T.R.N.C.

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

SWITCHABLE-POLARITY SOLVENT-LIQUID-LIQUID MICROEXTRACTION OF PIPERINE FROM BLACK AND WHITE PEPPER PRIOR TO ITS DETERMINATION BY HPLC

MAIS AL-NIDAWI

ANALYTICAL CHEMISTRY

MASTER OF SCIENCE THESIS

NICOSIA

2018

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

SWITCHABLE-POLARITY SOLVENT-LIQUID-LIQUID MICROEXTRACTION OF PIPERINE FROM BLACK AND WHITE PEPPER PRIOR TO ITS DETERMINATION BY HPLC

MAIS AL-NIDAWI

ANALYTICAL CHEMISTRY
MASTER OF SCIENCE THESIS

SUPERVISOR
ASSIST. PROF. DR. USAMA ALSHANA

CO-SUPERVISOR
PROF. DR. İHSAN ÇALIŞ

NICOSIA
2018

Insert Approval Page here

DECLARATION

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

Name, Last Name **:** MAIS AL-NIDAWI

Signature **:**

Date **:** 29 May 2018

ACKNOWLEDGEMENTS

I would like to express my biggest appreciation and thanks to my supervisor Assist. Prof. Dr. Usama Alshana who has guided me tirelessly to the right path in each step throughout this research, for being patient with me, and for giving me a positive energy to complete this thesis without whom it would not have been possible to complete. You were our father, elder brother and friend and not only a supervisor. I appreciate your efforts and everything you did. I am very grateful to you more than words can say.

I also would like to thank my co-supervisor Prof. Dr. İhsan Çalış for being helpful whenever I needed his advice despite his busy schedule and heavy responsibilities.

I want to thank Assoc. Prof. Dr. Hayati Çelik and Assist. Prof. Dr. Banu Keşanlı for giving their time, teaching and motivating me about life beyond MSc.; I appreciate your efforts.

I would like also to thank Duygu Yiğit Hanoğlu and Azmi Hanoğlu for giving me their time during my experiments at the Laboratory of Pharmacognosy; I am grateful for your help.

I would also like to thank Prof. Dr. Hasan Abou Gazar (Yusufoğlu) from the Department of Pharmacognosy, Faculty of Pharmacy, Prince Sattam bin Abdulaziz University, for analyzing the isolated piperine standard by FT-NMR.

My precious father who supported me morally and financially, I am grateful to you for the rest of my life for giving me the opportunity to chase my dream. I am proud to have you and will make you proud of me.

My mother, my soulmate, I do not know how to thank you; I am so lucky to have you in my life; without you I am nothing. You supported me whenever I needed, you motivated

me whenever I was mentally weak, you endured with me whenever I was in a bad mood, you did not and would not stop helping and supporting me and praying for me, you are always proud of me. I am truly thankful for having you.

My brothers; Mohammed and Ibrahim, my dear sister, my whole family and friends back in Iraq, I am grateful for your support. Thank you from the bottom of my heart for being a part of my life, I am lucky to have you.

Last but not the least, my friends and colleagues at Near East University, it was pleasure to know you and spend time with you, I am proud to have you and feel grateful to you all, specially Malik Hassan, my right hand in the laboratory; I appreciate your efforts and everything you did for me and Jude Caleb, you were always helping and supporting me mentally and giving me positive energy whenever I was mentally tired; thank you, I appreciate your help. Also I want to thank Semra Altunterim and Dr. Selin Işık for supporting and helping me in the writing of Turkish abstract. Thank you all again.

To my late grandfather Masoud Mustafa Al-Bayaty, who passed away few days before my graduation and left fingerprints of forgiveness, love, grace, generosity and kindness in our hearts and lives. Who did not stop encouraging me to get higher education and to open my own laboratory that carries my name. You will not be forgotten.

ABSTRACT

Al-Nidawi, Mais. Switchable-polarity solvent liquid-liquid microextraction of piperine from black and white pepper prior to its determination by HPLC.
Near East University, Institute of Health Science, Analytical Chemistry Program, Master of Science Thesis, Nicosia, 2018.

Piperine is an important alkaloid that has many medical and pharmaceutical benefits, it was extracted from *Piper nigrum L.* by switchable-polarity solvent which was synthesized from triethylamine (TEA)/water/CO_2 (1:1, excess, v/v) via proton transfer reaction and was used for the extraction of piperine from black and white pepper prior to its determination by high-performance liquid chromatography (HPLC). The proposed method was termed as switchable-polarity solvent-liquid-liquid microextraction (SPS-LLME). Optimum extraction conditions were found as follows: Extraction solvent, TEA; volume of TEA in the extraction solvent, 750 µL; and extraction time, 10s. Addition/ volume of organic modifier, centrifugation time, and ionic strength had no significant effect on the extraction efficiency. The final extract was suitable for direct injection into HPLC without any further pretreatment. Optimum HPLC conditions were found as follows: Column, Agilent Eclipse XDB C18 (4.6 mm ID × 15 cm, 5 µm); separation temperature, 20 °C; mobile phase, ACN:H_2O, 45:55 (%, v/v); flow rate, 1.1 mL min^{-1}, and injection volume, 5 µL. Piperine was monitored using a diode-array detector (DAD) at 346 nm. Limits of detection (LOD) and quantitation (LOQ), calculated based on $3S_b/m$ and $10S_b/m$, were found as 0.4-1.2 and 1.3-3.9 mg g^{-1}, respectively. Calibration graphs showed good linearity with coefficients of determination (R^2) higher than 0.9950. Piperine standard was isolated and characterized by 1D- (1H- and 13C-NMR) and 2D-NMR (COSY, HSQC and HMBC). SPS-LLME-HPLC was applied for the extraction and determination of piperine from seven black and one white pepper samples from different origins and percentage relative recoveries (%RR) were obtained within the range of 95.6% and 104.7%. The results proved that SPS-LLME can represent a simple, rapid and green alternative method for the extraction of piperine from black and white pepper prior to HPLC analysis in routine work.

