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.

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## ACKNOWLEDGEMENT

Foremost, I would like to gratefully and sincerely thank Prof. Dr. Filiz Meriçli for her guidance and assistance in getting my graduate career started on the right foot. Her understanding, patience, and most importantly, her motherly advices throughout my graduate studies at Near East University was excellent and superb. Also to Assist. Prof. Dr. Usama Alshana for his stylish and exceptional supervision of this thesis. His mentorship was paramount in providing a well-rounded experience consistent to my long-term career goals. He encouraged me not only to grow as an experimentalist and an analytical chemist but also as an instructor and an independent thinker. I am not sure many graduate students are given the opportunity to develop their own individuality and self-sufficiency by being allowed to work with such independence. It is worth mentioning here, to register my appreciation to him for introducing to me the DLLME. For everything you have done for me, Prof. Dr. Filiz Meriçli and Assist. Prof. Dr. Usama Alshana, I thank you very much for this superb supervision. I would also like to thank all of the members of the departments of Analytical Chemistry and Pharmacognosy at the faculty of Pharmacy, Near East University, especially Prof. Dr. Ali Meriçli for giving me the opportunity to learn a lot about the chemical constituents in many different plant species through experiments. Also to Prof. Dr. Ülkü Anik for inviting me to Muğla Sıtkı Koçman University to learn more about the electroanalytical methods. It is a memory that I will never forget and I am most grateful. Additionally, I am very grateful to Tuğba Ören, Jibrin Yusuf Bala, Yudum Tepeli, Sima and Semra Altunterim for their support and encouragements to bring this research to fruition.

At this point, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study. This accomplishment would not have been possible without them. Also to my family and friends for showing me love and support throughout my studies. Thank you very much.

Finally and most importantly, my sincere gratitude goes to the gov't of Kano State under the able leadership of Distinguished Senator Engr. Dr. Rabi'u Musa Kwankwaso (FNSE) for initiating and supporting the schorlaship scheme which I immensely benefited from. Thank you very much. To God be the glory.

***To my lovely parents,***
***Alh. Aliyu Isah Danmaraya and Haj. Aisha Aliyu Danmaraya***

## ABSTRACT

Caffeine is an important natural compound that occurs in many plant species, although it is mostly derived from guarana (*Paulinia cupana*), tea leaves (*Thea sinensis*), kola nuts (*Cola acuminata),* mate tea (*Ilex paraguariensis*), coffee beans (*Coffea arabica* and *Coffea robusta*), and cocoa beans (*Theobroma cacao).* Recently caffeine and caffeine-containing natural sources (e.g. Guarana, green tea, green coffee, mate, etc.) have been added to weight loss herbal products. Therefore, it is important to monitor caffeine in beverages, foods and some herbal products by establishing precise, simple, rapid and low-cost analytical method. Since the introduction of dispersive liquid–liquid microextraction (DLLME) in 2006, it has gained widespread acceptance as a simple, fast and miniaturized sample cleanup and analyte preconcentration technique. Owing to its simplicity of operation, rapidity, low cost, high recovery and low consumption of organic solvents and reagents, it has been applied for the determination of a vast variety of organic and inorganic compounds in different matrices. In this study, DLLME was applied prior to high-performance liquid chromatography (HPLC) to extract caffeine from red and white kola nuts (*Cola acuminata).* Optimum chromatographic conditions were achieved with an Agilent Eclipse XDB-C18, 4.6 mm ID x 150 mm (5 µm) column, a mobile phase of 70:30 (v/v) methanol/water (pH 7.0) at a flow rate of 1.0 mL $min^{-1}$, a temperature of 25 °C and sample injection volume of 20 µL. Caffeine was monitored using a diode-array detector at 273 nm. Optimum DLLME conditions were as follows: 250 µL chloroform (as extraction solvent), 0.5 mL methanol (as disperser solvent) and a 60 s extraction time. Back-extraction of caffeine from chloroform into a 40:60 (v/v) methanol/water solution within 90 s of vortexing time enabled direct injection of the extract into HPLC. The method's limit of detection (LOD) and limit of quantitation (LOQ) were determined as 0.56 and 1.86 µg $mL^{-1}$, respectively. The calibration graph was linear over the range of 1.86 to 30.0 µg $mL^{-1}$ with a coefficient of determination ($R^2$) of 0.9951 and %RSD lower than 3.3%. DLLME was demonstrated to be an efficient, cheap and effective for the extraction of caffeine from kola nuts prior to its quantitation by HPLC.



**Keywords**: Caffeine, DLLME, Extraction, HPLC, Optimization

# ÖZET

Kafein önemli bir doğal bileşik olup başlıca guarana (*Paulinia cupana*),çay yaprakları (*Thea sinensis*), kola tohumları (*Cola nitida, Cola acuminata*), mate çayı (*Ilex paraguariensis*), kahve çekirdeklerinde (*Coffea arabica, Coffea robusta*) ve az miktarda da kakao tohumlarında (*Theobroma cacao*) bulunmaktadır. Son yıllarda kafein ve kafein içeren droglar ( e.g. guarana, yeşil çay, yeşil kahve, mate, vb.) bitkisel zayıflama preparatlarına eklenmektedir. Bu nedenle, kafeinin yiyecek ve içecek vb ürünlerde, hassas, basit, hızlı ve düşük maliyetli bir analitik yöntem ile saptanması çok önemlidir.

Dispersif Sıvı-Sıvı Mikroekstraksiyon (DLLME) yöntemi, yayınlandığı 2006 yılından beri, basit, hızlı ve minimize numune temizleme ve ön zenginleştirme tekniği olarak yaygın kabul görmektedir. Yöntem, uygulama basitliği, hızlı, düşük maliyetli oluşu, az miktarda organik çözücü ve reaktif tüketimi ile, çok çeşitli organik ve inorganik bileşiklerin, farklı matrislerde belirlenmesinde uygulanmaktadır.

Bu çalışmada, yüksek performanslı sıvı kromatografi (HPLC) analizi için, kırmızı ve beyaz kola (*Cola acuminata*) tohumlarından kafein ekstraksiyonu DLLME yöntemi ile yapılmıştır.

Optimum Kromatografik koşullar; Agilent Eclipse XDB-$C_{18}$, 4.6 mm iç çap × 250 mm (5 µm) kolonda, 1.0 mL $dak^{-1}$'lik bir akış hızında, % 70 su (h/h), metanol (pH 7.0) bir hareketli faz ile elde edilmiştir (25 ° C sıcaklık ve 20 uL numune enjeksiyon hacmi). Kafein 273 nm'de diode-array dedektörü ile izlenmiştir. Optimum DLLME koşulları aşağıdaki gibidir: 250 µl kloroform (ekstraksiyon çözücü olarak), 0.5 µl metanol (dispersif çözücü olarak) ve ekstraksiyon süresi: 60 s. Kafeinin kloroformdan % 40 h/h metanol su ile 90 s dairesel çalkayarak geri ekstraksiyonu ile HPLC'ye doğrudan enjeksiyon yapılabilmiştir.

Yöntemin belirtme sınırı (LOD) ve saptama sınırı (LOQ) sırasıyla 0,56 ve 1,86 µg $mL^{-1}$ olarak belirlenmiştir. Kalibrasyon grafiği 1.86-30.0 µg $ml^{-1}$, bir belirleme katsayısı 0.9951 ($R^2$) ve %RSD daha düşük % 3.3 ile aralığında doğrusaldır. DLLME yönteminin HPLC analizi öncesi kola tohumlarından kafein ekstraksiyonunda etkili, ucuz ve etkin olduğu gösterilmiştir.



**Anahtar Kelimeler:** DLLME, Ekstraksiyon, HPLC, Kafein, Kola tohumu, Optimizasyon

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| ACRONYM | MEANING |
|---|---|
| **1-DO** | 1-Dodecanol |
| **1-UN** | 1-Undecanol |
| **AALLME** | Air assisted liquid-liquid microextraction |
| **BES** | Back-extraction solution |
| **CCE** | Column chromatographic extraction |
| **CE** | Capillary electrophoresis |
| **CF** | Chloroform |
| **CSD(s)** | Carbonated soft-drink(s) |
| **CTC** | Carbon tetrachroride |
| **DAD** | Diode-array detection |
| **DDSME** | Drop-to-drop solvent microextraction |
| **DI** | Deionized water |
| **DLLME** | Dispersive liquid-liquid microextraction |
| **DLLME-SFO** | DLLME based on solidifaction of floating organic droplet |
| **DDLLME** | Displacement DLLME |
| **DNA** | Deoxyribonucleic acid |
| **DPE** | Diphenyl ether |
| **ECD** | Electron capture detection |
| **EE** | Extraction efficiency |
| **EF** | Enrichment factor |
| **ESI-IMS** | Electrospray ionization-ion mobility spectrometry |
| **FDA** | Food and drug administration |
| **FID** | Flame ionization detection |
| **FT-IR** | Fourier transform-infrared spectrophotometry |
| **FLD** | Fluorescence detection |
| **GC** | Gas chromatography |
| **GC-MS/FID** | Gas chromatography-mass spectrometry/flame ionization detection |
| **GRAS** | Generally recognized as safe |

| | |
|---|---|
| **HF-LPME** | Hollow fiber liquid phase microextraction |
| **HPLC** | High performance liquid chromatography |
| **IC** | Ionic chromatography |
| **IL(s)** | Ionic liquid(s) |
| **IL-DLLME** | Ionic liquid based on DLLME |
| **IOC** | International olympic committee |
| **IUPAC** | International union of pure and applied chemistry |
| **LIF** | Laser-induced fluorescence |
| **LPE** | Liquid phase extraction |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantitation |
| **MS** | Mass spectrometry |
| **NAS** | National academy of sciences |
| **PB-EIMS** | Particle beam-electron ionization mass spectrometry |
| **SA-DLLME** | Surfactant assisted-DLLME |
| **SDME** | Single drop microextraction |
| **SFE** | Supercritical fluid microextraction |
| **SI-DLLME** | Sequential injection on-line DLLME |
| **SPE** | Solid phase microextraction |
| **ST-DLLME** | Solvent terminated-DLLME |
| **TLN** | Toluene |
| **UA-DLLME** | Ultrasound assisted DLLME |
| **UKFSA** | United kingdom food standard agency |
| **USFDA** | United states food and drugs administration |
| **UV** | Ultra-violet detection |
| **VA-DLLME** | Vortex assisted DLLME |

# CHAPTER 1

# INTRODUCTION

## 1.1 History and Occurrence of Kola nut

Kola nut is the fruit of the kola tree, an innate of tropical rainforests of Africa. The Kola nut is a caffeine-containing nut of evergreen trees of the genus *Cola*, essentially from the *Cola acuminata* and *Cola nitida* species. The nuts of these species are essential for their tonic and stimulating effects. Kola nut as a natural stimulant is typically consumed in different parts of the world because of these stimulatory properties [1]. There is a confirmation that intra-African trade of kola nuts dates back to at least the 14$^{th}$ Century, with strong written history of African exports to England and the U.S. which dates back to the mid-19$^{th}$ Century. It was in 1886 that the druggist, John S. Pemberton, from Atlanta, Georgia, formulated the brain tonic drink by combining erthroxylum coca leaves extract (ECLE) and cola seed extract for use as a headache and hangover remedy. Due to its cocaine contents and risk for addiction, ECLE was used as a brain tonic formulation which leads to the production of the popular soft drink, Coca Cola®. In its 1977 survey of industry on food additives in the U.S., the US National Academy of Sciences (NAS) indicated that the first reported use of kola nut extract was in 1935 (NAS, 1979) [2].

In Nigeria and some parts of west Africa, kola nuts are often used as a sign of peace, friendship, hospitality (to honour guests), and are also important in various social ceremonies and religious activities [1]. They have also been used in traditional medications such as aphrodisiac and appetite suppressants. The biological effects of the kola nuts extract have been credited to its caffeine content even when the caffeine content in the extract has not been characterized [1].

**Figure 1.** Red and white kola nut seeds *(Cola nitida)*

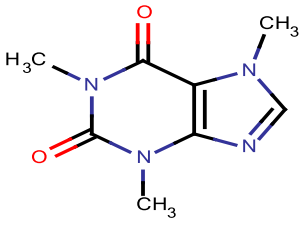
**Figure 2.** Bitter kola nut seeds *(Cola acuminata)*

## 1.2 Caffeine and Its Occurrence

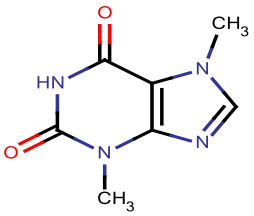
Caffeine has a systematic International Union of Pure and Applied Chemistry (IUPAC) name as 1,3,7-trimethylxanthine, and is the most widely consumed methylxanthine alkaloid [3]. Pure anhydrous caffeine exists as odorless, bitter taste white powder with a melting point of 235–238 °C (It has a molecular weight of 194.19 g $mol^{-1}$, the point at which caffeine sublimes is 178 °C at atmospheric pressure, has a pH of 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mm Hg at 178°C, vapor density 6.7). Caffeine is moderately soluble in water at room temperature (2 g per 100 mL), but very soluble in boiling water (66 g per 100 mL). It is also moderately soluble in ethanol (1.5 g per100 mL). It is weakly basic ($pK_a \approx 0.6$) requiring strong acid to protonate it. Caffeine does not contain any stereogenic centers and hence is classified as an achiral molecule [4].

The xanthine center of caffeine contains two fused rings, a pyrimidinedione and imidazole. The pyrimidinedione contain two amide functional groups that exist predominately in a zwitterionic resonance-the location from which the nitrogen atoms are double bonded to their adjacent amide carbon atoms. Thus, all the six atoms within the pyrimidinedione ring system are $sp^2$ hybridized and planar. Hence, the fused 5, 6 ring centers of caffeine contain a total of ten π-electrons, which according to Hückel's rule is aromatic. Paraxanthine, theophylline, and theobromine are the metabolites of caffeine.

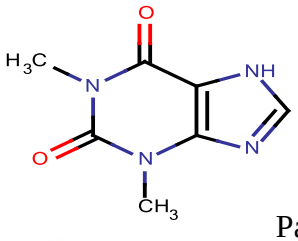
Caffeine occurs naturally in many plant species, although it is mostly derived from guarana (*Paulinia cupana*), tea leaves (*Thea sinensis*), kola nuts (*Cola acuminata)*, mate tea (*Ilex paraguariensis*), coffee beans (*Coffea arabica* and *Coffea robusta*) and cocoa beans (*Theobroma cacao)*. It is commonly spread in plant-derived and beverages. The acknowledged existence of caffeine in many plants played a major role in the age-old popularity of caffeine containing products [3].



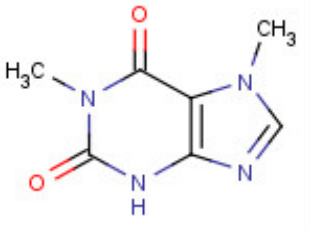

Caffeine




Theobromine




Paraxanthine




Theophylline

**Figure 3**: Structures of Caffeine and its Metabolites

### 1.3 Consumption of Caffeine

Historically, the first reported consumers of caffeine were from the Galla tribe in Ethiopia, about a century ago. Caffeine was isolated from coffee beans as a pure compound for the first time in 1819 by Friedlieb Ferdinand Runge [3]. Caffeine is recognized for its extensive applications in pharmacological preparations such as analgesics, diet aids and cold/flu remedies [5]. Caffeine is a commonly consumed food constituent. Therefore, for this reason, the major sources of the quantity consumed, and the demographics of consumers have long been of interest. While chocolate and other cocoa-containing foods contribute small amounts of caffeine to the diet, the majority of caffeine consumed comes from beverages [6].

Recently, a review about the functionality of caffeine indicates that, it is one of the most commonly consumed food component worldwide with tea and coffee being the most well-known sources in the diet, of which coffee is the second most commonly consumed beverage worldwide after water [3]. Caffeine intake varies across different types of beverages and in different population groups. Coffee naturally contains more caffeine than most other beverages and it is generally and extensively consumed [6]. According to a study conducted by Ahmad and co-workers [4], caffeine is added to beverages as a flavoring agent, or as part of the overall profile of beverages, which consumers enjoy for refreshment, taste and hydration. Most of the caffeine in cola drinks is added during the formulation process.

Carbonated soft drinks (CSDs), tea, energy drinks, energy shots, and some fruit or fruit-flavored and water beverages also contribute to total caffeine intake. Amongst children, CSDs had been shown to be the primary source of caffeine, compared to coffee in adults was perhaps the first to comprehensively investigate population-level data on newer categories of products such as energy drinks, energy shots, and other beverages containing caffeine. Some studies shows that such drinks may be more frequently consumed by young adults, teenagers, college students, athletes, and military personnel [6]. In the case of carbonated beverages, the variability in consumption occurs among brands, since most of the caffeine content in these products is added from other natural sources, i.e. less than 5% of the total present caffeine is from cola nuts. Cola-like drinks account for 80 to 90% of the caffeine added to foods today [7]. Caffeine and caffeine-containing natural sources have been added to weight loss herbal products [8].

## 1.4 Methods of Extraction and Determination of Caffeine

A number of analytical methods have been recommended for the separation and/or determination of caffeine in various sample matrices (including environmental, biological, plants, food, etc.). Among the conventional methods of hot water extraction for biological samples, many published researches have been reported for the determination of caffeine such as high performance liquid chromatography (HPLC) equipped with different types of columns and detection systems [9], gas chromatography–mass spectrometry/flame ionization detection (GC–MS/FID) [10], and Fourier transform-infrared spectrophotometry (FT-IR) [11]. FT-Raman spectrometry [12], electrospray ionization–ion mobility spectrometry (ESI–IMS) [13], capillary electrophoresis (CE) [14] and voltammetry [15] have also been applied.

However, in most of these methods, direct analysis of the caffeine in food and beverage samples is limited due to complex matrix effect. Therefore, in order to determine caffeine in these types of method, an extraction and/or a preconcentration step prior to the analysis is necessary. many extraction steps such as ultrasonic assisted extraction [5], soxhlet extraction [16], solid-phase extraction (SPE) [13], drop-to-drop solvent microextraction (DDSME) [17], single drop microextraction (SDME) [5] and hollow-fiber liquid phase microextraction (HF-LPME) [18] have been applied for this purpose. In addition to the above mentioned pretreatment methods, an efficient and green extraction/preconcentration technique was introduced by Assadi et al. (2006) named as dispersive liquid–liquid microextraction (DLLME) [19]. This method provides high recovery and enrichment factor within a very short time (a few min).

