can be found in most laboratories, hence the preference for isocratic elution.

Despite the fact that neither theobromine nor theophylline were found in the real coffee samples analyzed, HPLC conditions were optimized for the three analytes, making this method readily applicable for samples containing the three analytes. The total extraction time of DLLME was 60 s, while the back-extraction time was 60 s making the total extraction time to be 120 s while HPLC analysis time was 8 min.

All of the 24 different brands of Turkish coffee samples analyzed contained caffeine at varying concentrations in the wide range of 0.89-15.40 μg $g^{-1}$. Theobromine and theophylline were not detected in any of the studied samples.

Since only 100 μL of chloroform were required per sample for extraction of caffeine, this method can be considered as environmentally friendly. Considering the figures of merit and the “clean” chromatograms obtained with real samples, DLLME-HPLC can be considered as highly selective, sensitive and reproducible. These results proved that DLLME combined with a simple back-extraction step prior to HPLC could be of great

interest in the determination of caffeine in foods and beverages in routine food analysis laboratories.

## REFERENCES


1. Frizzarin RM, Maya F, Estela JM, Cerda V. Fully-automated in-syringe dispersive liquid-liquid microextraction for the determination of caffeine in coffee beverages. *Food Chemistry.* 2016;212:759-767.
2. Nawrot P, Jordan S, Eastwood J, Rotstein J, Hugenholtz A, Feeley M. Effects of caffeine on human health. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment.* 2003;20(1):1-30.
3. Jabbar SB, Hanly MG. Fatal Caffeine Overdose A Case Report and Review of Literature. *American Journal of Forensic Medicine and Pathology.* 2013;34(4):321-324.
4. Fabrizio C, Desiderio M, Coyne RF. Electrocardiogram Abnormalities of Caffeine Overdose. *Circulation-Arrhythmia and Electrophysiology.* 2016;9(7).
5. Heckman MA, Weil J, de Mejia EG. Caffeine (1, 3, 7-trimethylxanthine) in Foods: A Comprehensive Review on Consumption, Functionality, Safety, and Regulatory Matters. *Journal of Food Science.* 2010;75(3):R77-R87.
6. Wikoff D, Welsh BT, Henderson R, et al. Systematic review of the potential adverse effects of caffeine consumption in healthy adults, pregnant women, adolescents, and children. *Food and Chemical Toxicology.* 2017;109:585-648.
7. Algharrawi KHR, Summers RM, Subramanian M. Production of theobromine by N-demethylation of caffeine using metabolically engineered E. coli. *Biocatalysis and Agricultural Biotechnology.* 2017;11:153-160.
8. Mitchell ES, Slettenaar M, Van der Meer N, et al. Differential contributions of theobromine and caffeine on mood, psychomotor performance and blood pressure. *Physiology & Behavior.* 2011;104(5):816-822.
9. Fernandes TAP, Aguiar JP, Fernandes AI, Pinto JF. Quantification of theophylline or paracetamol in milk matrices by high-performance liquid chromatography. *Journal of Pharmaceutical Analysis.* 2017;7(6):401-405.
10. Shannon M. Life-threatening events after theophylline overdose - A 10-year prospective analysis. *Archives of Internal Medicine.* 1999;159(9):989-994.

11. Salinas-Vargas ME, Canizares-Macias MP. On-line solid-phase extraction using a C-18 minicolumn coupled to a flow injection system for determination of caffeine in green and roasted coffee beans. *Food Chemistry.* 2014;147:182-188.
12. Bi W, Zhou J, Row KH. Decaffeination of coffee bean waste by solid-liquid extraction. *Korean Journal of Chemical Engineering.* 2011;28(1):221-224.
13. Rezaee M, Assadi Y, Hosseinia MRM, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid-liquid microextraction. *Journal of Chromatography A.* 2006;1116(1-2):1-9.
14. Quigley A, Cummins W, Connolly D. Dispersive Liquid-Liquid Microextraction in the Analysis of Milk and Dairy Products: A Review. *Journal of Chemistry.* 2016.
15. Alshana U, Goger NG, Ertas N. Dispersive liquid-liquid microextraction combined with field-amplified sample stacking in capillary electrophoresis for the determination of non-steroidal anti-inflammatory drugs in milk and dairy products. *Food Chemistry.* 2013;138(2-3):890-897.
16. Leong MI, Huang SD. Dispersive liquid-liquid microextraction method based on solidification of floating organic drop for extraction of organochlorine pesticides in water samples. *Journal of Chromatography A.* 2009;1216(45):7645-7650.
17. Skrlikova J, Andruch V, Balogh IS, Kocurova L, Nagy L, Bazel Y. A novel, environmentally friendly dispersive liquid-liquid microextraction procedure for the determination of copper. *Microchemical Journal.* 2011;99(1):40-45.
18. Zhang PP, Shi ZG, Yu QW, Feng YQ. A new device for magnetic stirring-assisted dispersive liquid-liquid microextraction of UV filters in environmental water samples. *Talanta.* 2011;83(5):1711-1715.
19. Alshana U, Ertas N, Goger NG. Determination of parabens in human milk and other food samples by capillary electrophoresis after dispersive liquid-liquid microextraction with back-extraction. *Food Chemistry.* 2015;181:1-8.
20. Bahadir Z, Bulut VN, Mermer A, Demirbas N, Duran C, Soylak M. The separation-preconcentration and determination of ultra-trace gold in water and solid samples by dispersive liquid-liquid microextraction using 4-ethyl-1(2-(4-(4-

nitrophenyl)piperazin-1-yl)acetyl)thiosemicarbazide) as chelating agent and flame atomic absorption spectrometry. *Journal of the Iranian Chemical Society.* 2018;15(6):1347-1354.