Keywords: Black Pepper, Determination, HPLC, Piperine, Switchable-Polarity Solvent-Liquid-Liquid Microextraction, White Pepper

ÖZET

Al-Nidawi, Mais. Siyah ve Beyaz Biberden Piperinin Değiştirilebilir Polarite Çözücülü - Sıvı-Sıvı Mikroekstraksiyonu ve HPLC ile Tayini.
Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Analitik Kimya Programı, Yüksek Lisans Tezi, Lefkoşa, 2018.

Birçok tıbbi ve farmasötik faydaya sahip önemli bir alkaloid olan piperin, bu tez çalışmasında, trietilamin (TEA)/su/CO_2 (1:1, fazla, h/h) ile sentezlenen değiştirilebilir polariteli çözücü kullanılarak *Piper nigrum L.*'den (kara ve beyaz biber) ekstrakte edilmiş ve proton transfer reaksiyonu ile yüksek performanslı sıvı kromatografi (HPLC) kullanılarak tayin edilmiştir. Önerilen yöntem; değiştirilebilir polarite çözücülü-sıvı-sıvı mikroekstraksiyonu (SPS-LLME) olarak adlandırılmıştır. Optimum ekstraksiyon koşulları aşağıdaki gibi bulunmuştur: Ekstraksiyon çözücü, TEA; ekstraksiyon çözücüsündeki TEA hacmi, 750 µL; ve ekstraksiyon süresi, 10 s. Organik modifiye edicinin varlığı ve hacmi, santrifüjleme süresi ve iyonik şiddeti ekstraksiyon verimi üzerinde önemli bir etkiye sahip olmadığı tespit edilmiştir. Son ekstrakt, başka bir ön işlem yapılmadan HPLC'ye doğrudan enjeksiyon için uygun olarak değerlendirilmiştir. Optimum HPLC koşulları aşağıdaki gibi bulunmuştur: kolon, Agilent Eclipse XDB C18 (4.6 mm ID × 15 cm, 5 µm); ayırma sıcaklığı, 20 °C; mobil faz, ACN:$H_2$0, 45:55 (%, v/v); akış hızı, 1.1 mL min^{-1} ve enjeksiyon hacmi, 5 µL. Piperin, 346 nm'de bir diyod dizisi detektörü (DAD) kullanılarak izlenmiştir. $3S_b$/m ve $10S_b$/m'ye göre hesaplanan teşhis (LOD) ve tayin (LOQ) sınırları sırasıyla 0.4-1.2 ve 1.3-3.9 mg g^{-1} olarak bulunmuştur. Kalibrasyon grafikleri, 0.9950'den yüksek olan tamamlayıcılık katsayısı (R^2) ile iyi bir doğrusallık göstermiştir. Piperin standardı izole edilerek 1D- (1H- ve 13C-NMR) ve 2D-NMR (COSY, HSQC ve HMBC) ile karakterize edilmiştir. Farklı orijinlerden yedi adet siyah ve bir adet beyaz biber örneklerinden elde edilen piperinin ekstraksiyonu ve tayini için SPS-LLME-HPLC uygulanmış ve % 95,6 ile % 104,7 aralığında nispi geri kazanımlar (%RR) elde edilmiştir. Sonuçlar, SPS-LLME'nin, rutin çalışmada HPLC analizinden önce siyah ve beyaz biberden piperin ekstraksiyonu için basit, hızlı ve çevreci alternatif bir yöntemi temsil edebileceği kanıtlanmıştır.

Anahtar kelimeler: Beyaz biber, Değiştirilebilir Polarite Çözücülü-Sıvı-Sıvı Mikroekstraksiyonu, HPLC, Karabiber, Piperin

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Definition
ACN	Acetonitrile
AP	Acceptor phase
BES	Back-extraction solvent
CF	Chloroform
DI	Direct injection
DLLME	Dispersive liquid-liquid microextraction
DMCHA	Dimethylcyclohexylamine
DP	Donor phase
EF	Enrichment factor
EVAP	Evaporation-to-dryness
FT-NMR	Furrier transform-nuclear magnetic resonance
GC	Gas chromatography
HF-LPME	Hollow fiber liquid-liquid microextraction
HLLE	Homogeneous liquid-liquid extraction
HPLC	High-performance liquid chromatography
IE	Ion-exchange
ILUAE	Ionic liquid-based ultrasonic-assisted extraction
LC/MS	Liquid chromatography/mass spectrometry
LDR	Linear dynamic range
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LOD	Limit of detection
LOQ	Limit of quantitation
LPME	Liquid-phase microextraction
LP-SOX	Low pressure-based Soxhlet extraction
LP-UAE	Low pressure-based ultrasound-assisted extraction
NMR	Nuclear magnetic resonance spectroscopy
NP	Normal phase