## 1.5 Health Effects of Caffeine

Caffeine is considered as a bioactive material which in moderation has several positive effects on the body. It boosts alertness, functions as a bronchial dilator, stimulates metabolism and influences an increase in dopamine levels in the blood, which in return improves mood [7]. Natural and artificial sources of caffeine have similar physiological effects on the human body with a relatively short half-life of 5 hours. This can lead to serious negative physiological symptoms like headache, insomnia, indigestion, anxiety, and diuresis especially among individuals not habituated to caffeine [3]. Caffeine has rapid effects on the central nervous

system. It also dilate blood vessels, increases heart beat rate and raises the levels of free fatty acids and glucose in the plasma. It has a tendency to increase alertness, reaction times and alleviate tiredness by stimulating the central nervous system [20]. According to Nawrot et at. (2003) [21], caffeine is believed to be a risk factor for cardiovascular diseases and may affect behavior effects of depression. Caffeine also motivates the stomach to discharge large amounts of acid, which in return leads to burning of the pits of stomach and intensifies peptic ulcers of the stomach and duodenum [7]. It also increases blood sugar level as a consequence of speedy respiration. It also decreases blood flow to the brain by causing the brain's blood vessels to constrict. It may also induce benign (non-cancerous) breast diseases and may worsen premenstrual symptoms in women who are abused to it. Caffeine overlaps the placenta and go into the fetal circulation, which pharmacologically has been linked to low birth weight [7].

Over dosage of caffeine can result into a condition of central nervous system over-stimulation known as caffeine intoxication. Clinically, caffeine intoxication may result in hypertension, vomiting, cardiac arrest, adverse mutation effects, such as inhibition of DNA repair and cyclic AMP phosphodiesterase activity [22]. It can also cause cancer, heart diseases, increase heart beat rate, control the release of neuro-transmitters like dopamine and glutamate [23] and complications in pregnant women and aging [22]. Caffeine intoxication normally occurs only after ingesting large amounts of caffeine, well over the amounts found in typical caffeinated beverages and caffeine tablets (e.g. >400–500 mg at a time). However, only a few fatal caffeine intoxication cases have been reported in the medical literature [24]. Caffeine intake is related to undesired effects such as anxiety, tremors, headache and gastrointestinal irritation [25]. When consumed in a moderate amount, it reduces a desire for sweets by simulating the production of adrenal hormones which cause blood sugar to be increased. The weakness, depression and discomfort from excess of alcohol can be lost out with black coffee or hypodermic injections of caffeine [26].

Caffeine was classified as a drug of abuse by the international Olympic committee (IOC) when appear in urine at concentration levels of more than 12 µg $mL^{-1}$. As for any food, the composition of soft drinks is regulated by legislations. According to Directive 2000/13/EC, quinine and/or caffeine used as a flavoring in the production or preparation of a foodstuff must be mentioned by name in the list of ingredients immediately after the term "flavoring".

Moreover, according to Directive 2002/67/EC of 18 July 2002, drinks containing caffeine in excess of 150 mg $L^{-1}$must also provide a warning message on the label followed by an indication of the caffeine content such that "High caffeine content (X mg per 100 mL)" [21]

Caffeine, being one of the most comprehensively studied ingredients in the food supply, with centuries of safe consumption in foods and beverages, was in 1959 designated (in cola drinks) as "Generally Recognized As Safe" (GRAS) by the United State Food and Drug Administration (USFDA). The FDA considers caffeine safe for all consumers, including children. In 1987, after a general review, the FDA found no evidence to show that the use of caffeine in carbonated beverages would make these products injurious to health. More than 140 countries have specifically considered the safety of caffeine and allow its use in beverages at various levels [2].

Positive health consequences related to caffeine intake have been reported in studies assessing coffee consumption, including counter associations between regular caffeine consumption and risk of developing type 2 diabetes, Parkinson's Disease, and gliomas [3].

It has been shown that caffeine is capable of reducing the pharmacological activity of a number of aromatic anticancer drugs [27]. Caffeine citrate was placed on the world health organization (WHO) model list of essential medicines in 2007 which is used in medical treatment, including short-term treatment of apnea of prematurity. However, at high levels it can cause restlessness, insomnia and anxiety. In cases of overdosing and in combination with alcohol, narcotics and some other drugs, these compounds result to toxic effect, sometimes with lethal outcome [28].

### 1.6 Recommended Caffeine Intake

Human consumption of caffeine is mostly in liquid form and the absorption into the body occurs from aqueous solutions [29]. For adults, moderate caffeine intake of nearly 300 mg per day is well accepted, with some evidence of potential health benefits. In 2008, the UKFSA recommended that caffeine consumptions by pregnant women should not be above 200 mg per day due to modern evidence associated with health risks to the unborn child [3].

Canada is among the few countries worldwide to have set recommendations for caffeine intake and includes specific guidance for children, at 45 mg per day for 4–6 years, 62.5 mg per day for 7–9 years, and 85 mg per day for 10–12 years [3]. Since 2002, European Union (EU) rulings for

caffeine labeling requires a caffeine-containing beverages in excess of 150 mg $L^{-1}$, and intended for consumption without modification or after reconstitution, to be clearly labeled as ‘‘High caffeine content’’ [3]. This directive includes most caffeinated energy drinks. However, tea and coffee-based beverages are exempted so long as the product is clearly labeled as containing caffeine. There has also been an introduction of a greater variety of beverages in the marketplace. The introduction of functional beverages such as energy drinks, energy shots, as well as a range of special coffees, also stresses the importance of characterizing more recent beverage consumption patterns and caffeine intake that may have developed over the last decade. Therefore, the determination of caffeine compounds in soft and energy drinks for assurance of food safety and quality control is much needed [6].

A recent study has shown that regular caffeine consumption in the form of coffee (seven cups per day) can considerably lower the risk of clinical type 2 diabetes. Conversely, consuming high amounts of caffeine has been shown to create some negative effects upon premenstrual syndrome and pregnancy and to some extent promote infertility and even cancer. Therefore, it is important to develop more reliable, simpler and faster methods to determine the amounts of caffeine from different sources as a way to evaluate their contents in order to find a more precise relationship between the amounts of consumed caffeine and its physiological effects [30].

### 1.7 Clinical and Biological Activities of Caffeine

Among individuals, epidemiological studies showed that caffeine intake is inversely associated with the incidence of Alzheimer’s or Parkinson’s disease [31]. Since caffeine intake is in liquid form, there are several known mechanisms of action to explain the effects of caffeine. The most well-known mechanism is to reversibly block the action of adenosine on its receptor, which in return blocks the onset of drowsiness induced by adenosine [32]. The stimulatory effect of caffeine generally results in an increased ability for mental activity and muscular work [26]. It increases the basal metabolic rate and acts as a mild central nervous system stimulant, myocardial stimulant and smooth muscle relaxant [26]. It has also been reported to have an effect on several biological processes on the cell levels. Caffeine is also known to increase the secretion of epinephrine, which can lead to a range of secondary metabolic changes that can positively affect physical or mental performance [27]. Despite its popularity and ergogenic properties

during sports performance, caffeine is generally acknowledged as having a mild diuretic effect. The USFDA recommend that caffeine has diuretic properties and advises its users to drink more water to avoid dehydration during exercise in the heat [33].

Caffeine can have both positive and negative health effects depending on the amount consumed. Severe side effects are infrequent when fairly low doses are consumed. Minimum information concerning its chronic effects has been reported. A few studies have suggested that chronic caffeine intake decreases inflammatory injury and chronic inflammation in the liver and brain. High concentrations of caffeine are now being added to energy drinks or are being taken as dietary supplements [24]. Moreover, the overpowering effects of caffeine have been reported severally, but to a small extent, it affects the heart muscle, gastric secretion and diuresis. Remarkably, caffeine is consumed daily by many individuals and is exclusive to be a potent drug, considered to be a part of human normal diet [31].

### 1.8 Pharmacology and Pharmacodynamics of Caffeine

Caffeine applies pharmacological actions at various sites, both centrally and peripherally, principally due to antagonism of endogenous adenosine, with A1 and A2A receptors seeming to be the main targets [34]. In the absence of caffeine in a wakeful state and alert, little adenosine is present in central nervous system (CNS) neurons. With a continued wakeful state over time, it gathers in the neuronal synapse, in turn binding to and activating adenosine receptors found on certain CNS neurons. When activated, these receptors produce a cellular response that eventually increases drowsiness [32].

When caffeine is consumed, it antagonizes adenosine receptors. In other words, caffeine prevents adenosine from activating the receptor by blocking the location on the receptor where adenosine attaches to it. As a result, caffeine momentarily prevents or relieves drowsiness, and as a result maintains or restores alertness. Caffeine's primary mechanism of action is as an antagonist of adenosine receptors in the brain.

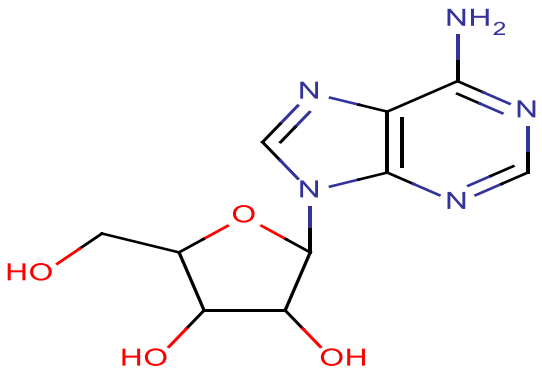



**Figure 4:** Structure of Adenosine

**Receptor and Ion Channel Targets**

Caffeine is a receptor antagonist at all adenosine receptor sub-types (A1, A2A, A2B, and A3 receptors) [35]. Antagonism at these receptors stimulates the medullary vagal, vasomotor, and respiratory centers, which increases respiratory rate, reduces heart beat rate and constricts blood vessels. Adenosine receptor antagonism also promotes neurotransmitter release (e.g., monoamines and acetylcholine), which provides caffeine with its stimulant effects, Adenosine acts as an inhibitory neurotransmitter that suppresses activity in the central nervous system [32].

Because caffeine is both water- and lipid-soluble, it readily crosses the blood–brain barrier that separates the bloodstream from the interior of the brain. Once in the brain, the principal mode of action is as a nonselective antagonist of adenosine receptors. The caffeine molecule is structurally similar to adenosine and is capable of connecting to adenosine receptors on the surface of cells without activating them, thus acting as a competitive inhibitor [32].

**Enzyme Targets**

Caffeine, like other xanthines, also acts as a phosphodiesterase inhibitor. As a competitive nonselective phosphodiesterase inhibitor, caffeine raises the cellular concentrations of cyclic adenosine monophosphate (CAMP), activates protein kinase A,TNF-alpha and leukotriene synthesis and reduces inflammation and innate immunity. Caffeine is also considerably linked in cholinergic system where it inhibits enzyme acetylcholinesterase [21].

**Performance-Enhancing Mechanism**

Many probable mechanisms have been projected for the athletic performance-enhancing effects of caffeine. It may increase fat utilization and decrease glycogen utilization. Caffeine changes metabolism across several complex mechanisms and can help to enable fat loss and post-exercise glycogen re-synthesis. Caffeine mobilizes free fatty acids from fat and/or intramuscular triglycerides by increasing circulating epinephrine levels. The increased availability of free fatty acids increases fat oxidation and spares muscle glycogen, thereby enhancing endurance performance. In the nervous system, caffeine may reduce the sensitivity of effort by lowering the neuron activation threshold, making it easier to recruit the muscles for exercise [36].

**Metabolite Pharmacodynamics**

Caffeine experiences hepatic metabolism through N-demethylation, acetylation and oxidation with less than 5% of the caffeine taken being excreted in urine. Metabolites of caffeine also contribute to the caffeine's effects. Paraxanthine is responsible for an increase in the lipolysis process, which releases glycerol and fatty acids into the blood to be used as a source of fuel by muscles. Theobromine is a vasodilator that increases the amount of oxygen and nutrient flow to the brain and muscles. Theophylline acts as a smooth muscle relaxant that primarily affects bronchioles and acts as a chronotrope and inotrope that increases heart rate and force of contraction [26].

## 1.9 Pharmacokinetics of Caffeine

After consumption, caffeine is quickly and essentially absorbed completely from the gastrointestinal tract into the bloodstream [21]. Absorbed caffeine is immediately distributed all over the body. Peak blood concentration is reached within 30–120 min. Caffeine is eliminated by first-order kinetics. Caffeine can also be absorbed rectally, evidenced by suppositories of ergotamine tartrate and caffeine (for the relief of migraine). Similarly, chlorobutanol mixed with caffeine is used for the treatment of hyperemesis [37].

Caffeine's biological half-life – the time needed for the body to eliminate one half of a dose – commonly differs among individuals according to factors such as pregnancy, other drugs, liver enzyme function level (needed for caffeine metabolism) and age. Caffeine is metabolized in

the liver by the cytochrome P450 oxidase enzyme system (CYP1A2) isozyme, into three dimethylxanthines, each of which has its own effects on the body:

**Paraxanthine (84%):** Increases lipolysis, leading to elevated glycerol and free fatty acid levels in blood plasma.

**Theobromine (12%):** Dilates blood vessels and increases urine volume. Theobromine is also the principal alkaloid in the cocoa bean (chocolate).

**Theophylline (4%):** Relaxes smooth muscles of the bronchi and is used to treat asthma. The therapeutic dose of theophylline, however, is many times greater than the levels attained from caffeine metabolism.

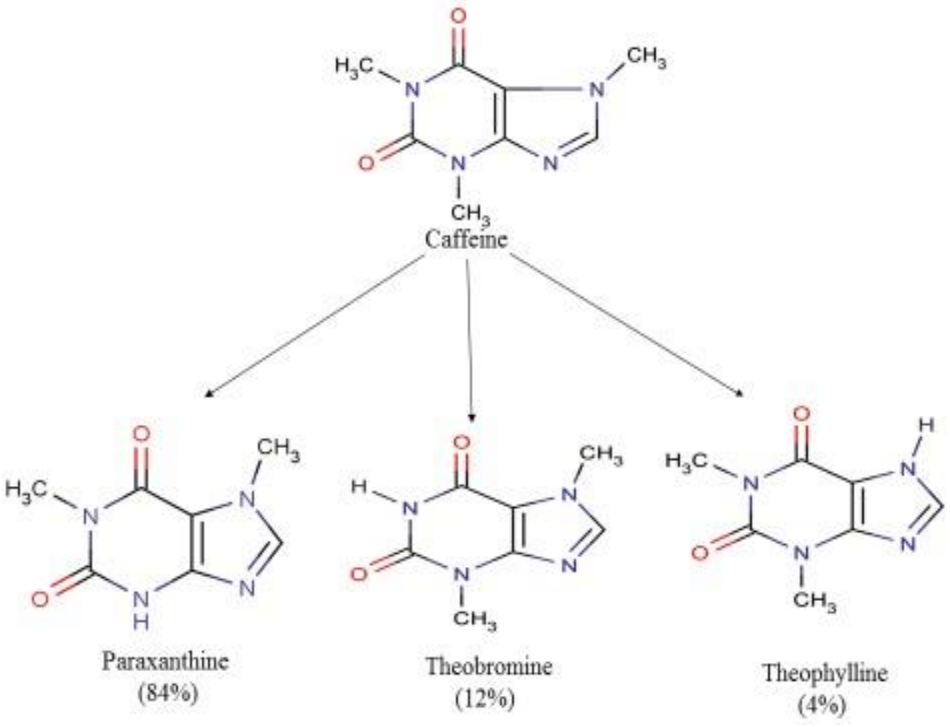



**Figure 5:** Primary Metabolites of Caffeine

## 1.10 Aim of the Research

The aim of this study is to develop a green, simple, fast, low cost, sensitive and efficient method for the determination of caffeine in kola nuts. DLLME was applied and optimized for extraction and preconcentration of caffeine in red and white kola nuts. The important parameters of DLLME were studied using one parameter-at-a-time approach. The preconcentrated caffeine was analyzed using HPLC equipped with a diode array detector (DAD).

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Dispersive Liquid-Liquid Microextraction (DLLME)

Sample pretreatment is perhaps the most important step in analysis. Both extraction of analytes from the matrix and their preconcentration are important aspects of this process. Moreover, it is important to clean up samples of complex matrices prior to analysis [38]. Extraction can differ in degree of selectivity, speed and convenience. This depends not only on the method and conditions used but also on the geometric configurations of the extraction phase [19].

Classical sample pretreatment techniques like liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are generally slow and labor demanding. Most often, hazardous organic solvents are used and sample volumes can be greater than 1 L for liquid samples. The main disadvantage of LLE in ultra-trace analysis is the requirement of using large amounts of pure solvents and their subsequent evaporation is an unavoidable step in obtaining significant preconcentration. Therefore, this technique is both expensive and environmentally unfriendly [38]. SPE might be considered as better alternative, as smaller amounts of organic solvents are generally used. However, SPE cartridges are not only expensive but also generate a great deal of waste. After use, SPE cartridges are discarded because it should be used only once in ultra-trace analysis. Both LLE and SPE are environmentally unfriendly though these sample preparation techniques are commonly used, even today.

Development and improvement of modern sample pretreatment techniques in the past decade was primarily driven based on miniaturization, simplification and automation in order to reduce the costs of both materials and personnel, as the main aim of sample preparation is to clean up and to concentrate the target analyte(s) and finally to analyze it in a well-matched and desired analytical instrument. LLE, soxhlet extraction, distillation and absorption are conventional practices for sample preparation that suffer from different drawbacks, such as being time consuming, tedious, consume large amount of toxic solvents and to some degree, complications in automation. As a result of these drawbacks, many novel microextraction techniques were

developed [e.g., solid-phase microextraction (SPME), single-drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME), etc.]. Most of these techniques are simple, fast and consume less extraction solvents than conventional ones [4]. Interest in miniaturizing sample pretreatment techniques began with the introduction of SPME by Arthur and his co-workers [39]. Subsequently, a number of other microextraction techniques have been developed. Nevertheless, all these extraction techniques have both advantages and disadvantages.

DLLME was introduced by Rezaee et al. in 2006 [19], as a result of the demand for rapid, economical and environmentally friendly sample pretreatment techniques. DLLME was developed for water samples, but was later applied to other matrices, such as soil and foodstuff. The extraction mechanism is based on different affinities of the analytes to the aqueous sample and the organic extractant [4]. The main advantages of this powerful sample preparation and preconcentration technique include simplicity, minimal use of harmful solvents, short extraction time and low cost. This technique is one of the most remarkable due to the large number of publications since its inception [4]. The main aims of this technique are: (1) To overcome the disadvantages of conventional techniques in order to reduce both personnel and material expense; and, (2) To achieve promising results in terms of recovery and enrichment factors (EF).