21. Mahmoudpour M, Pilevar Z, Javanmardi F, Taram F, Mousavi MM. PAHs in toasted bread: determination using microwave-assisted extraction and dispersive liquid-liquid microextraction followed by high-performance liquid chromatography. *Analytical Methods.* 2018;10(20):2398-2404.
22. Mirparizi E, Rajabi M, Asghari A. Simple determination of some antidementia drugs in wastewater and human plasma samples by tandem dispersive liquid-liquid microextraction followed by high-performance liquid chromatography. *Journal of Separation Science.* 2018;41(10):2214-2220.
23. Wang J, Shi YL, Cai YQ. A highly selective dispersive liquid-liquid microextraction approach based on the unique fluorous affinity for the extraction and detection of per- and polyfluoroalkyl substances coupled with high performance liquid chromatography tandem-mass spectrometry. *Journal of Chromatography A.* 2018;1544:1-7.
24. Bazregar M, Rajabi M, Yamini Y, Arghavani-Beydokhti S, Asghari A. Centrifugeless dispersive liquid-liquid microextraction based on salting-out phenomenon followed by high performance liquid chromatography for determination of Sudan dyes in different species. *Food Chemistry.* 2018;244:1-6.
25. Kamankesh M, Shahdoostkhany M, Mohammadi A, Mollahosseini A. Fast and sensitive low density solvent-based dispersive liquid-liquid microextraction method combined with high-performance liquid chromatography for determining cholecalciferol (vitamin D3) in milk and yogurt drink samples. *Analytical Methods.* 2018;10(9):975-982.
26. Shen X, Liang J, Zheng LX, Lv QZ, Wang H. Application of dispersive liquid-liquid microextraction for the preconcentration of eight parabens in real samples and their determination by high-performance liquid chromatography. *Journal of Separation Science.* 2017;40(22):4385-4393.

27. Yan HY, Wang H. Recent development and applications of dispersive liquid-liquid microextraction. *J Chromatogr A.* 2013;1295:1-15.
28. Trujillo-Rodriguez MJ, Rocio-Bautista P, Pino V, Afonso AM. Ionic liquids in dispersive liquid-liquid microextraction. *Trac-Trends in Analytical Chemistry.* 2013;51:87-106.
29. Haeri SA, Abbasi S, Sajjadifar S. Bio-dispersive liquid liquid microextraction based on nano rhaminolipid aggregates combined with magnetic solid phase extraction using Fe3O4@ PPy magnetic nanoparticles for the determination of methamphetamine in human urine. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences.* 2017;1063:101-106.
30. Gaborieau M, Castignolles P. Size-exclusion chromatography (SEC) of branched polymers and polysaccharides. *Analytical and Bioanalytical Chemistry.* 2011;399(4):1413-1423.
31. Zhang CH, Rodriguez E, Bi C, et al. High performance affinity chromatography and related separation methods for the analysis of biological and pharmaceutical agents. *Analyst.* 2018;143(2):374-391.
32. Arai K, Terashima H, Aizawa S, et al. Simultaneous Determination of Trigonelline, Caffeine, Chlorogenic Acid and Their Related Compounds in Instant Coffee Samples by HPLC Using an Acidic Mobile Phase Containing Octanesulfonate. *Analytical Sciences.* 2015;31(8):831-835.
33. Belguidoum K, Amira-Guebailia H, Boulmokh Y, Houache O. HPLC coupled to UV-vis detection for quantitative determination of phenolic compounds and caffeine in different brands of coffee in the Algerian market. *Journal of the Taiwan Institute of Chemical Engineers.* 2014;45(4):1314-1320.
34. Liu HC, Shao JL, Li QW, Li YG, Yan HM, He LZ. Determination of Trigonelline, Nicotinic Acid, and Caffeine in Yunnan Arabica Coffee by Microwave-Assisted Extraction and HPLC with Two Columns in Series. *Journal of Aoac International.* 2012;95(4):1138-1141.

35. Rezaee M, Yamini Y, Faraji M. Evolution of dispersive liquid-liquid microextraction method. *Journal of Chromatography A.* 2010;1217(16):2342-2357.
36. Alshana U, Goger NG, Ertas N. Ultrasound-assisted emulsification microextraction for the determination of ephedrines in human urine by capillary electrophoresis with direct injection. Comparison with dispersive liquid-liquid microextraction. *Journal of Separation Science.* 2012;35(16):2114-2121.
37. Xia Z, Ni Y, Kokot S. Simultaneous determination of caffeine, theophylline and theobromine in food samples by a kinetic spectrophotometric method. *Food Chemistry.* 2013;141(4):4087-4093.
38. Hadad GM, Salam RAA, Soliman RM, Mesbah MK. Rapid and simultaneous determination of antioxidant markers and caffeine in commercial teas and dietary supplements by HPLC-DAD. *Talanta.* 2012;101:38-44.
39. Rostagno MA, Manchon N, D'Arrigo M, et al. Fast and simultaneous determination of phenolic compounds and caffeine in teas, mate, instant coffee, soft drink and energetic drink by high-performance liquid chromatography using a fused-core column. *Analytica Chimica Acta.* 2011;685(2):204-211.
40. Rahim AA, Nofrizal S, Saad B. Rapid tea catechins and caffeine determination by HPLC using microwave-assisted extraction and silica monolithic column. *Food Chemistry.* 2014;147:262-268.