Abbreviation	Definition
ODS	Octadecyl silica gel
PLE	Pressurized liquid extraction
RP	Reversed phase
RR	Relative recovery
SD-LPME	Single-drop liquid phase microextraction
SE	Size exclusion
SFE	Supercritical fluid extraction
SOE	Salting-out extraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SPS	Switchable-polarity solvent
SPS-LLME	Switchable-polarity solvent liquid-liquid microextraction
SS	Switchable solvent
TEA	Triethylamine
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
TRNC	Turkish Republic of Northern Cyprus
UV	Ultra-violet

CHAPTER 1

INTRODUCTION

1.1 Piperine

Herbs and spices have an old history in food processing and medicinal preparation because of their health benefits and charming flavor which make them necessary and essential in our daily food. In addition, they are used in the preparation of many medications due to their pharmacological characteristics. Black peppers are formed from green immature berries of pepper plant. After picking these berries, they are left under heat treatment for drying and for browning enzymes to be secreted out of the cell walls, while white peppers are formed when the berries are mature and dried with the removal of the outer shell. Green peppers are collected immature and left to dry either by air or freeze-drying. Red pepper is collected when matured [1]. Different types of berries pepper are shown in **Figure 1.1**.

Figure 1.1: Different types of berries pepper.

Black pepper is largely used as spice worldwide. It has been called the “king of Spices”, and was used in the past in many fields, extended to the present time and its use is predicted to continue in the future. The pungency and flavor of the pepper is associated with the presence of the naturally existing alkaloid, known as piperine in addition to some volatile oils. Generally, the amount of piperine in black and white pepper ranges between 2 and 7.4% in *Piper nigrum L*., about 4% of long pepper (*Piper longum L*.), and 4.5% of Balinese long pepper fruits (*Piper retrofractum Vahl*). Whereas, the volatile oils, which are responsible for the odor of the pepper, form about 0.4 to 7%. Other compounds such as oleoresin, fats and starch form about 4.4-12%, 1.9-9%, and 28-49% of the black body, respectively [2].

The amount of piperine changes in the plants according to the *piperaceae* family, and it is also affected by various environmental determinants like, weather, growing circumstances and place of origin [3]. Christian Ørsted was the first chemist who isolated piperine from black pepper in 1819. The extracted piperine was a yellow crystalline compound, having a melting point of 128 to 130 ºC. The chemical structure is recognized as piperoylpiperidine, with a molecular formula of $C_{17}H_{19}NO_3$, and its IUPAC name is 1-(5-[1,3-benzodioxol5-yl]-1-oxo-2,4-pentadienyl) piperidine. Because piperine tends to be a weak base, it decomposes through acid or alkali hydrolysis to a volatile basic compounds, piperidine ($C_5H_{11}N$) and piperic acid ($C_{12}H_{10}O_4$). There are four isomeric structures for piperine: piperine (trans-trans isomer), isopiperine (cis-trans isomer), chavicine (cis-cis isomer), and isochavicine (trans-cis isomer) [4], the structures of which are shown in **Figure 1.2**.