## 2.2 Classification of DLLME

Numerous research articles have been published since the introduction of DLLME. Several new advances occurred in time to overcome the possible drawbacks of the process, thus leading to different modifications in DLLME. Most often for each modification, a different acronym was assigned by the researcher. Occasionally, there are more than two or three acronyms for the same DLLME method, which often makes it difficult to differentiate them and this leads to some complications. Ahmad and his co-workers [4] have made an effort to arrange all those acronyms in four general groups, as shown in Fig. 6. The four bases of the classification are:

(i) Mixed mode extraction;

(ii) Extraction based on assisting dispersion;

(iii) Extraction based on use of ionic liquids (ILs); and

(iv) Extraction based on solvent density in its acronym, and any other type not fitting into the other three groups.

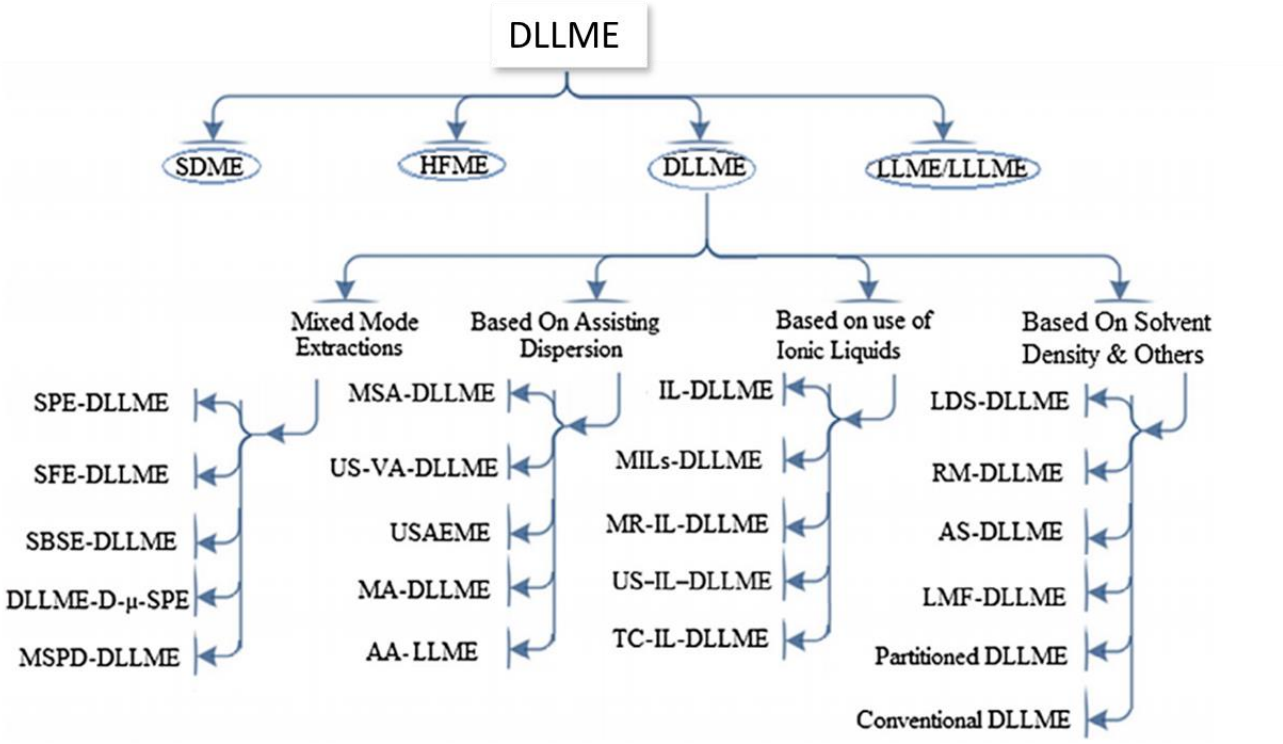



**Figure 6:** Classification of DLLME

DLLME as a miniaturized LLE using microliter volumes of extraction solvent is based on the equilibrium distribution process of the target analyte(s) between sample solution and extraction solvent. Distribution coefficient *(K)* is defined as the ratio between the analyte concentrations in the extraction solvent to that in the sample solution. The distribution coefficient of a solute A, in an aqueous/organic system is shown in equation (1) below:

$$K = \frac{[A]_{org}}{[A]_{aq}} \quad \text{..... (1)}$$

where $[A]_{org}$ and $[A]_{aq}$ are the concentrations of solute A in the organic and aqueous phase, respectively.

A certain volume of the sample solution is placed in a screw cap test tube with conic bottom, followed by the rapid injection of a mixture of disperser solvent containing an extraction solvent

into the aqueous sample solution with a syringe or pipette. Then, the ternary mixture is gently shaken, thereby making a cloudy solution (water/disperser solvent/extraction solvent) in the test tube. At this point, the surface area between the extraction solvent and the aqueous phase (sample) is infinitely large, thus the mass transfer of the analyte from the aqueous phase (sample) to the extraction phase is fast. Subsequently, equilibrium state is achieved quickly, resulting in a very short extraction time, which is the remarkable advantage of DLLME compared with other techniques. Finally, the dispersed fine droplets of the extraction phase are sedimented in the bottom conical test tube into injected tube through centrifugation. A certain volume of the sedimented phase is injected into a suitable analytical instrument (e.g. GC) for further analysis or the whole of it is withdrawn and the analyte is back-extracted into the aqueous phase that is compatible with the instruments (e.g. HPLC, CE, etc.). Fig. 8 shows the process.

### 2.3 Calculations in DLLME

In DLLME, the enrichment factor (EF) and percentage extraction recovery (%ER) should be taken into consideration to evaluate the efficiency of the method. Rezaee et al. [19] defined EF as shown in equation (2). In this study, since the analyte was back-extracted into an aqueous back-extraction solution (BES), this equation and other equations were modified below:

$$EF = \frac{C_0}{C_{sed}} \quad \text{or} \quad EF = \frac{C_0}{C_{BES}} \quad \text{..... (2)}$$

where $C_{sed}$ is the analyte concentration in the sedimented phase and $C_0$ is the initial analyte concentration in the sample. %ER is defined as the percentage ratio of the amount of analyte in the sedimented phase to the initial concentration in the sample:

$$\%ER = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed} \times V_{sed}}{C_0 \times V_0} \times 100 \quad \text{or} \quad \%ER = \frac{n_{BES}}{n_0} \times 100 \text{ ..... (3)}$$

$$= \frac{C_{BES} \times V_{BES}}{C_0 \times V_0} \times 100$$

where $n_{sed}$ and $n_{BES}$ are the amounts of the analyte in the sedimented phase or BES respectively, $n_o$ is the initial amount of the analyte in the sample, $V_{sed}$ and $V_{BES}$ are the volumes

of the sedimented phase or BES respectively, and $V_0$ is the volume of the sample solution. ER can also be calculated from the BES calibration.

$$ER = \frac{V_{sed}}{V_0} \times EF \times 100 \quad \text{..... (4)}$$

The relative recoveries (RR) can be calculated from the equation:

$$RR = \frac{C_{found} - C_{real}}{C_{added}} \quad \text{..... (5)}$$

where $C_{found}$ is the analyte concentration measured from the sample after analyte addition, $C_{real}$ is the native analyte concentration and $C_{added}$ is the amount of the analyte that was added to the sample solution.

## 2.4 Applications of DLLME

As a novel sample preparation method, DLLME was coupled with GC [5, 20, 40], HPLC [41], CE [42-46], and atomic absorption spectroscopy (AAS) [47, 48], for the analysis of different samples. It has been widely applied to the analyses of simple and complex sample matrices such as urine, milk, blood, for the determination of pharmaceuticals, pesticide residues, heavy metals, and so on [4, 49, 50]. Some applications are illustrated in Table 1.

According to Ahmad and his co-workers [4], about 50 papers were devoted to development of DLLME for the analysis of pesticides. Such a great number of papers concerning one group of analyte(s) can be attributed to both high interest in this field connected with food and environmental pollution caused by these compounds and the great variety of different pesticides belonging to several classes. The pesticides were analyzed mainly in aqueous samples.

DLLME was also used for preconcentrating many other organic compounds. Many researches were devoted to the determination of both phenols and pharmaceuticals [4, 38, 50, 51]. Two groups of phenols were analyzed in the water samples – chlorophenols and endocrine-disrupting phenols i.e. bisphenols and alkyl-phenols. Also, volatile phenols were analyzed in wine samples. However, pharmaceuticals were extracted mainly from both water and urine samples. Different classes of drugs were analyzed with no stress put on any particular group.

Other groups of compounds that were of interest included polyaromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs). These compounds are persistent environmental pollutants that are toxic even at low concentration levels, so different matrices were tested for their presence. PCBs were extracted from water, fish and soil sample. PBDEs were analyzed in water, plants and animal tissues. Finally, PAHs were extracted from water and marine sediments. Naturally, solid samples have to be subjected to extraction with appropriate solvents that could be used in the second extraction step as a constituent of a ternary solvent mixture in the DLLME procedure [4, 39, 50, 51].

Many more organic compounds were extracted using different DLLME procedures. Metal analyte(s) were present in many researches and are the second most popular group after pesticides (i.e. ligand or chelating agents). Fig. 7 shows the number of publications describing the usage of DLLME for the extraction of organic analyte(s) and metal ions [38]. The DLLME procedures used for metals required the use of an appropriate organic compound to transfer these analyte(s) to the extracting solvent. Of the different chelating agents proposed, the most frequently used were ammonium pyrrolidinedithiocarbamate (APDC) and sodium diethyldithiocarbamate (DDTC). Metals were extracted mostly from aqueous samples. However, several solid or semi-solid samples (e.g., food or soil) were also analyzed. An appropriate digestion method had to be used before metals could be extracted from the samples using DLLME. The most frequently analyzed metals are Cu, Pb and Cd [4, 39, 50, 51].

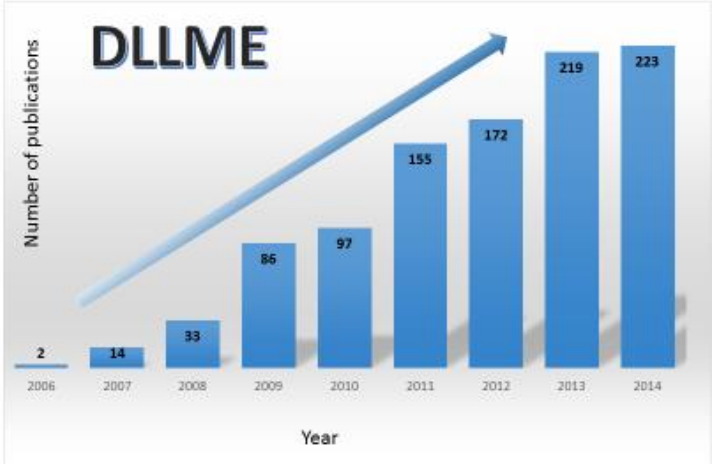



**Figure 7:** Number of publications on DLLME

**Table 1:** Some Applications of DLLME

| Analyte | Matrix | Extraction Solvent (volume) | Disperser Solvent (volume) | Analytical Technique | EF |
|---|---|---|---|---|---|
| Polycyclic aromatic hydrocarbon | Rivers water, well & surface water | $C_2Cl_4$ (8.0 µL) | Acetone (1.0 mL) | GC-FID | 0.007-0.030 |
| Chlorobenzene | River, tap and well water | $C_6H_5Cl$ (9.5 µL) | Acetone (0.5 mL) | GC-ECD | 0.0005-0.05 |
| Mononitrotoluenes | River, tap and well water | $C_6H_5Cl$ (10 µL) | Acetonitrile (0.5 mL) | GC-FID | 0.5 |
| UV filters | River, pool, and waste water | $C_6H_5Cl$ (60 µL) | Acetone (1.0 mL) | GC-MS | LOQ 0.002-0.014 |
| Ochratoxin A | Wine | $CHCl_3$ (100 µL) | Acetone (1.0 mL) | HPLC-MS-MS | 0.005 |
| Ochratoxin A | White, rose, and red wine | $CHCl_3$ (660 µL) | Acetonitrile (940 mL) | HPLC-LIF | 0.0055 |
| Carbaryl, Triazophos | Fruit juice and water sample | $C_2H_2Cl_4$ (15.0 µL) | Acetonitrile (1.0 mL) | HPLC-FLD | 0.012-0.016 |
| Chloramphenicol, Thiamphenicol | Honey | $C_2H_2Cl_4$ (30.0 µL) | Acetonitrile (1.0 mL) | HPLC-UV | 0.1-0.6 |
| Macrocyclic lactones | Milk | $CHCl_3$ (200 µL) | Acetonitrile (2.0 mL) | HPLC-DAD, HPLC-MS | 0.03-0.72 |
| Phenols | Wastewater | $CS_2$ (165 µL) | Acetone (2.50 mL) | HPLC-DAD | 0.01- 1.3 |
| Opium Alkaloids | Urine | $CHCl_3$ (88 µL) | Acetone (1.0 mL) | HPLC-UV | 0.2-10 |
| Sertraline | Urine | $CH_2Cl_2$ (30 µL) | Acetone (0.2 mL) | CE-UV | 0.76 |
| Volatile constituents of tea | Green, black, oolong, and white tea | $CHCl_3$ (27 µL) | Methanol (0.6 mL) | GC-FID | 300 |
| Chlorophenols | Rain, tap, and lake water | Toluene (250 µL) | Methanol (0.5 mL) | HPLC-UV | 0.016-0.084 |
| Sulfonylurea herbicides | Soil | Chlorobenzene (60 µL) | Acetonitrile (1.0 mL) | CE-DAD | 0.5-1.0 |
| Parabens | Mouth rinse solution, sauces, and tomato paste | Octanol (20 µL) | Acetone (0.5 mL) | GC-FID | 5-15 |
| Benzoate, sorbate | Yoghurt drinks | Octanol (60 µL) | Ethanol (0.45 mL) | HPLC-UV | 0.06-0.15 |

## 2.5 Limitations of DLLME

Conventional DLLME utilizes solvents that are denser than water. The number of these solvents is limited, and they are mostly halogenated and hazardous [4]. Although the performance of single-step DLLME in aqueous samples is excellent, it is not yet suitable in complex matrixes such as biological samples which is generally overcome by the use of another extraction step prior to DLLME. Therefore, it needs further improvements in the future. Another disadvantage of DLLME is the consumption of relatively large volumes (in mL) of disperser solvents which usually decreases the partition coefficient of the analyte(s) into the extractant solvent. To avoid this problem, some improvements have suggested the use of either ultrasonic energy or surfactants to disperse the extraction solvent instead of disperser solvents [51].

Combination of DLLME with other extraction techniques such as solvent extraction, SPE and supercritical fluid extraction (SFE) and salting-out extraction have been reported for complex matrix samples [43]. More applications in the analysis of samples with complex matrices are needed to show the ability and applicability of DLLME to the analysis of such samples. Furthermore, owing to the high clean-up efficiency of the combined extraction methods, the direct analysis of the extracted analyte(s) with selective detection systems such as MS and fluorescence detection is desirable [49].

## 2.6 Automation of DLLME

DLLME is primarily accomplished manually. Automation is needed to achieve a rapid extraction process with higher sample amount, an enhanced reproducibility, lower contamination, less sample/reagent consumption. Automation of DLLME is one of the current challenges of DLLME possibly because the technique usually requires phase separation by centrifugation. Automation of DLLME was first reported by Anthemidis and Ioannou (2009) for the determination of copper and lead in water samples by flame atomic absorption spectroscopy (FAAS) [52].

In this method, which was named as online sequential injection DLLME (SI-DLLME), the disperser solution was injected into the moving sample solution to form a cloudy solution of fine droplets of the extraction solvent (xylene). For phase separation, the organic phase was retained

in a micro column packed with PTFE turnings instead of centrifugation. In the next step, analytes were eluted using 300 μL of isobutyl methyl ketone and transferred to the FAAS system for their determination [52].

## 2.7 Analytical Techniques for the determination of Caffeine

Caffeine is a very attractive compound for analytical chemists. Due to this recognition, novel and perspective analytical methods providing rapid, sensitive and reliable detection and determination of caffeine are desirable. It is therefore needful to find methods that are appropriate for the determination of caffeine in sample matrix. Numerous reported studies were aimed at the development of analytical methods for determination of caffeine in sample matrix (such as beverages, food, environmental, biological samples etc.) [53]. Most of the researches have focused on using chromatographic methods for identifying the methylxanthine content in different sample matrix [30].

To date, several analytical techniques have been reported for the determination and analysis of caffeine in different samples, which include HPLC coupled with ultraviolet detection (UV) [20], electrochemical detection (ECD) [39], mass spectrometry (MS) [54] and particle beam/electron ionization mass spectrometry (PB/EIMS). GC-MS [10, 17], Fourier transform near infrared reflectance (FT-NIR) spectroscopy [55], electrochemical methods [56, 57], and spectrophotometric methods [11, 58, 59]. Many laboratories prefer to use HPLC–UV, which was found to be comparably less costly, convenient to operate and suitable for routine analysis for the determination of many analytes including caffeine in many samples [20].

## 2.8 HPLC Methods

HPLC method was described for determining the amount of caffeine in related real samples such as chocolates, candies, breads and energy drinks. The method was validated for caffeine in those matrices. Liquid samples were appropriately diluted with warm water and caffeine from solid samples was extracted using hot water and carrez solution followed by centrifugation, The concentration of caffeine was determined by HPLC with $C_{18}$ column (4.6 × 150 mm, 5 μm) and methanol:acetic acid:water (20:1:79 %, v/v/v) as the mobile phase. The recovery, linearity, limit

of detection (LOD), limit of quantitation (LOQ) and relative standard deviation (RSD) were satisfactory for this method [60].