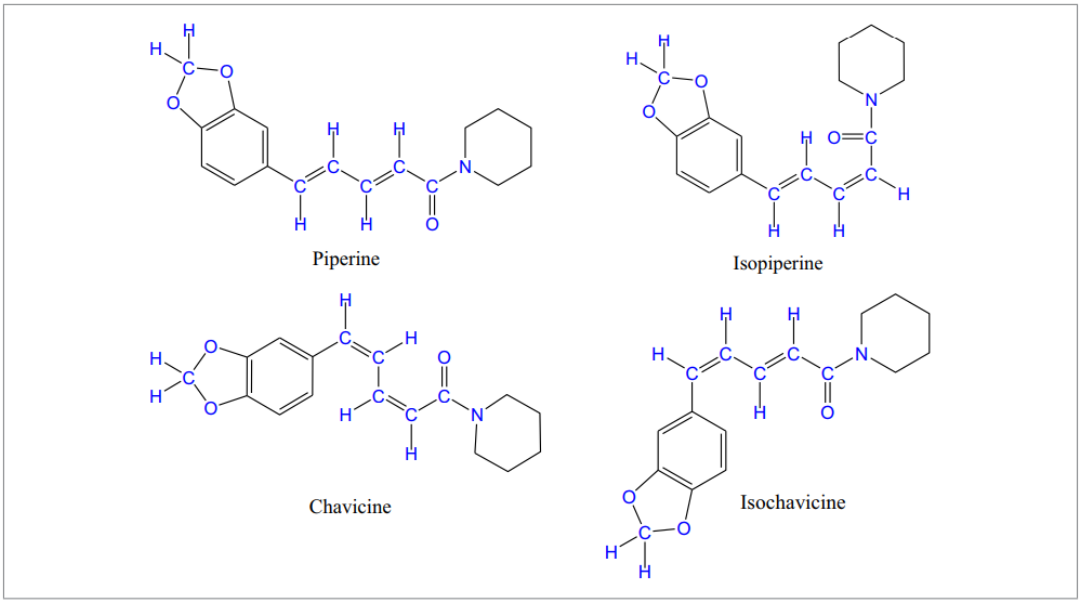


Figure 1.2: Isomeric structures of piperine

Nevertheless, the three other geometric isomers of piperine do not exhibit pungency. Other alkaloids have been found in black pepper like, piperanine, piperettine, piperylin A, piperolein B and pipericine (**Figure 1.3**). All of these alkaloids have some degree of pungency in the pepper, but the overall contribution of these alkaloids is small. Piperine is the main compound which is responsible for the pungency of pepper; it is about 98% of the total alkaloids in pepper. Piperine and its similar compounds are used to measure the total pungency of pepper [4].

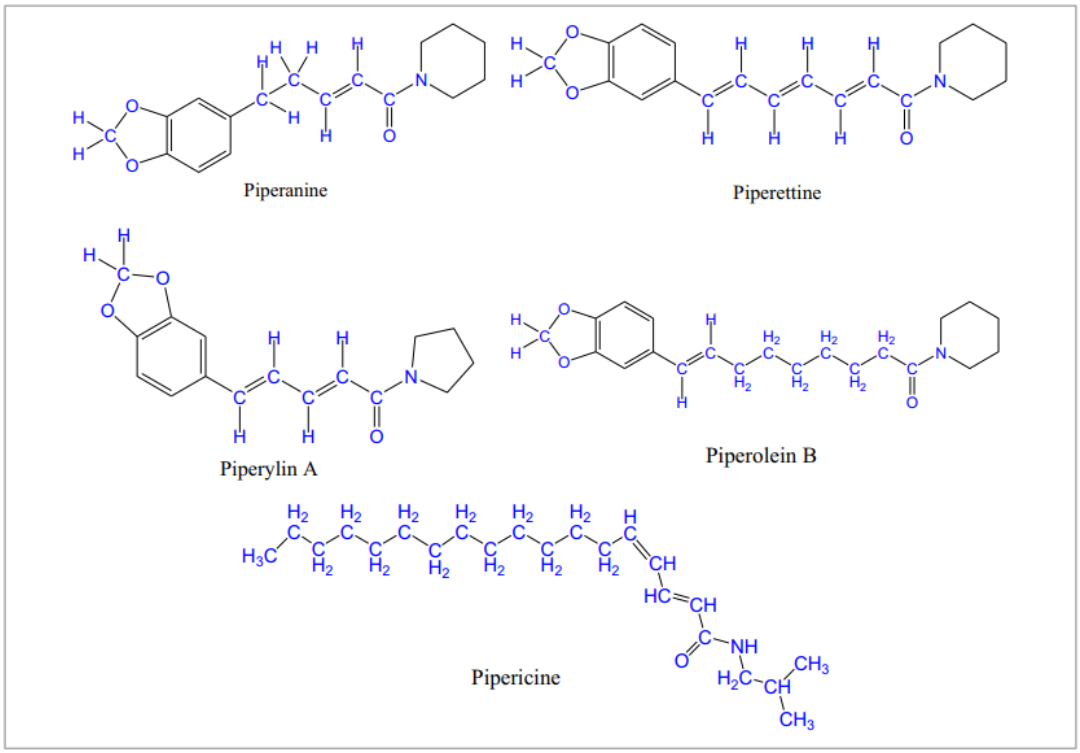


Figure 1.3: Different isomers of piperine.

Black pepper has been used in ancient Chinese and Indian medicine for treatment and relief of cold, influenza, rheumatism, fever, chills, and muscular pain as a natural medicinal product. Nevertheless, black pepper is also believed to have been used as tea for serious headache, sore throat, bad digestion and even coma. It has also been used to increase the flow of saliva, enhance the circulation of blood, and activate appetite [4]. Black pepper is also used in many fields like food processing due to its specific bitter quality which is related to piperine [5], as food preservatives, in cosmetic products and pharmaceutical [6]. In addition to the previously mentioned points, it has been reported lately that piperine and its essential oils have physiological effects such as antioxidant activities, chemoprevention, immunomodulatory, anti-carcinogenic, hepatoprotective, anti-inflammatory, stimulatory [7], antiulcer activities [8,9]. Black pepper has shown positive effects on swallowing reflexes in elderly dysphagia people [10], protection against oxidative damage and reduced lipid peroxidation [11].

Piper nigrum Linn. and *Piper longum Linn,* which are known as Black pepper and Long pepper, are shown in **Figure 1.4** and **Figure 1.5** respectively. They are the oldest and most important spices in the world, which are cultivated and native to the hot and moist part of India.

Figure 1.4: *Piper nigrum Linn*., known as black pepper.

Figure 1.5: *Piper longum Linn.*, known as long pepper.

In addition to the fact that piperine is highly photosensitive in solution, it may be decomposed under light, heat, oxygen and other environmental conditions and affect the long term storage stability [12].