A HPLC method was developed for the simultaneous determination of caffeine, theobromine and theophylline, present in synthetic and real samples. In this method, the separation was performed with an Agilent Zorbax eclipse XDB-$C_{18}$ column (250 mm × 4.6 mm, 5 µm) with an Agilent Zorbax high pressure reliance cartridge guard-column ($C_{18}$, 12.5 mm × 4.6 mm, 5 µm) and a DAD at wavelength at 320 nm. All samples and their standard were injected into the monolithic column via the autosampler of the HPLC instrument (injection volume of 30 µL for all samples). A mixture of methanol and phosphoric acid (0.1%) was used as the mobile phase and the flow rate was set at 1.0 mL $min^{-1}$ at ambient temperature. All analytes were detected at 271 nm, and the retention times for theophylline, theobromine and caffeine were 3.9 min, 5.8 min and 7.8 min respectively. Peak area was used for signals evaluation. Each sample was filtered through 0.45 membrane nylon filters before injection. This method therefore, is a potential alternative to the commonly used HPLC technique; it is low-cost, and uses easily available chemicals and instrumentation [59].

The applicability of DLLME followed by a back-extraction step as an efficient sample clean-up and preconcentration technique for the determination of caffeine in red and white kola nuts (*Cola nitida*) in this study was demonstrated to be an effective sample preparation technique which offers a rapid extraction with minimum consumption of organic solvents and short extraction time of analysis in the HPLC instrument. Other advantages such as simplicity, low cost and ease of operation make the method user-friendly.

### 2.9 Future work

- The proposed method can further be optimized to include the metabolites of caffeine.
- The optimized DLLME conditions for caffeine and its metabolites would be applied with CE which is considered to be a green technique.
- Other microexteaction techniques such as USEME and HLLME would be applied for the determination of caffeine in food samples and these methods could be compared with the proposed method.

# CHAPTER 3

# EXPIREMENTAL

## 3.1 Chemicals and Reagents

HPLC grade methanol, acetone and chloroform with purity higher than 99% were from Sigma-Aldrich, Germany. Ethanol was from EMSURE® (Darmstadt, Germany). Carbon tetrachloride, chlorobenzene, and sodium chloride were also used in this research. 1-undecanol, 1-dodecanol, dichloromethane and diphenylether were obtained from Sigma-Aldrich (Steinheim, Germany). The pH of the solutions was adjusted (where applicable) with 0.10 M NaOH. Caffeine standard (≥ 99.0%) was from Fluka (AG, USA). A stock solution of caffeine with a concentration of 1000 mg $L^{-1}$ was prepared in methanol, stored at room temperature and protected from light. Working caffeine standard solutions ranging from 0.5–50 mg $L^{-1}$ were freshly prepared in deionized (DI) water by appropriate dilutions of the stock standard solution and used for evaluation and optimization of the DLLME method. All other reagents and solvents used were at least of analytical reagent grade.

## 3.2 Apparatus

Centrifugation was performed by a Hettich Zentrifugen D-78532 (Tuttlingen, Germany). Ultrasonic water bath with a temperature control and a timer from Bandelin Sonorex digitec, type: DT 102 H-RC (Berlin, Germany) was used after hot water extraction. Mixing/shaking and extraction were performed using vortex machine from Heldoph Instruments, type: REAX top D-91126 (Schwabach, Germany). A gradient HPLC system equipped with diode-array detector (DAD) (Agilent Technologies, Waldbronm, Germany) was used. The chromatographic separations were performed on an analytical column: Agilent Eclipse XDB-$C_{18}$. 4.6 mm ID × 150 mm (5 µm) with column temperature adjusted at 25 °C. Filtrations were performed through cellulose filters 0.45 µm. Hot plate from Heldoph Instruments type: MR Hei-standard D81126 (Schwabach, Germany) was used for hot water extractions. Micropipette BIOHIT-Proline eppendorf (100-1000 µL) and (10-100 µL) (Deutschland, Germany) were used for microliter

measurements. Data were processed by an HP Compaq (Pentium dual-core inside) E185HCSP with 18.5"W LED monitor and collected through an HP lesser jet P2014 printer.

### 3.3 Sample Collection and Preparation

Red and white kola nuts (*Cola nitida*) were purchased from a local market in Kano City (Kano State, Nigeria). The nuts were dried and ground to smaller particles and stored in tightly closed jars at room temperature until analysis.

### 3.4 Preparation of Kola nut samples (Hot water extraction)

0.10 g (± 0.001 g) of ground red and white kola nuts were weighed separately and transferred into 50 mL beakers. Hot water extraction was carried out by adding approximately 45 ml of hot DI water to the sample, and the mixture was heated on the hot plate till boiling. The solution was allowed to boil for 5 min. After cooling down, the supernatant solution was transferred to a 50 mL volumetric flask and the volume was made up to mark with DI water. Aliquots of this solution were transferred into a screw-cap 15 mL conical centrifuge graduated polypropylene test tubes and were centrifuged for 2 min at 6000 rpm. The supernatant solution, hereafter referred to as sample solution, was used for the DLLME procedure.

### 3.5 Caffeine Isolation

4.0 g of black tea samples (collected from a local market in Nicosia, TRNC) were weighed and made basic with 7.5 mL of 10% $Ca(OH)_2$. Alkaloids were extracted with 10 mL chloroform after stirring for 10 min. The chloroform extract was collected and filtered through a cotton wool into a funnel. The filtrate was then collected into a capsule and evaporated using a water bath. After fully evaporating the chloroform, an inverted glass funnel was used to close the capsule mounted on a sand-filled aluminium container on electric heater (250-300 $^{o}C$) for 1 h. Caffeine crystals then formed at the edges of the inverted glass funnel.

### 3.6 DLLME Procedure

3.0 mL of sample solutions were transferred into a conical test tube and made basic with 10 µL of 6.0 M NaOH. Then, the volume was completed to 9.0 mL with DI water. 200 µL chloroform (as the extraction solvent) were added. 1.0 mL methanol (as the disperser solvent) was rapidly

injected into the test tube using a syringe. The mixture was vortexed for 1 min. Accordingly, a cloudy solution (consisting of tiny droplets of chloroform dispersed into the aqueous phase) was formed. At this point, extraction of caffeine from aqueous phase into chloroform took place. After centrifugation of the cloudy solution (2 min, 6000 rpm), the extractant (chloroform) containing caffeine sedimented at the bottom of the test tube. Further treatment is needed (back extraction) prior to analysis with HPLC.

### 3.7 DLLME of Kola Nuts

In order to plot standard calibration graphs, 3.0 mL of sample solutions of kola nuts were transferred into six test tubes and made basic with 10 µL of 6.0 M NaOH. The first tube was not spiked with the caffeine standard solution. The rest were spiked with increasing concentrations as 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mg $L^{-1}$ from the 1000 mg $L^{-1}$ caffeine standard solution or 100 mg $L^{-1}$ intermediate stock when necessary. The volumes were then completed to 9.0 mL with DI water. A mixture of 250 µL of chloroform and 0.5 mL acetonitrile was rapidly added and vortexed for 60 s and centrifuged (2 min, 6000 rpm). The sedimented phase in each test tube was then back-extracted with 40:60% (v/v) MeOH/$H_2O$ (BES) and injected into the HPLC instrument as shown in Fig. 8.

In the back extraction step, the sedimented phase (chloroform) was all transferred into a 1.5 mL snaplock microtube (eppendorf) and 200 µL of the back extraction solution (BES) made of MeOH/$H_2O$ (60:40%, v/v) were added and vortexed for 30 s, followed by centrifugation at 6000 rpm for 2 min. This resulted into a two phase system and the upper phase was then collected for the analysis with HPLC.

Finally, 20 µL of the upper phase (containing the analyte) was injected into the HPLC instrument using the initial HPLC conditions shown in Table 2. The performance and robustness of the procedure were examined by changing different parameters in both DLLME and HPLC procedure using the one parameter-at-a-time approach.

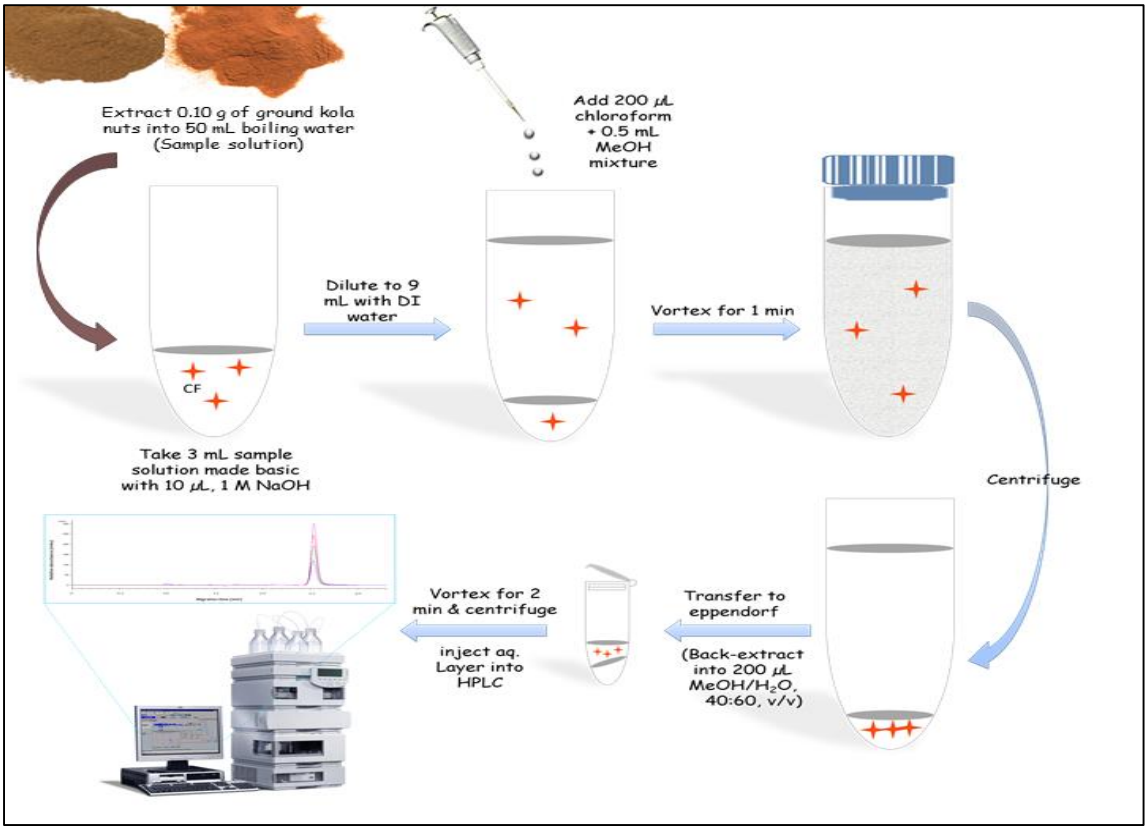



**Figure 8:** Steps in DLLME protocols

**Table 2:** Initial HPLC Conditions

| | | |
|---|---|---|
| **Physical parameters** | Column | Agilent Eclipse XDB-C18. 4.6 mm ID x 150 mm (5 µm) |
| | Flow Rate | 1.0 mL $min^{-1}$ |
| | Temperature | 25 °C |
| | Detector/wavelength | UV. 273 nm (BW 4). Reference 360 nm (100 BW) |
| | Injection volume | 20 µL |
| **Chemical parameters** | Mobile phase | MeOH:$H_2O$ 70:30 (%, v/v) |
| | pH of MP | Not adjusted |

# CHAPTER 4

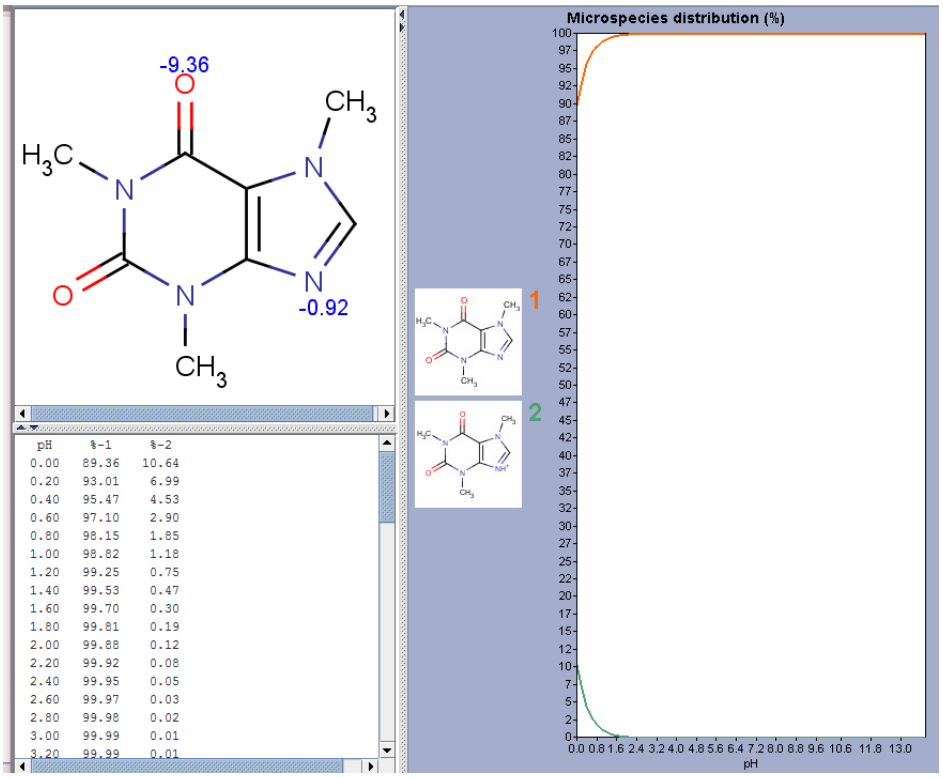
# RESULTS AND DISCUSSION

## 4.1 Optimization of DLLME Parameters

In order to obtain the best performance and conditions of the extraction procedure, the effective parameters of the proposed method were investigated and optimized. Peak area, giving a better reproducibility than peak height, was used to evaluate the influence of the parameters- including the type and volume of the extraction and disperser solvents, pH and volume of sample and back-extraction solutions and ionic strength, on the extraction efficiency of the technique.

### 4.1.1 Effect of pH

A literature survey based on the influence of pH on the extraction efficiency of caffeine showed that the most of investigations have been carried out at the natural pH of samples, but in some studies, the results have been carried out at pH 7.0 [13], while in some other researches, the experiments have been carried out at pH value 12.5 [18]. In this study, the experiments were performed at basic pH adjusted with 10 µL of 6 M NaOH, since the neutral form of caffeine is dominant at basic pH range as shown in Fig. 9.




| pH | %-1 | %-2 |
|---|---|---|
| 0.00 | 89.36 | 10.64 |
| 0.20 | 93.01 | 6.99 |
| 0.40 | 95.47 | 4.53 |
| 0.60 | 97.10 | 2.90 |
| 0.80 | 98.15 | 1.85 |
| 1.00 | 98.82 | 1.18 |
| 1.20 | 99.25 | 0.75 |
| 1.40 | 99.53 | 0.47 |
| 1.60 | 99.70 | 0.30 |
| 1.80 | 99.81 | 0.19 |
| 2.00 | 99.88 | 0.12 |
| 2.20 | 99.92 | 0.08 |
| 2.40 | 99.95 | 0.05 |
| 2.60 | 99.97 | 0.03 |
| 2.80 | 99.98 | 0.02 |
| 3.00 | 99.99 | 0.01 |
| 3.20 | 99.99 | 0.01 |

**Figure 9:** The Microspecies Distribution (% vs. pH Curve of Caffeine). Drawn using MarvinSketch (vesion: 5.3.8, ChemAxon, Budapest, Hungary)

### 4.1.2 Selection of Disperser Solvent

Miscibility of the disperser solvent with the extraction solvent and aqueous phase is a fundamental factor in DLLME which affects the selection of the disperser solvent. The disperser solvent has to be highly miscible with both water and the extraction solvent [19]. The miscibility of disperser solvent in both organic solvent and water is the main reason behind the emulsification of the extraction solvent [46]. Extraction solvents with high interfacial tension make the formation of tiny droplets more tasking by shaking. Thus, using a disperser makes the surface area between the extraction solvent and the aqueous phase (sample) is infinitely large, thereby increasing the extraction efficiency [51].

One of the main disadvantages of DLLME is that its efficiency is restricted by the use of large volumes (in the milliliter range) of a polar solvent such as methanol or acetonitrile to disperse the extraction solvent into the aqueous solution. The use of an organic disperser solvent usually

decreases the partition of analytes into the extractant solvent. Additionally, the use of relatively large volumes of a harmful solvent is environmentally unfriendly. According to Saraji and Boroujeni (2010), different strategies in DLLME methods on the use of low-toxicity and more efficient disperser agents, to reduce the disperser volume, and to use DLLME without a disperser agent are reported [49]. The most commonly used disperser solvents include methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), acetone, propan-2-ol, and tetrahydrofuran [46, 50].

MeOH, EtOH and ACN were examined for this purpose. The tests were performed according to the initial HPLC conditions and the proposed DLLME procedure. The results in Fig. 10 indicated that EtOH gave the highest extraction efficiency. However, considering error bars, the three solvents gave almost similar results. ACN was chosen as the disperser solvent for the subsequent experiments owing to its lower toxicity and better reproducibility as also dictated by its smallest error bar.

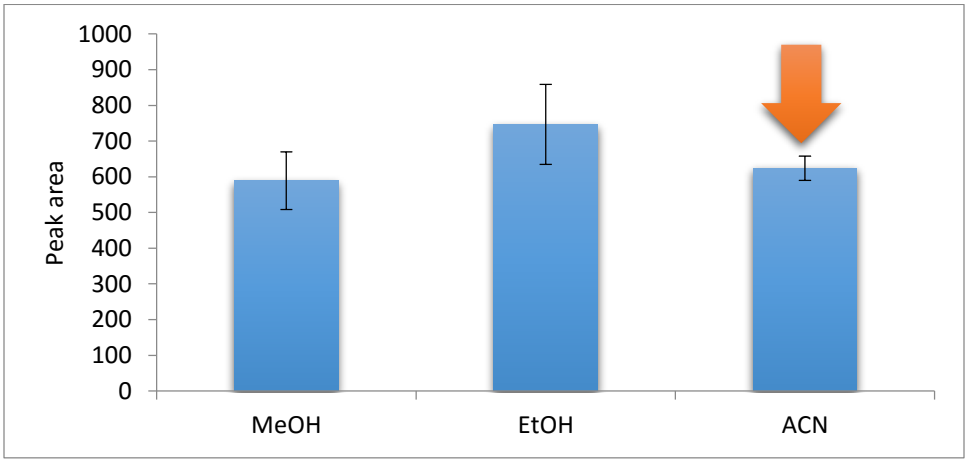



**Figure 10:** Effect of Disperser Solvent Type on the Peak area

### 4.1.3 Effect of Disperser Solvent Volume

The volume of disperser solvent can directly affect the formation of cloudy solution, the degree of dispersion of the extraction solvent in the aqueous phase and subsequently the extraction efficiency. Low volumes of disperser solvent may not be able to disperse the extraction solvent properly, and therefore, a cloudy solution may not form well. On the other hand, at high

volumes, the solubility of the analytes in the aqueous phase increases by increasing the volume of the disperser solvent leading to low extraction efficiency may be low [51].