1.2 Liquid-Phase Extraction

Generally, the analysis of any compound in complex sample need two steps of sample processing. The first step includes extraction, separation, purification and pre-concentration. The second step consists of identification, qualitative and quantitative analysis. With the development in analytical methods and techniques due to the evolution in computer, information and instrument technologies, sample pre-treatment is lagging behind. Nevertheless, it was only until recently that sample pre-treatment has received attention in an effort to reduce procedure steps, and reduce or eliminate using solvents for extraction or alternative solvents that are environmentally friendly. Flexibility of sampling and automation is also a huge area of interest [13]. Such a development helps especially with neutral product analysis which have a high complexity, low levels of active ingredients and multiple components. Therefore, sample pre-treatment is necessary to separate and remove any ineffective ingredient and impurities because figures of merit such as precision, accuracy and limit of detection (LODs) can be affected by any part of the extraction procedure. Thus, it is very important, and fundamental for any successful analytical method, which includes mainly three parts: (1) sample matrix simplification and replacement, (2) Analyte enhancement and preconcentration, and (3) sample clean-up [13].

The ideal goal of extraction techniques is to minimize the use of toxic solvents and combine sampling, extraction, preconcentration and even conversion of the analytes into another suitable form by derivatization in a single step [14,15].

Sample preparation is always considered as the bottleneck of any analytical protocols before determination of any substance in complicated matrices [16]. The major classical

extraction methods that have been used in many fields are liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [17,18]. But they are still not satisfying due to the use of large volumes of organic solvents, boring, time consuming and complex mass transfer in two phase systems. Shortcomings associated with LLE such as emulsion formation, use of large sample volumes and toxic organic solvents make it labor-intensive, expensive, time-consuming, and environmentally-unfriendly. Although SPE uses much less solvent than LLE, it can still be considered significant, and normally an extra step is needed to preconcentrate the analytes further into smaller volumes. SPE is also time-consuming and relatively expensive [19]. Recently, much research has been directed toward efficient, economic and ‘‘green’’ miniaturized extraction techniques. Liquid–liquid microextraction (LLME) with its different operating modes, such as single-drop microextraction (SDME) [20], hollow fiber-based liquid-phase microextraction (HF-LPME) [21], solvent-bar microextraction (SBME) [22], and dispersive liquid–liquid microextraction (DLLME) [23], among others, has attracted increasing attention as novel sample preparation techniques.

1.3 Liquid-Phase Microextraction

Liquid-phase microextraction (LPME) is a miniaturized form of LLE, which has been used in separation and extraction procedure to overcome the obvious disadvantages of LLE time consuming and solvent toxicity in addition to laborious application [24]. In LPME, a few microliters are used as the extraction solvent which is a water-immiscible solvent, also called acceptor phase (AP), in contact with the aqueous phase, also called the donor phase (DP), which contains the analyte.

SPME is a miniaturized form of SPE that has also been developed to offer advantages such as simplicity, ease of application, requirement of short time for the extraction, relatively low analysis cost, high enrichment factor, minimum volumes of the extractant and agreement with the green analytical chemistry approach [25]. The main disadvantage of LLME is using toxic and many cases volatile solvents which are denser than water,

like halogenated solvents that cause danger to the analyst and the environment. The requirement for an extraction solvent in ordinary LLME procedure matches the improvement of "green extraction agents" which are require the reduction of toxicity and volatility of extraction solvents and the hazard to the analysts, hence, the increased attention toward this method in recent years [26].

In order to enhance the selectivity, sensitivity, to protect analytical columns, and to reduce matrix effect, the pretreatment of the sample is very important, specifically in the pharmaceutical and biomedical analysis [27]. Simple procedures are needed to prepare the sample for the analysis in pharmaceutical analysis such as dissolution, filtration and dilution used for dosage form assays [28]. But, in biological samples, the matrix is much more complicated and the analyte is present at a much lower concentration, making it necessary to develop more specific and sensitive methods [29,30], in conjunction with common methods such protein precipitation [31,32].

SDME was first introduced in 1996 by Jeannot and Cantwell [20] followed by chromatographic analysis. This technique relies on the suspension of a few microliters of the extraction solvent at the tip of a microsyringe needle above (head-space) or immersed directly (direct-immersion) into the sample solution that contains the analyte. After the extraction has taken place, the microdrop is withdrawn into the syringe as shown in **Figure 1.6,** and injected directly to electrophoretic or chromatographic system [33]. Despite the various advantages of this method, which include low consumption of organic solvents, high enrichment factors, it suffers from several limitations such as imbalance and dislodge of the drop during extraction step, the need for filtration after the extraction due to adsorption of other components of the complex matrix, low precision due to low surface area of contact between the drop and sample solution, and unsuitability for routine analysis.

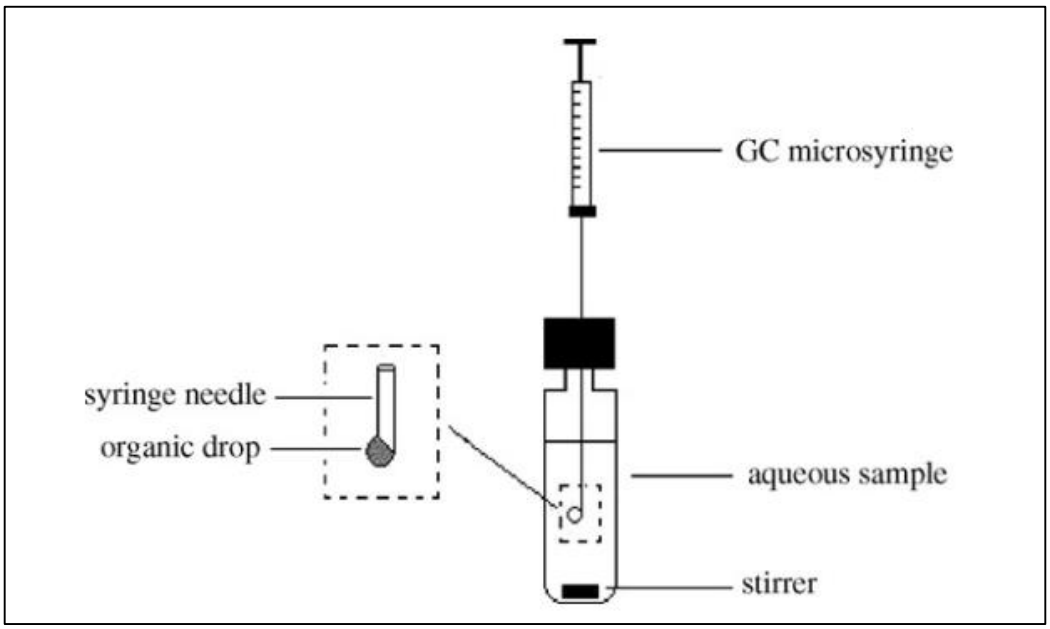


Figure 1.6: Direct-immersion SDME.

HF-LPME [21]was performed first introduced 1999 by Pedersen-Bjergaard and co-workers and has been, since then, applied for various analytes and samples [34]. In its simplest mode, two-phase-HF-LPME, it is performed by placing the sample solution containing the analyte into a small vial and a hollow fiber, made of porous polypropylene, is soaked in an organic solvent, is placed inside the sample solution. Hollow fiber is covered with a layer of 200 μm immiscible organic solvent to ensure the solvent does not leak to the sample solution during extraction **(Figure 1.7)**.

pH of the sample solution is adjusted so that the analyte is present in its neutral form. The analyte then diffuses from the sample solution through the pores containing an organic solvent inside its lumens, into the acceptor solution, which is withdrawn and analyzed. There are several limitations of HF-LPME, the most important being the long extraction time ranging between 30 to 60 minutes and the high probability for the hollow fiber pores to be blocked during extraction [35].

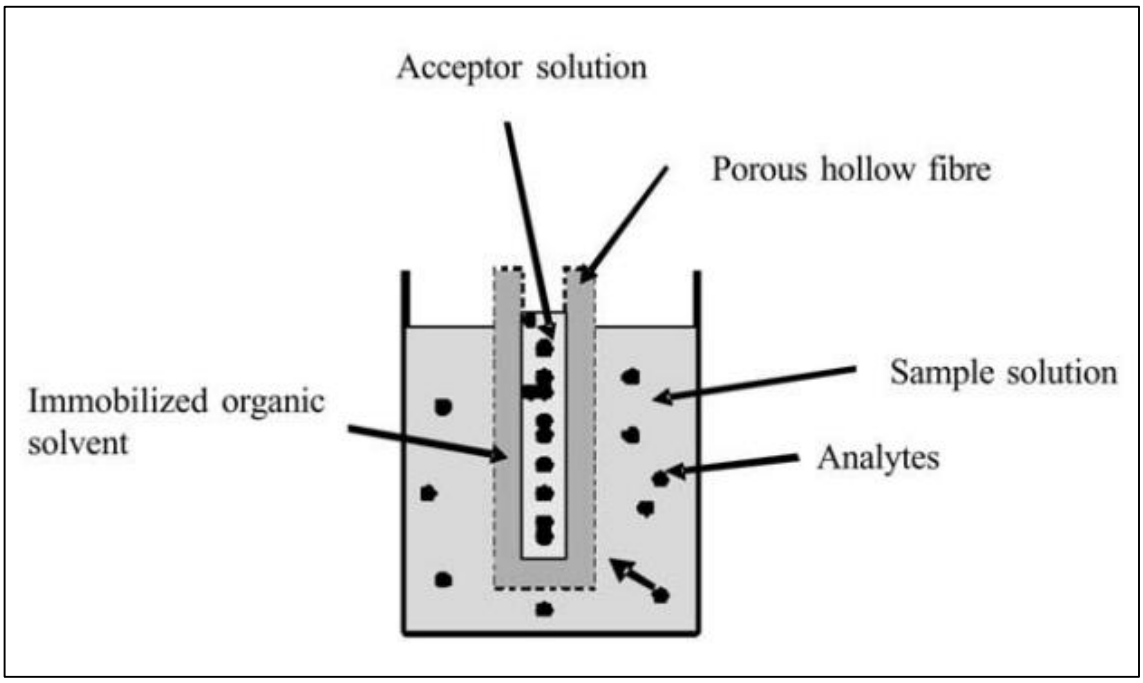


Figure 1.7: HF-LPME.