To evaluate the effect of this parameter, various volumes of ACN (i.e., 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mL) were examined in accordance with the initial HPLC and DLLME procedure. The results presented in Fig. 11 show that at low volumes, extraction efficiency was low which was attributed to the observation that tiny droplets of the extraction solvent were not dispersed effectively and thus the cloudy state was not well formed. This therefore, decreased the extraction recovery. On the other hand, at higher volumes, the solubility of caffeine in aqueous phase increases and the transfer into CF (extraction solvent) decreases resulting into a lower efficiency. The maximum extraction efficiency based on the peak area was achieved at 0.5 mL. Therefore, 0.5 mL of ACN was chosen for further experiments.

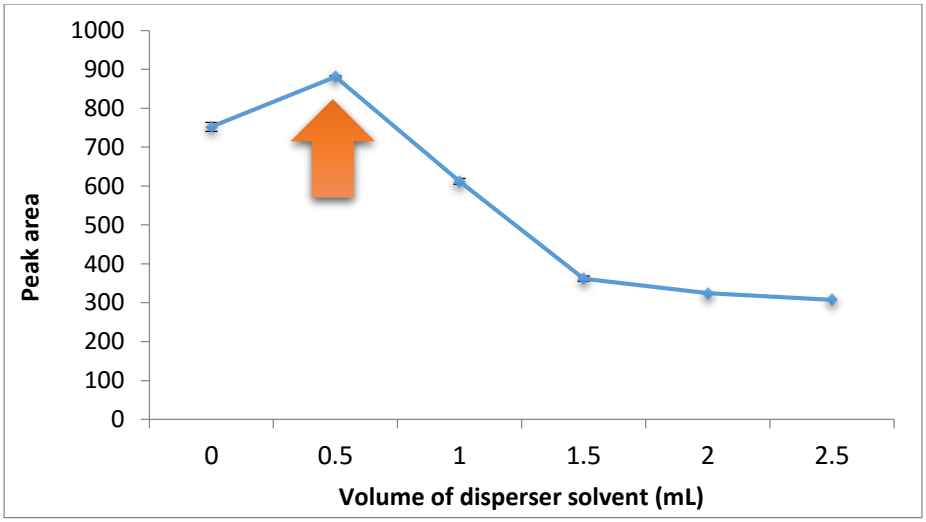



**Figure 11**: Effect of Disperser Solvent Volume on the Peak area

### 4.1.4 Selection of Extraction Solvent

The extraction solvent plays a very important role in DLLME. A conventional DLLME extraction solvent should satisfy four conditions: should have a higher density than that of water, which makes it possible to separate extraction solvent from the aqueous phase by centrifugation; should be immiscible with water and have the capability to extract the analyte; should have a good chromatographic behavior, and low solubility in water [46]. Some applications of lower density solvents including ones with melting points near room temperature have been proposed

[49, 51]. The distribution coefficient and selectivity are the most valuable parameters that control extraction solvent selection. Selectivity means the ability of the solvent to pick up the analyte from the sample solution as compared to other components. In general, the extracting solvent must be able to extract the analytes well, while its solubility in water must be low. For extractants less dense than water, the recovery step is relatively tedious. However, several methods have been developed for this purpose, including solidification of the floating organic drop [45], adsorption by nanoparticles, centrifugation and collection of organic phase in special apparatus [49, 51].

When the density of extraction solvent is lower than that of water, an auxiliary solvent can be used to make the mixture's density higher than that of water [51]. Therefore, phase separation can be performed by centrifugation without the use of special apparatus. Chloroform (CF), carbon tetrachloride (CTC), 1-undecanol (1-UN), 1-dodecanol (1-DO), toluene (TLN), diphenyl ether (DPE) and dichloromethane (DCM) are the most commonly used extraction solvents in DLLME [46, 50] Halogenated hydrocarbons, such as chlorobenzene, chloroform, carbon tetrachloride and tetrachloroethane, are usually selected as the extraction solvents because of their high density. It is important to note that halogenated solvents are toxic and environmentally unfriendly [49].

To overcome the drawback of the use of halogenated solvents in conventional DLLME, low toxicity solvents have been proposed, including brominated/iodinated solvents with toxicity lower than that of chlorinated solvents. A research was conducted by Saraji and Boroujeni (2010), about the extraction performance of 5 chlorinated solvents and 13 brominated/iodinated solvents in the extraction and preconcentration of polycyclic aromatic hydrocarbons (PAHs) from water samples. The results showed that some of the brominated/iodinated solvents have better extraction efficiency than chlorinated solvents. In addition, for polar organic compounds, a novel DLLME method was established by using tri-n-butylphosphate as the extractant [49].

Considering the characteristics of the extraction solvent, chloroform (CF) (density: 1.48 g $mL^{-1}$), dichloromethane (DCM) (density: 1.326 g $mL^{-1}$), diphenylether (DPE) (density: 1.08 g $mL^{-1}$), 1-undecanol (UN) (density: 0.83 g $mL^{-1}$) and 1-dodecanol (DO) (density: 0.833 g $mL^{-1}$) were tested in this study. The results shown in Fig. 12 demonstrated that among these solvents, CF

provided the highest extraction efficiency for caffeine. Therefore, CF was considered as the extraction solvent in the subsequent experiments.

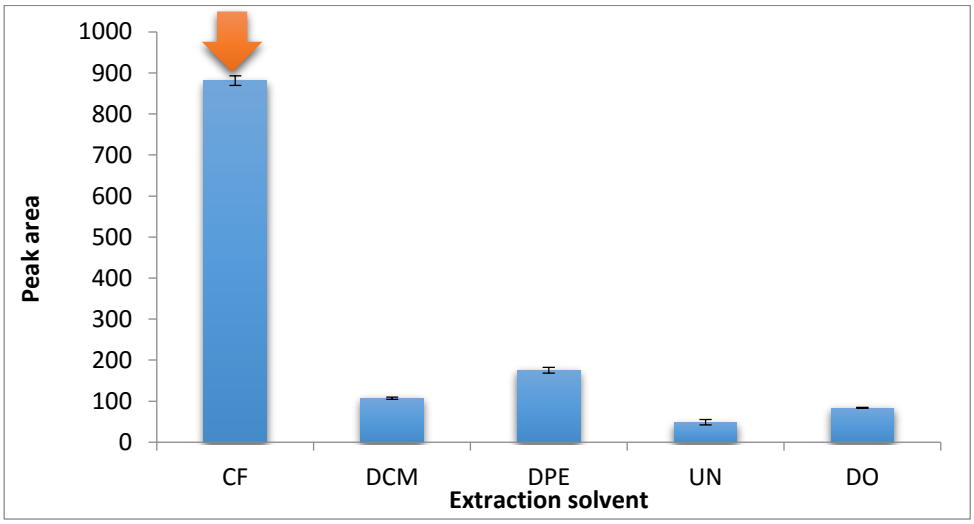



**Figure 12:** Effect of Extraction Solvent Type on Peak Area

### 4.1.5 Effect of Extraction Solvent Volume

The volume of extraction solvent is another key parameter that greatly affect the extraction performance. With the increase of the extraction solvent volume, the final organic phase obtained by centrifugation increases, resulting in a decrease of the concentration of the target analyte in the organic phase. Although the extraction recovery remains almost constant, the enrichment factor will decreases, leading to a decrease in the sensitivity of the method for determining the analytes. Therefore, the optimal volume of extraction solvent should ensure both high enrichment factors and enough volume for the subsequent determination after centrifugation [50, 51].

To study the effect of extraction solvent volume on the extraction efficiency, different volumes of CF (150, 200, 250, 300 and 350 µL) were studied based on the proposed procedure. With the volumes lower than 150 µL, insufficient sedimented extractant at the bottom of the conical test tube formed. This was attributed to the partial solubility of CF in water. Fig. 13 shows that the

highest chromatographic peak area was obtained when the volume of CF was 250 µL. At volumes greater than 250 µL, the dilution effect became predominant over extraction capacity and thus the extraction efficiencies were low. Considering the above discussion, 250 µL was chosen as the optimum volume of CF for further experiments in this study.

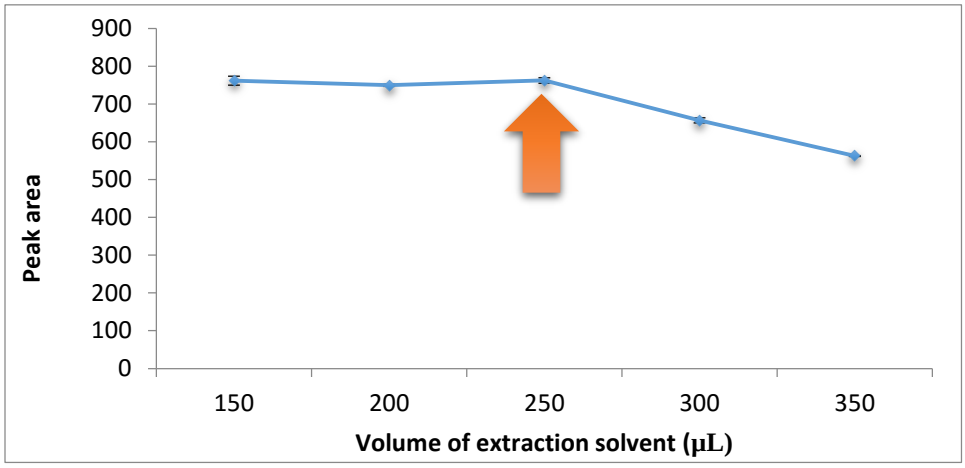



**Figure 13:** Effect of Extraction Solvent Volume Peak Area

### 4.1.6 Effect of Salt Addition

The addition of salt to aqueous sample solutions may have different effects on extraction (salting-out, salting-in or no effect). Addition of salt to the aqueous solutions increases the polarity of water in the aqueous solution. Depending on the target analyte, if the analyte is polar, it will favor the polar phase (consisting of the salt and water). This process is known as salting-in of analyte. However, if the analyte is non-polar, addition of salt will make the analyte partition itself more into the non-polar organic phase. This process is known as salting-out of analyte. The solubilities of the target analyte and organic extraction solvent in aqueous phase usually decreases with increase in the ionic strength due to salting out effect and this is favorable for reaching high recovery [50]. On the other hand, the volume of the obtained organic phase increases with increase in the salt concentration and therefore, both the target analyte concentration and the enrichment factor decreases. In some investigations, salt addition did not have effective influence on the efficiency of extraction [50, 51].

In order to study the effect of salt concentration on the performance of the proposed method, different concentrations of NaCl (0, 2, 4, 6, 8 and 10%, w/v) were examined and the results were plotted in Fig. 14. The experimental results shown in Fig. 14 indicated that peak area decreased with increasing NaCl concentration from 0 to 10% (w/v). It was also noted that the cloudy state did not form well when 8.0% and above were added to the sample solution due to the decrease of ACN miscibility with water. Consequently, no salt was added in the subsequent experiments.

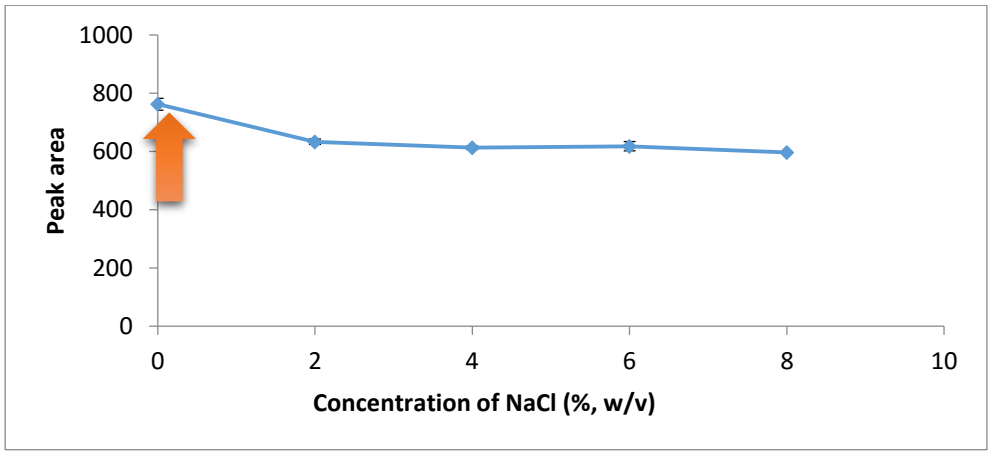



**Figure 14:** Effect of Salt Addition on Peak Area

### 4.1.7 Effect of Extraction Time

In DLLME, 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 [19, 45] , which corresponded to the time of vortex mixing in this study. It was reported that the extraction time generally has little effect on the extraction efficiency of DLLME because the extraction solvent can be evenly be dispersed after the formation of the cloudy solution, the transition of the analyte from aqueous phase (sample) to extraction phase can be very fast, and the equilibrium state can be subsequently achieved very quickly, resulting in a very short extraction time needed for equilibrium to be reached. Short extraction time is a remarkable advantage of the DLLME technique [50]. The very short time of DLLME is most likely attributed to an infinitely large surface area between the extraction solvent and the aqueous phase [51].

The effect of extraction time on the extraction efficiency was examined in the range of 30–210 s under constant experimental conditions. The results showed that the highest extraction efficiency was obtained at 60 s after which it remained constant as shown in Fig. 15. Therefore, 60 s was used as the extraction time for further experiments.

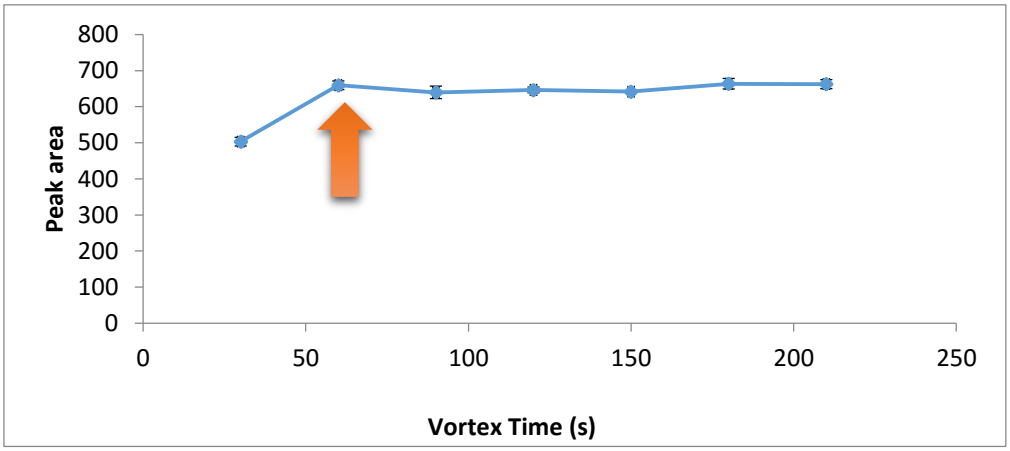



**Figure 15:** Effect of Extraction Time on Peak Area

## 4.2 Optimization of Back-Extraction Parameters

Back extraction is a further sample clean up prior to injection into HPLC instrument. This step was important in order to avoid damaging the column of the instrument due to immiscibility of CF with the mobile phase.

### 4.2.1 Effect of MeOH concentration in BES

Under optimized DLLME parameters and initial HPLC conditions, different volumes of MeOH (i.e., 40, 50, 60, 70, 80 and 90% v/v) in DI water were tested separately. The results indicated that the highest peak area was obtained using 40% MeOH in water as shown in Fig. 16. Therefore, 40:60 MeOH/$H_2O$ (%, v/v) was chosen as the BES for further experiments.

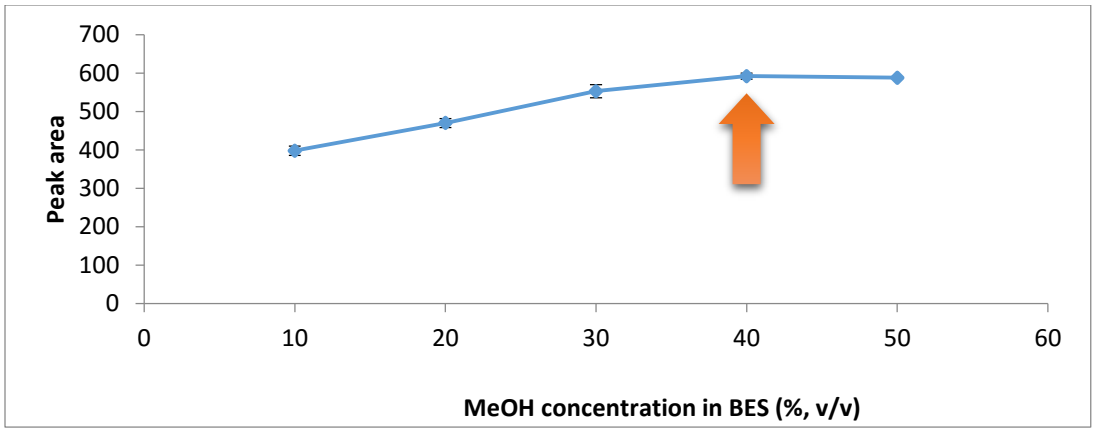



**Figure 16:** Effect of BES on Peak Area

### 4.2.2 Effect of BES Volume

The effect of BES volume on the extraction efficiency was studied over the range of 100-800 µL of 40:60 MeOH/$H_2O$ (% v/v). The extraction efficiency increased up to 200 µL were it decreased gradually upon increasing BES volume due to dilution of the analyte (Fig. 17). Nevertheless, lower volumes than 100 µL could not be used due to loss of phase separation. Hence, subsequent experiments were performed using 200 µL of 40:60 MeOH/$H_2O$.

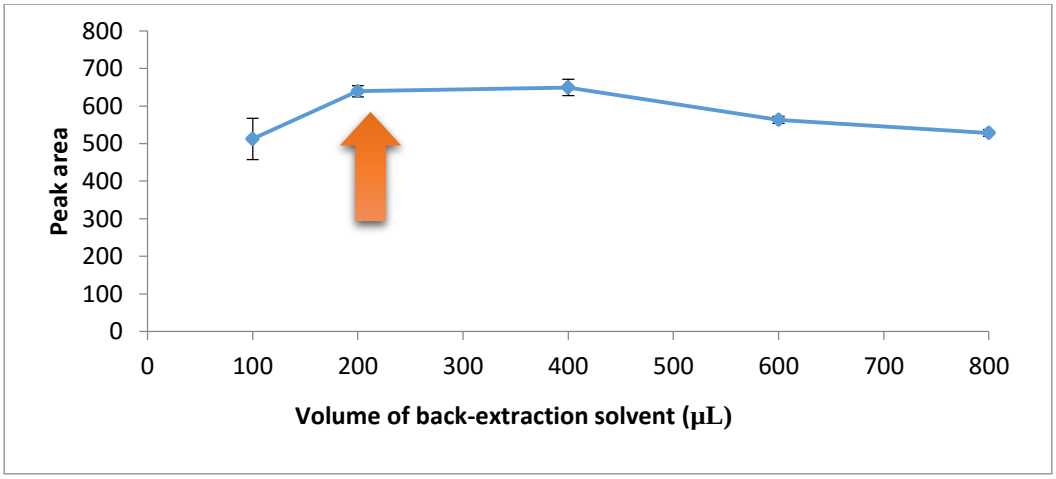



**Figure 17:** Effect of BES Volume on Peak Area

### 4.2.3 Effect of Back-Extraction Vortex Time

Under optimized DLLME parameters and initial HPLC conditions, the back extraction vortex time (i.e., 30, 60, 90, 120, 150, 180 and 210 s) were tested. The results obtained showed that the back-extraction vortex time has no much effect on the peak area from 60 s and above. However, 30 s was not enough to extract all the analyte from the CF as shown from the peak area in Fig. 18, indicating that 60 s were required to reach equilibrium.

Table 3 summarizes the optimum DLLME and back-extraction conditions, and Fig. 19 shows a representative chromatogram obtained under these conditions.

**Table 3:** Optimized DLLME Parameters

| **NAME OF PARAMETER** | **OPTIMIZED PARAMETER** |
|---|---|
| **DLLME PARAMETERS** | |
| Type of disperser solvent | ACN |
| Disperser solvent volume (mL) | 0.5 |
| Type of extraction solvent | CF |
| Extraction solvent volume (µL) | 250 |
| Salt addition (%, w/v) | 0% NaCl |
| Extraction time (s) | 60 |
| **BACK-EXTRACTION PARAMETERS** | |
| MeOH concentration in BES (%, v/v) | 40:60 MeOH/$H_2O$ |
| BES volume (µL) | 200 |
| Back-extraction vortex time (s) | 60 |

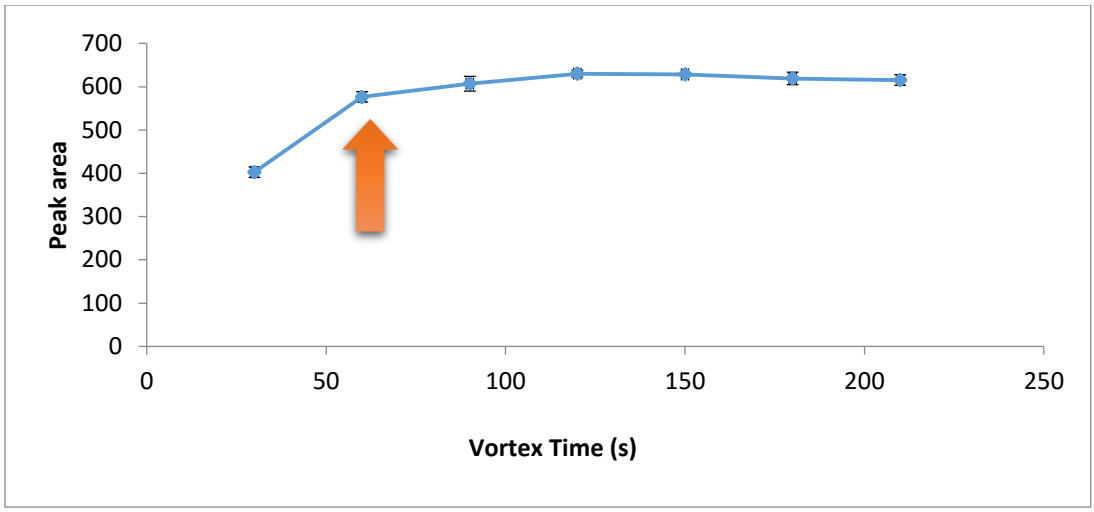



**Figure 18:** Effect of BES Vortex Time on Peak Area

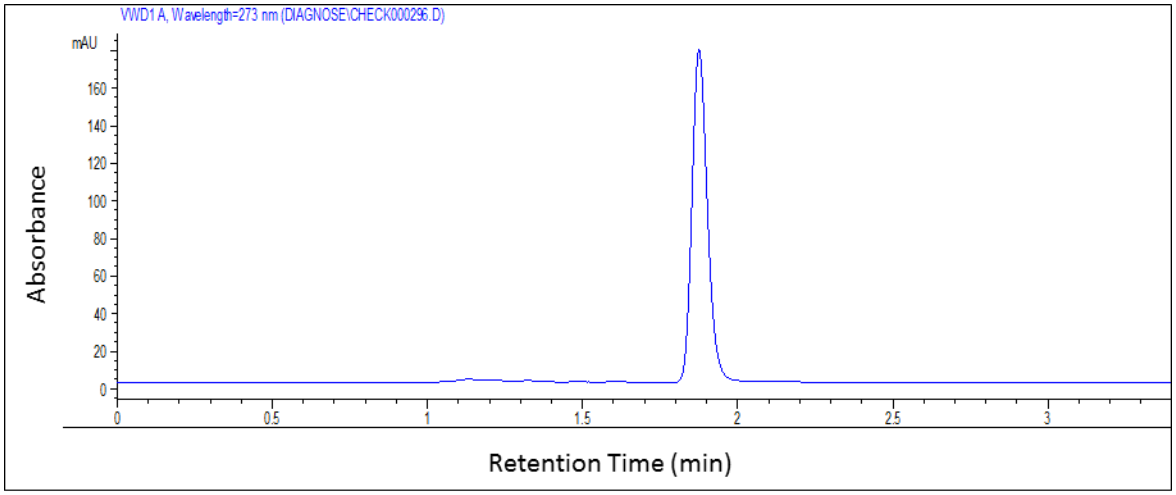



**Figure 19:** A Representative Chromatogram under Optimum DLLME Conditions

## 4.3 Optimization of HPLC Conditions

The major HPLC parameters which include the column type, type of mobile phase and its composition and flow rate were tested to get the optimum chromatogram of the analyte from the instrument.

### 4.3.1 Effect of Column Type

Two HPLC columns were examined using 20.0 mg $L^{-1}$ standard solution without extraction to get the chromatographic efficiency according to the initial HPLC conditions. The peaks obtained from the experiments are in Fig. 20.

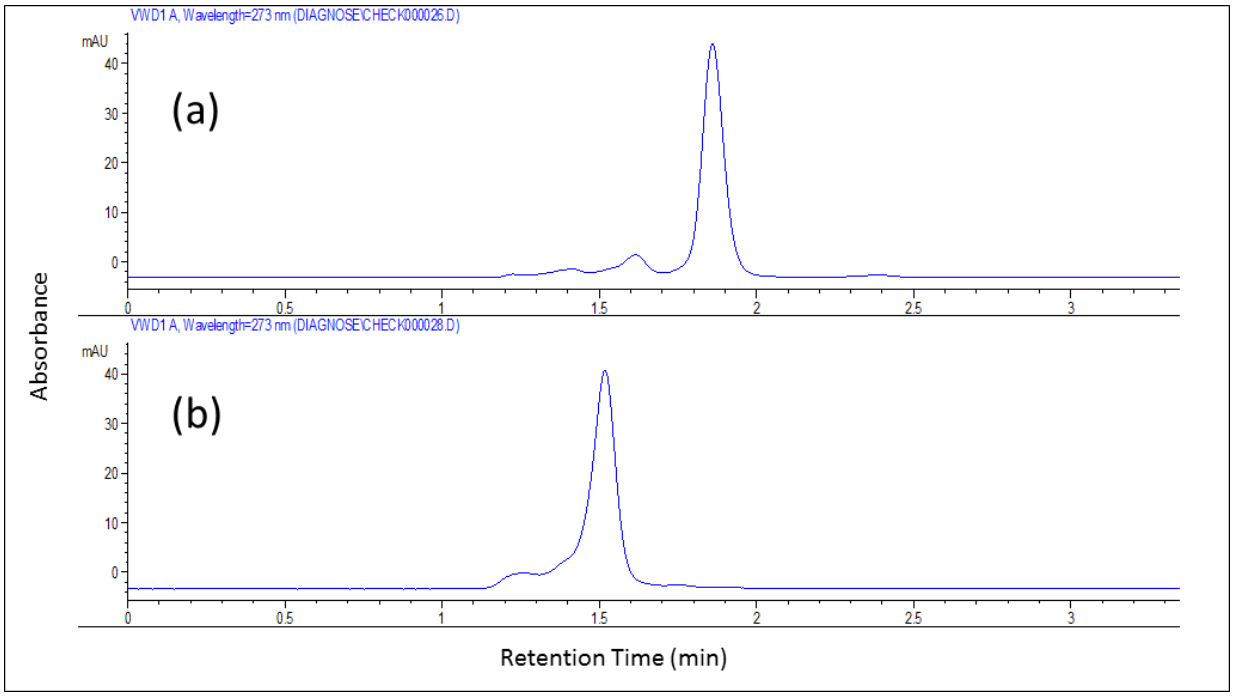



**Figure 20:** Representative Chromatograms obtained with (a) Agilent Eclipse XDB-$C_{18}$. 4.6 mm ID × 150 mm (5 µm) and (b) Zorbax $C_{18}$. 4.6 mm ID × 150 mm (5 µm)

From the peaks obtained by using the two different columns, Agilent Eclipse XDB-C18. 4.6 mm ID × 150 mm (5 µm) was found to give a better chromatogram and was therefore selected for further experiments in this research.

### 4.3.2 Effect of Mobile Phase Composition

Different compositions of MeOH/$H_2O$ were examined by increasing the concentration of MeOH in the range of 50-90% (v/v). At 80 and 90% (v/v), peak shape deteriorated as indicated by poor peak symmetry (Appendix 10). Better peak shape was obtained with 50, 60 and 70% MeOH. Although the maximum peak area was obtained with 50% MeOH, this volume was not preferred

as the maximum $H_2O$ content from this column (as recommended by the manufacturer was 40% $H_2O$). Therefore, 70% MeOH was selected as the optimum value.

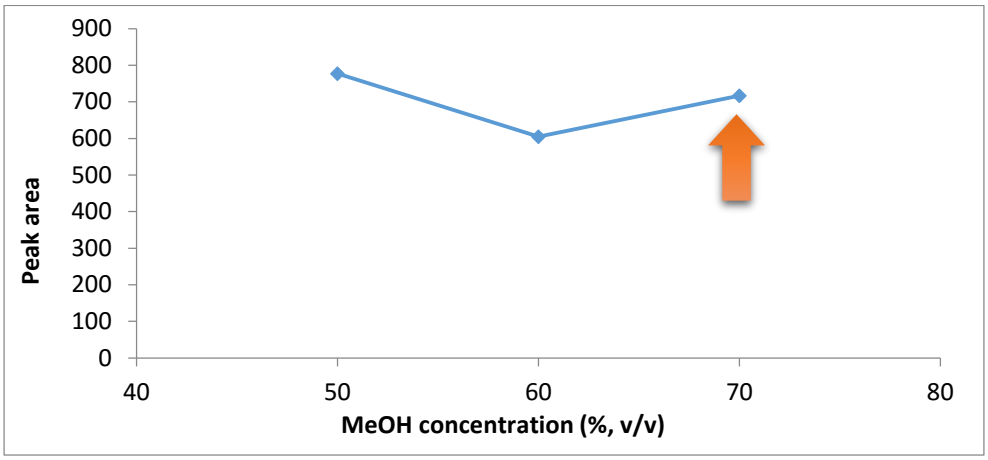



**Figure 21:** Effect of Mobile Phase Composition on Peak Area

### 4.3.3 Effect of Flow Rate

Flow rate was studied in the range of 0.6-1.4 mL $min^{-1}$. It can be seen from Fig. 22 and Fig. 23 that increasing the flow rate decreases the peak area and retention time. As such, 1.0 mL $min^{-1}$ was chosen as a compromise between the retention time and peak area.

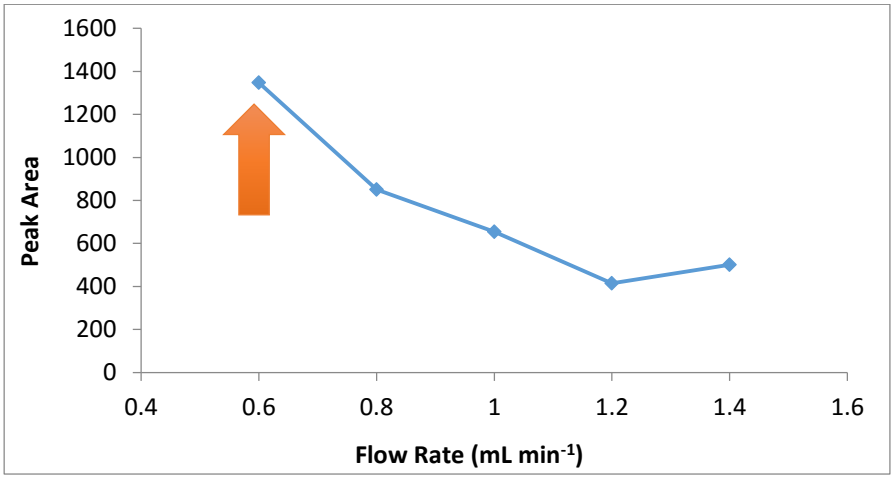



**Figure 22:** Effect of Flow Rate on Peak Area

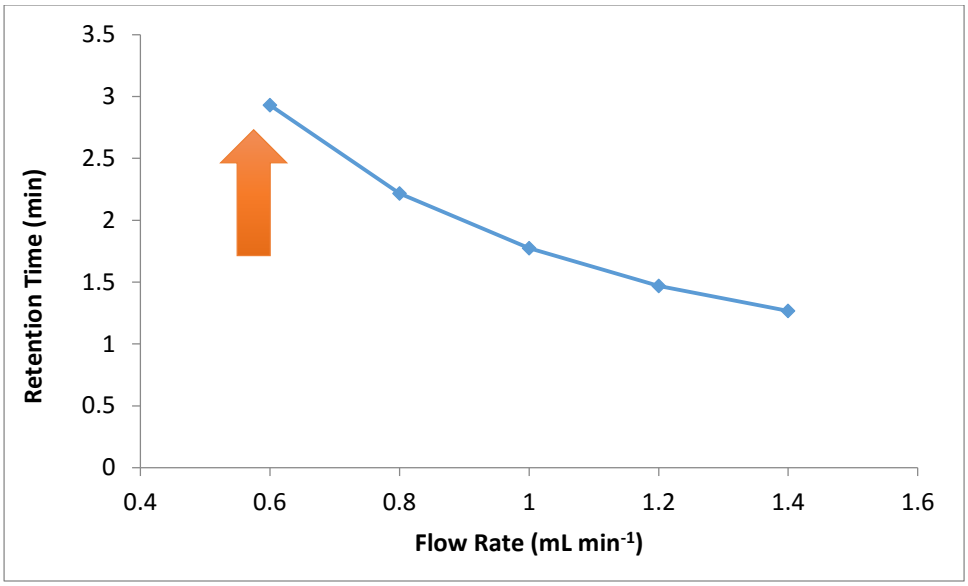



**Figure 23:** Effect of Flow Rate on Retention Time

## 4.4 Analytical Performance and Figures of Merit

Under the optimal HPLC and DLLME operating conditions, the analytical figures of merit consisting of linear dynamic range (LDR), limit of detection (LOD), limit of quantitation (LOQ), coefficient of determination ($R^2$) and relative standard deviation (RSD) for the determination of caffeine in the kola samples using DLLME were obtained to evaluate the method.

### 4.4.1 Standard Calibration in BES

Under optimized HPLC conditions, different caffeine concentrations ranging from 0-30 mg $L^{-1}$ prepared in the BES was injected (without extraction) and the results of the peak areas obtained were plotted (Fig. 24). 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 (equations 1-5). 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.

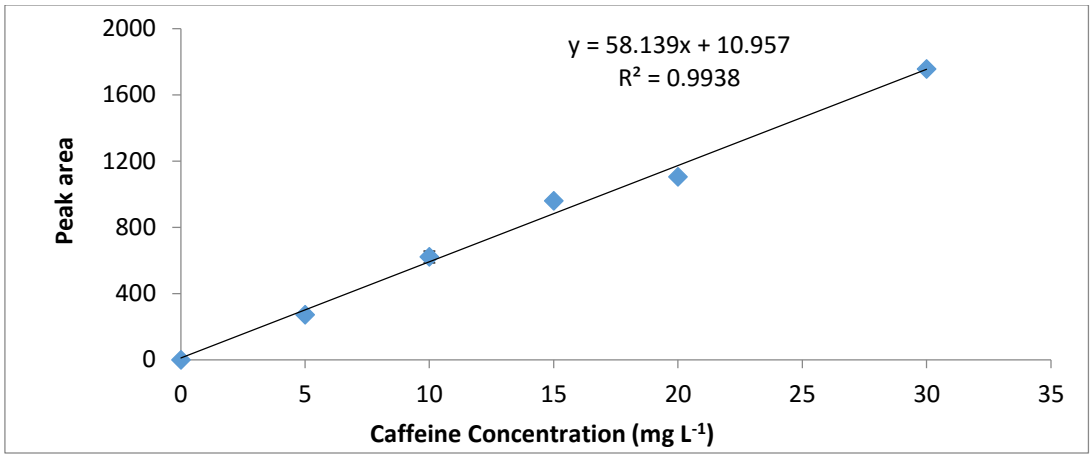



**Figure 24:** Standard Calibration in BES

### 4.4.2 Standard Addition Calibration for Red Kola

Under optimum HPLC conditions, the DLLME procedure (as described in section 3.7), was used after hot water extraction to obtain the standard addition curve for red kola.