DLLME was invented by Assadi and his co-workers in 2006 [23]. The main principle of this method is the use of a tertiary solvent system that consists of an aqueous sample solution, an extraction solvent and a disperser solvent. Upon the addition of a mixture of the extraction and disperser solvents to the aqueous sample solution, an emulsion forms (**Figure 1.8**); the extraction solvent, which is abundant with the analyte(s), is precipitated at the bottom or floated at the surface of the solution upon centrifugation, depending on the density of the organic extraction solvent used. This solvent is collected and injected into the instrument for analysis, or is further pretreated before injection depending on its compatibility with the instrument used. DLLME has found wide acceptance as an outstanding technique for its simplicity, cost effectiveness and ability to provide high extraction efficiencies within a very short time due to the extensive surface contact between the droplets of the extraction solvent and the sample solution. However, it has some limitations which still need to be overcome: (1) three solvents are needed; (2) relatively long steps; (3) centrifugation is necessary; (4) limited compatibility with complex matrix composition [35,36].

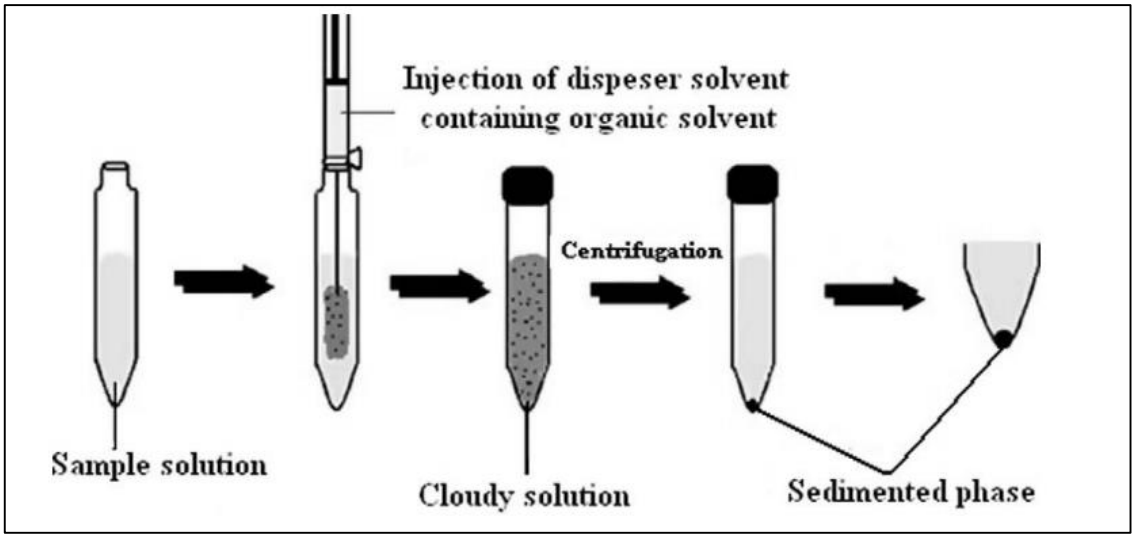


Figure 1.8: DLLME.

1.4 Switchable-Polarity Solvents (SPS)

Despite their low volumes, the most commonly used extraction phases in almost all of the above mentioned techniques, are volatile organic solvents that may cause health problems to the analyst and environment, such as toxicity, smog formation, inhalation difficulties and flammability. Greener solvents include ionic liquids, CO_2, water and bio-derived organic solvents have been focused on as alternatives to achieve "environmental damage reduction".

Since their initial discovery in 1914, ionic liquids (ILs) have been widely studied in multiple chemistry disciplines. They possess melting points at or below 100 °C. In most cases, ILs are composed of an organic cation and an organic or inorganic anion. It has been estimated there can be up to 1018 possible combinations of ILs [37]. A number of ILs exhibit beneficial characteristics, such as high thermal stability, negligible vapor pressure, and non-flammability, in addition to varying viscosities, conductivity, and miscibility in different solvents. These characteristics can also be finely tuned to meet

specific requirements by imparting different functional groups and/or varying the combinations of cations and anions in the ILs.

Switchable-polarity solvents (SPS) "also called smart solvents" were first invented in 2005 by Philip G. Jessop [38]. The author reported that certain environmentally-friendly organic solvents such as tertiary amines show complete solubility in water in the presence of carbon dioxide. An acid-base reaction occurs between tertiary amines and hydrated CO_2 in water to produce protonated SPS and the bicarbonate salt which are completely soluble in water. Upon removal of CO_2, the polarity of the SPS can be switched off toward the non-polar form of the tertiary amine by converting it into its original water-immiscible non-polar form. Thus, this property makes it possible to extract relatively hydrophobic compounds from aqueous media. The analyte-rich tertiary amine phase, can be easily collected, from the surface of the sample solution, and analyzed by the instrument for the presence and/or concentration of the analyte.

Based on this reversible reaction shown in **Figure 1.9** [39], it is possible to produce a new generation of ionic liquids that can have switchable polarity behavior in the presence and absence of CO_2.