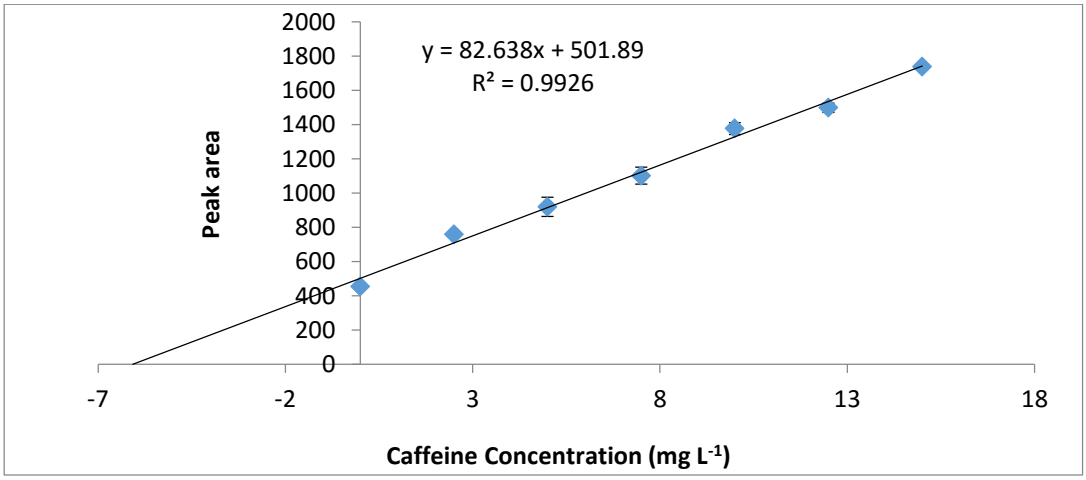



**Figure 25:** Standard Addition Calibration for Red Kola

Therefore, from the standard addition calibration curve for Red Kola (Fig. 25), the concentration of caffeine in the sample solution of red kola was found to be 6.072 mg $L^{-1}$ indicating that red kola contained 202 (±5.3) µg $g^{-1}$ of caffeine.

### 4.4.3 Standard Addition Calibration for white Kola

Under optimum HPLC conditions, the DLLME procedure (as described in section 3.7), was used after hot water extraction to obtain the standard addition curve for white kola.

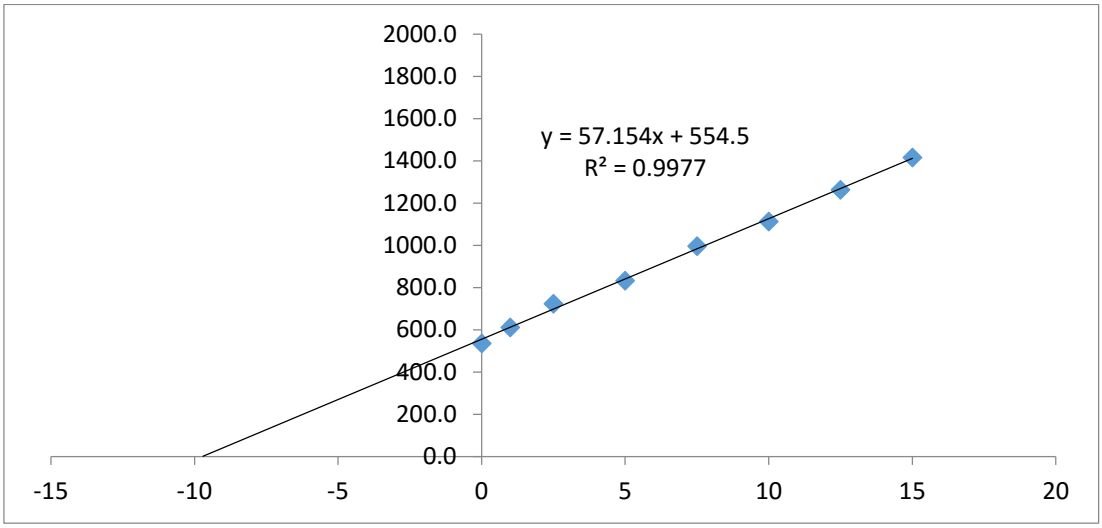



**Figure 26:** Standard Addition Calibration for White Kola

Therefore, from the standard addition calibration curve for white kola (Fig. 26), the concentration of caffeine in white kola was found to be 9.70 mg $L^{-1}$ indicating that white kola contained 323 (±9.3) µg $g^{-1}$ of caffeine.

**Table 4:** Analytical Performance of DLLME-HPLC

| Matrix | Regression equation[a] | LOD[b] | LOQ[c] | LDR[d] | $R^2$ | RSD (%)[e] Intra-day | Inter-day |
|---|---|---|---|---|---|---|---|
| Red Kola | y = 82.638x + 501.89 | 0.56 | 1.33 | 1.9-30 | 0.9926 | 3.3 | 3.4 |
| White Kola | y = 57.154x + 554.5 | 0.35 | 1.2 | 1.2-30 | 0.9977 | 3.1 | 4.2 |

[a] Peak area = slope (± SD) × [CF concentration (μg $mL^{-1}$)] + intercept (± SD).

[b] Limit of detection

[c] Limit of quantitation

[d] Linear dynamic range

[e] Percentage relative standard deviation, n = 3.

$$LOD = \frac{3SD_b}{m}$$

$$LOQ = 3.33 \times LOD = \frac{10SD_b}{m}$$

**Table 5:** Relative Recoveries of Caffeine from Kola Samples

| Matrix | Added (μg $mL^{-1}$) | Found (μg $mL^{-1}$, ±SD, n=3) | % RR[a] |
|---|---|---|---|
| Red Kola | - | 6.07 (±0.16) [202 ± 5.3][b] | - |
| | 5.0 | 10.6 (±0.35) | 95.8 |
| | 10.0 | 16.0 (±0.33) | 99.6 |
| White Kola | - | 9.70 (±0.28) [323 ± 9.3][b] | - |
| | 5.0 | 14.2 (±0.44) | 96.6 |
| | 10.0 | 19.1 (±0.56) | 97.0 |

[a] % Relative recovery, percentage value obtained considering yields from standard addition calibrations.

[b] Concentration of caffeine (in μg $g^{-1}$) in the original sample.

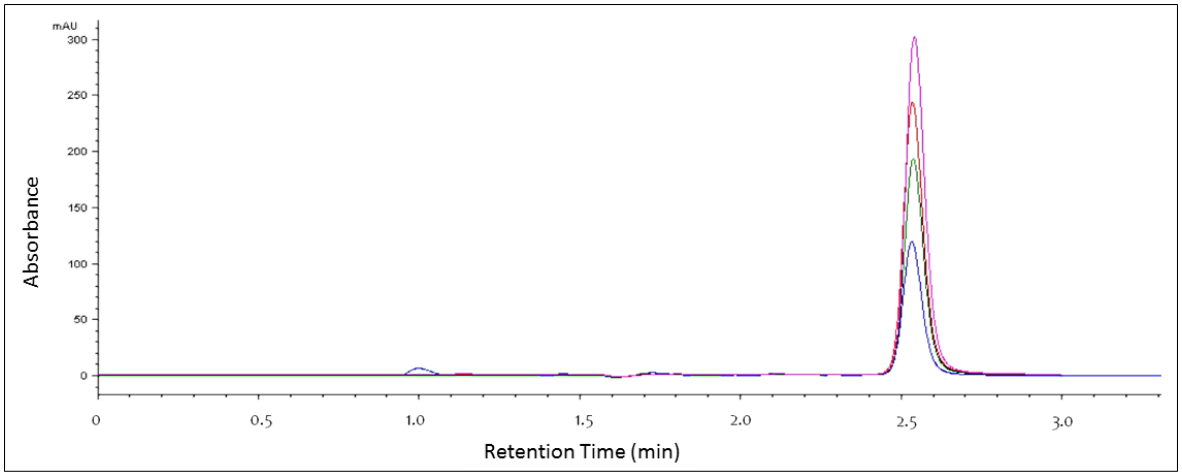



**Figure 27.** Representative chromatograms of the samples.

From down to top: unspiked red kola sample ( ); unspiked white kola ( ); red kola spiked at 10.0 µg $mL^{-1}$ of CF ( ); and white kola spiked at 10.0 µg $mL^{-1}$ of CF ( ).

## 4.5 Comparison with other Preconcentration Methods

Efficiency of the presented DLLME–HPLC method for the selected analyte was compared with other reported HPLC methods taking into account parameters such as extraction time, total volume of organic solvents consumed per sample, LOD and LDR. In comparison with other methods, the main advantages of this extraction method were rapidness, simplicity and cost effectiveness. As listed in Table 6, the extraction time was only 2.5 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 only 0.5 mL of organic solvents for analysis which is much less compared with other methods. LODs and LDRs achieved were comparable with others except for those suggested in [60] and [61].

**Table 6:** Comparison with Other HPLC Methods

| Extraction method | Detector/ λ (nm) | Extraction time (min) | $V_{org.}$[a] (mL) | LOD[b] (μg $mL^{-1}$) | LDR[c] (μg $mL^{-1}$) | REF |
|---|---|---|---|---|---|---|
| LLE[d] | UV (271) | 240 | 50 | 0.30 | 0.4–8.2 | [59] |
| UAE[e] | DAD (212) | 30 | 17.5 | 0.0028 | 1–500 | [60] |
| UAE[e] | DAD (270) | 20 | ~33 | 0.004 | – | [61] |
| MAE[f] | UV (280) | 10 | 12.5 | 0.17 | 0.5–80 | [62] |
| DLLME | DAD (273) | 2.5 | 0.5 | 0.56 (red kola) 0.35 (white kola) | 1.2–30 | 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 5

## CONCLUSIONS AND RECOMMENDATIONS

In this study, the applicability of DLLME followed by a back-extraction step as an efficient sample clean-up and preconcentration technique for the determination of caffeine in red and white kola nuts (*Cola nitida*) was demonstrated to be an effective sample preparation technique which offers a rapid extraction with minimum consumption of organic solvents. Other advantages such as simplicity, low cost and ease of operation make the method user-friendly. Despite the complexity of the matrices studied, good recoveries, high reproducibility and interference-free chromatograms were achieved in about 2.5 min analysis times. The results indicated 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.

Future work will focus on the extension of the proposed DLLME-HPLC method to include the main metabolites of caffeine (i.e. paraxanthine, theobromine and theophylline) in other food and beverages samples such as Turkish coffee and tea as well as soft and energy drinks on the Turkish market.

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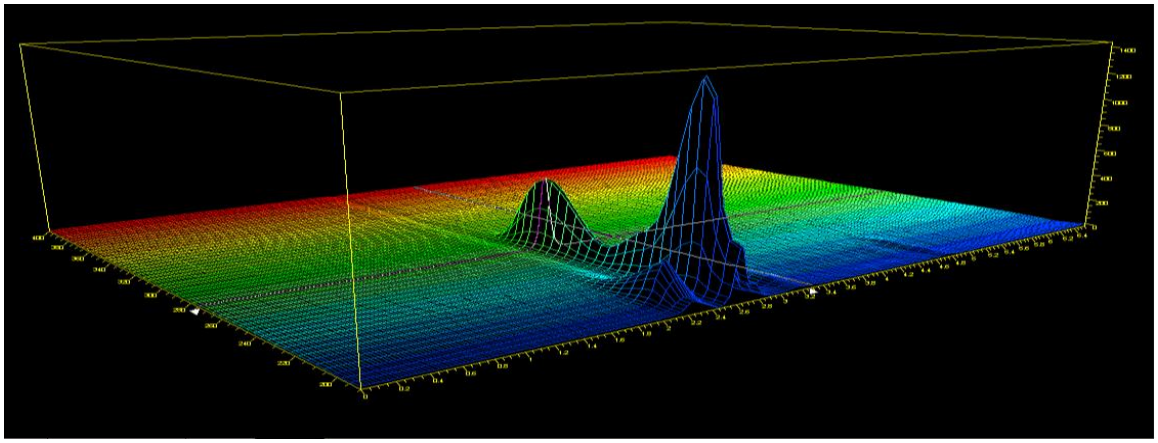
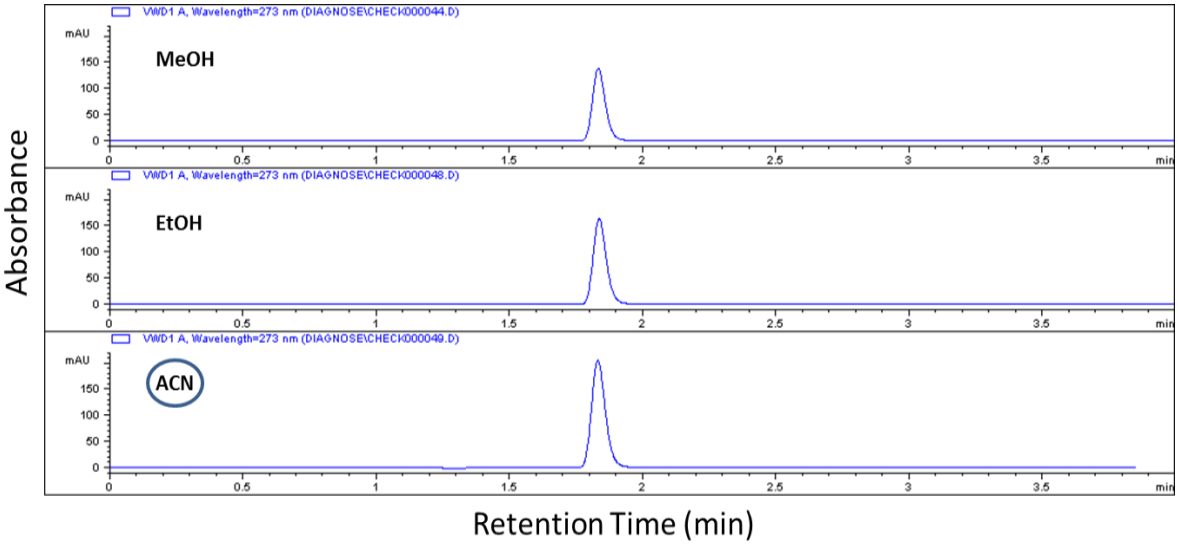
# APPENDICES

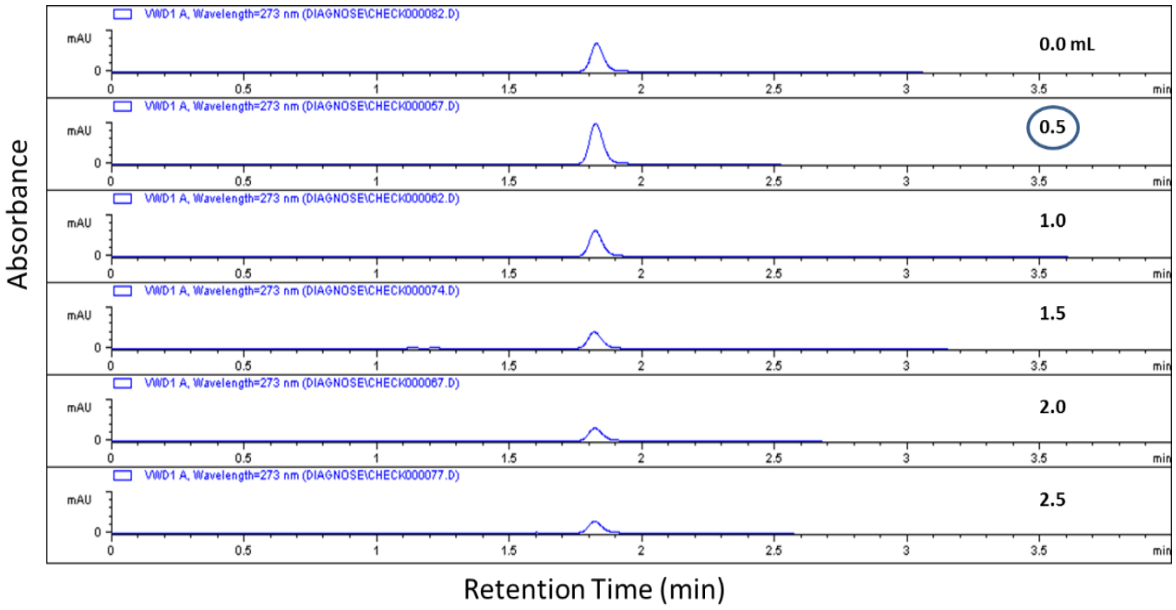
**APPENDIX 1:** Optimization of wavelength detection



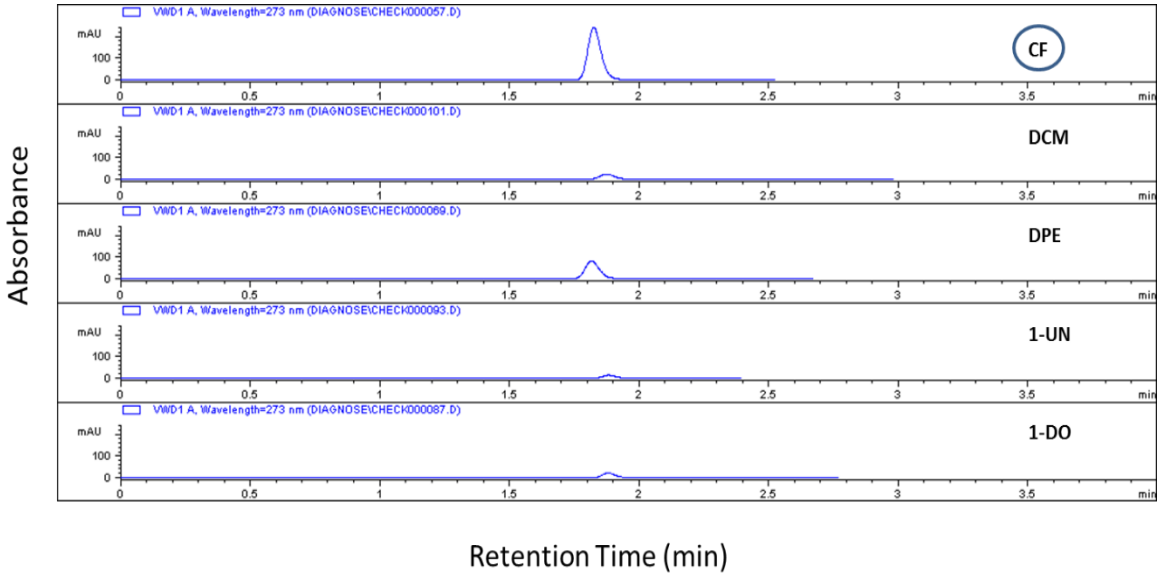
**APPENDIX 2:** Chromatograms for the effect of the type of disperser solvent



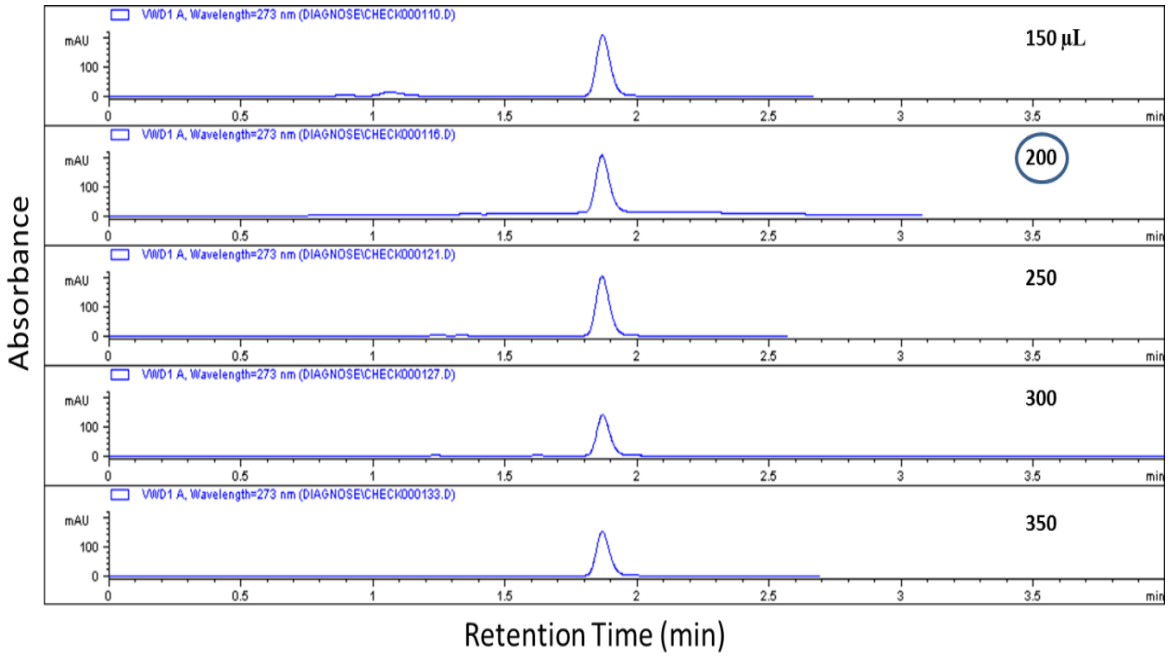
**APPENDIX 3:** Chromatograms for the effect of Disperser solvent volume



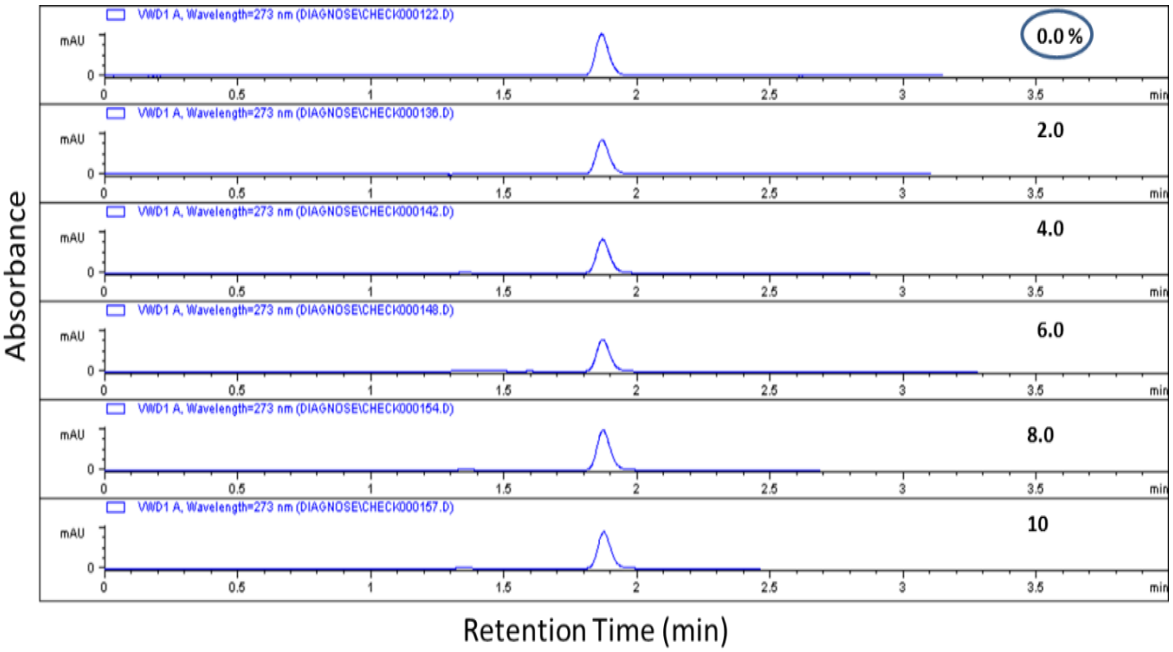

**APPENDIX 4:** Chromatograms for the effect of the type of extraction solvent



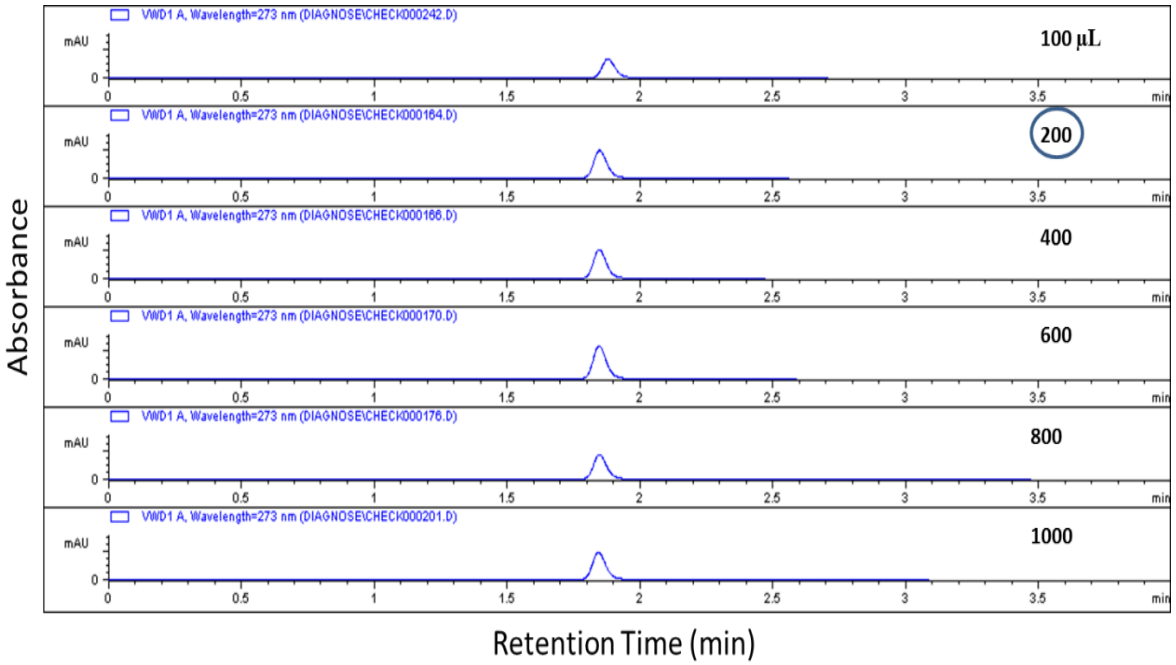
**APPENDIX 5:** Chromatograms for the effect of the volume of extraction solvent



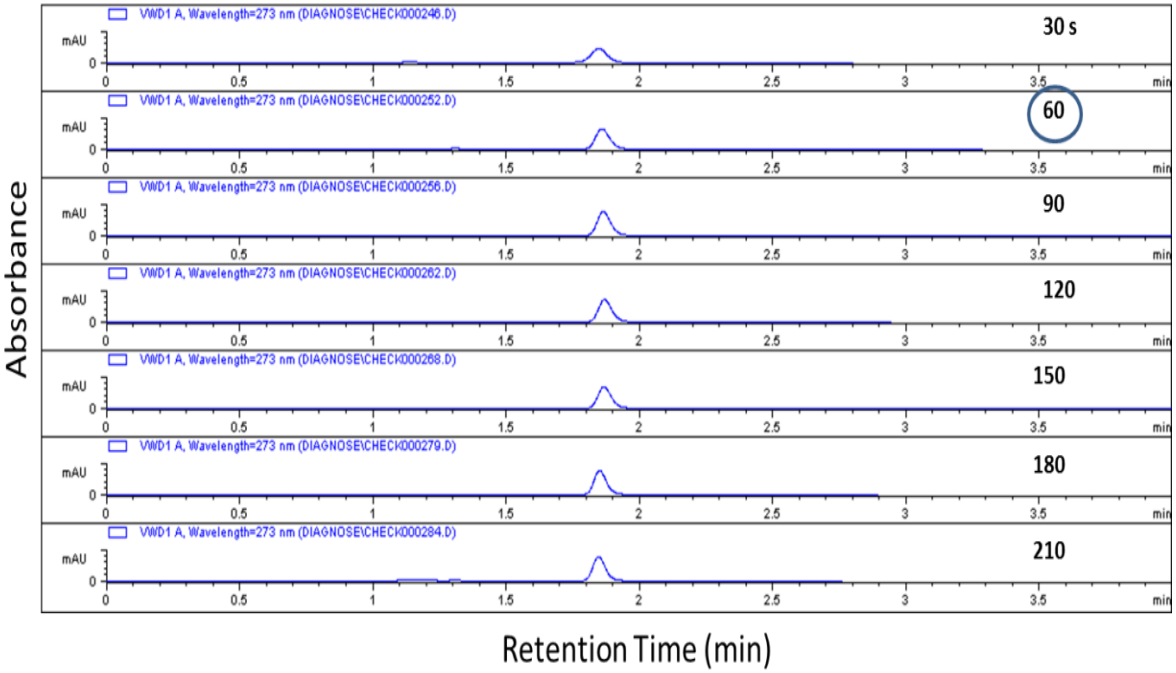

**APPENDIX 6:** Chromatograms for the effect of salt addition



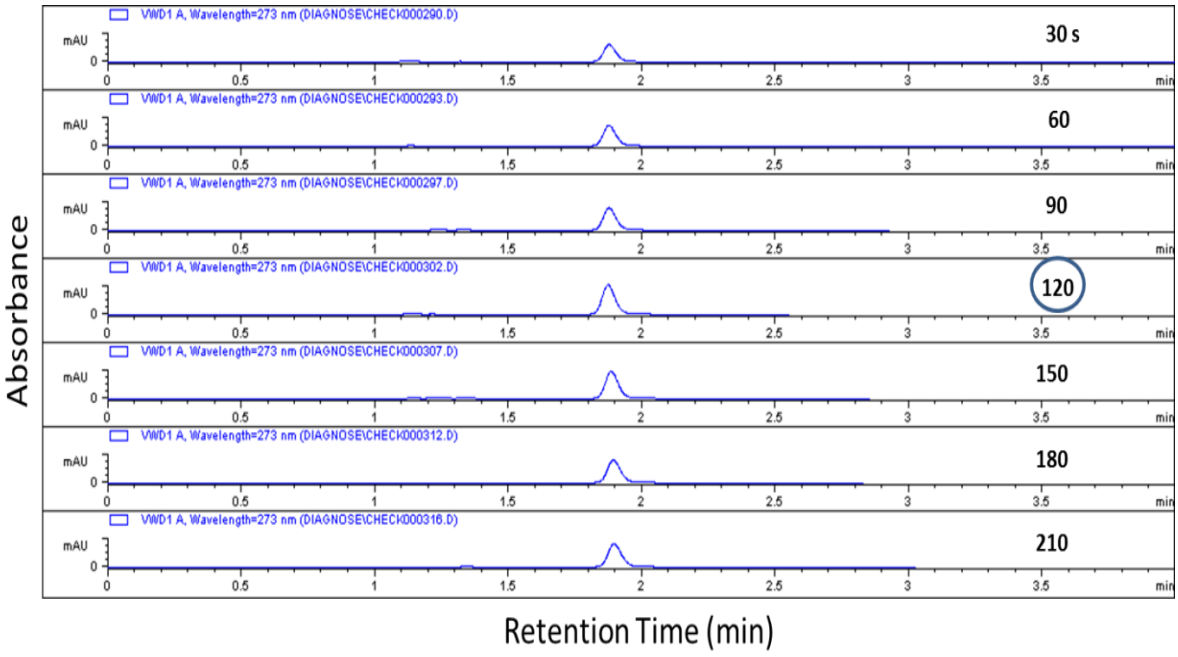
**APPENDIX 7:** Chromatograms for the effect of back-extraction volume



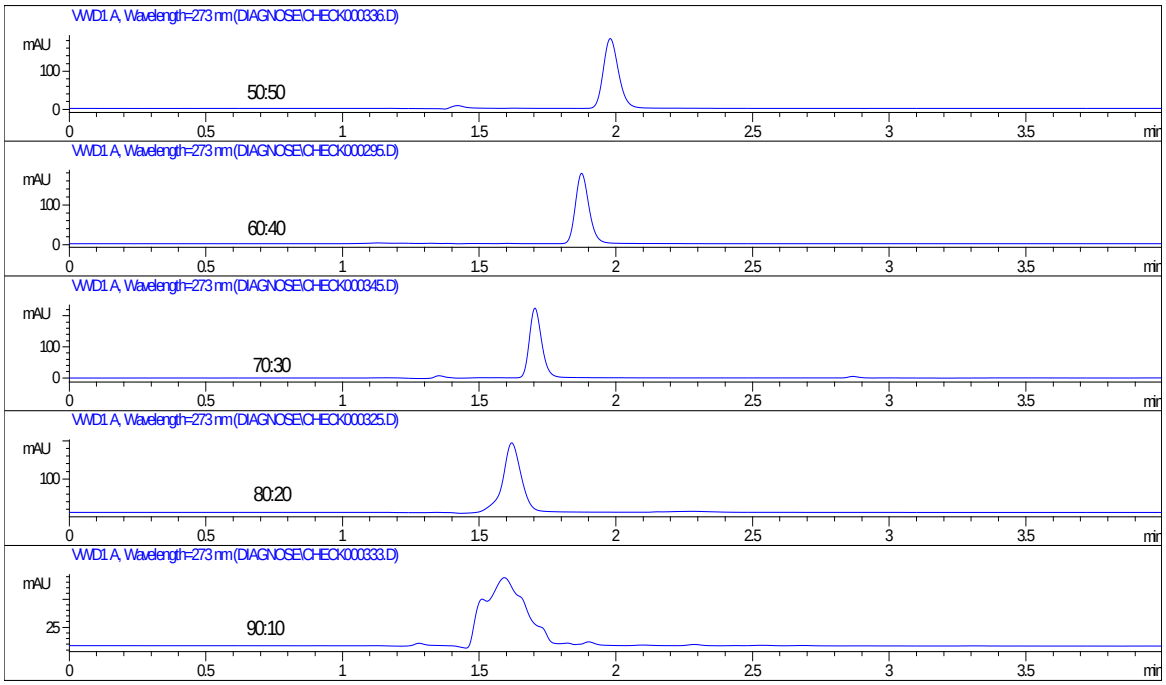

**APPENDIX 8:** Chromatograms for the effect of DLLME vortex time



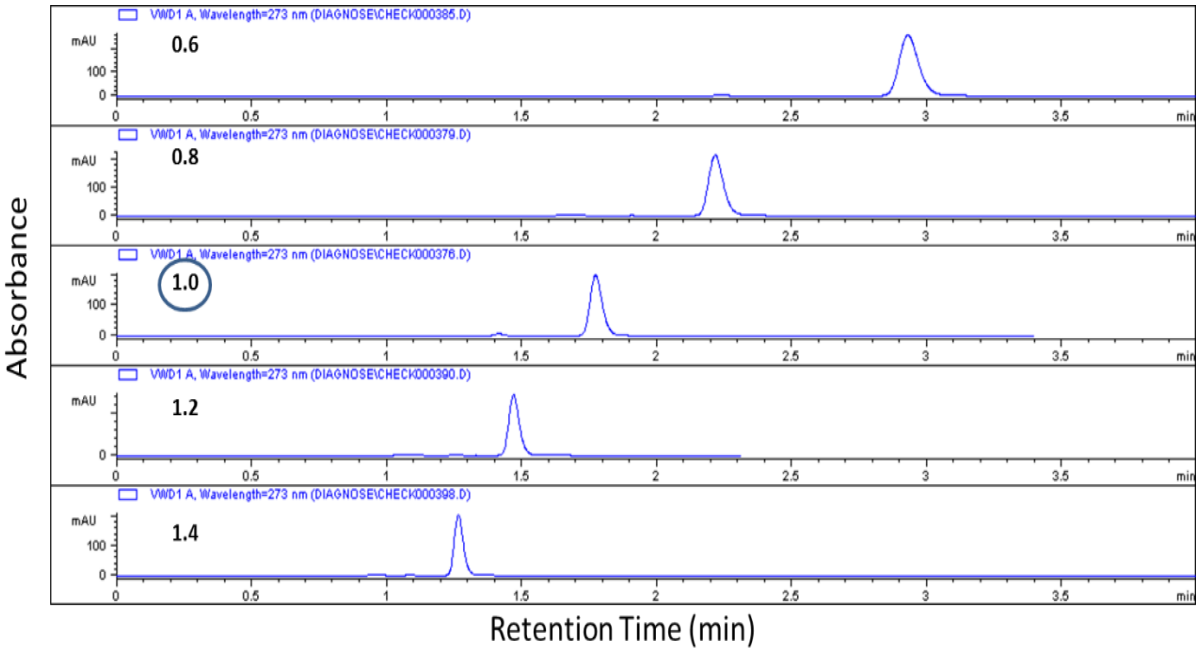
**APPENDIX 9:** Chromatograms for the effect of BES vortex time




**APPENDIX 10:** Chromatograms for the effect of mobile phase composition



**APPENDIX 11:** Chromatograms for the effect of flow rate




**APPENDIX 12:** Chromatograms for the effect of MeOH concentration in BES (%, v/v)