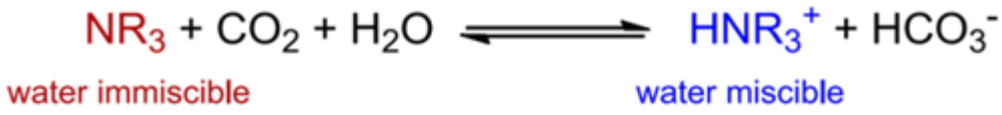


Figure 1.9: Reversible reaction of amines with CO_2.

Because not all solvents have the ability to change or switch their polarities in the solution, this is a special and new class of solvents for analytical applications. Such organic solvents should be able to form two layers "biphasic" with water (immiscible) before the reaction and should for a "monophasic" mixture (immiscible) upon the addition of CO_2. The process for forming SPS is shown in **Figure 1.10**.

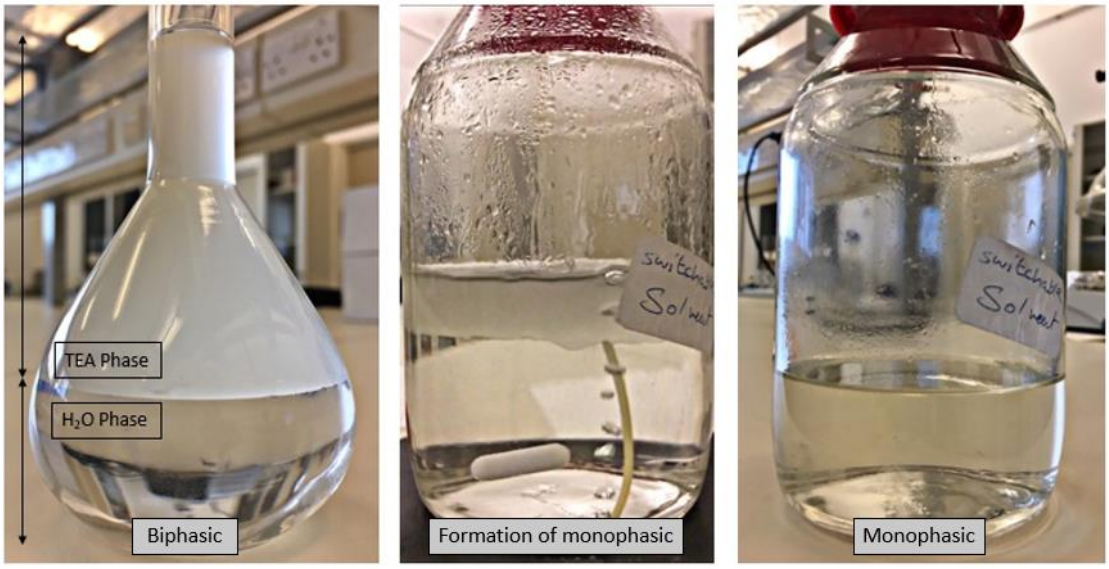


Figure 1.10: Process for formation of SPS.

The most common solvents tested for SPS behavior are amines, amidines and guanidines. However, it should be noted that not all classes of these solvents have this property. In addition, amidines are expensive, not easily found in nature and are difficult to synthesize [40,41]. A list of amines, amidine and guanidines tested for their SPS behavior is shown in **Table 1.1**.

Table 1.1: Design and evaluation of switchable-polarity solvents.

Behavior	Compound	Ratio of compound to water (v:v)	LogK_{ow}	pK_a
Monophasic	Triethanolamine	1:1	-1.51	7.85
Monophasic	N,N,N′,N′-Tetramethylethylenediamine	1:1	0.21	9.2
Monophasic	N,N,N′,N′-Tetramethylguanidine	2:1	0.30	13.6
Monophasic	N-Ethylmorpholine	1:1	0.30	7.70
Monophasic	1,8-Diazabicycloundec-7-ene	2:1	1.73	12
Monophasic	N-Hexyl-N′,N′-dimethylacetamidine	2:1	2.94	12
Irreversible	N″-Hexyl-N,N,N′,N′-tetramethylguanidine	2:1	2.82	13.6
Irreversible	N″-Butyl-N,N,N′,N′-tetraethylguanidine	2:1	3.52	13.6
Irreversible	N″-Hexyl-N,N,N′,N′-tetraethylguanidine	2:1	4.43	13.6
Switchable	Triethylamine	1:1	1.47	10.7
Switchable	N,N-Dimethylbutylamine	1:1	1.60	10.0
Switchable	N-Ethylpiperidine	1:1	1.75	10.5
Switchable	N-Methyldipropylamine	1:1	1.96	10.4
Switchable	N,N-Dimethylcyclohexylamine	1:1	2.04	10.5
Switchable	N-Butylpyrrolidine	1:1	2.15	10.4
Switchable	N,N-Diethylbutylamine	1:1	2.37	10.5
Switchable	N,N-Dimethylhexylamine	1:1	2.51	10.2
Switchable	N,N,N′-Tripropylbutanamidine	2:1	4.20	12.0
Switchable	N,N,N′-Tributylpentanamidine	2:1	5.99	12.0
Biphasic	N,N-Dimethylaniline	1:1	2.11	5.1
Biphasic	N,N-Diisopropylethylamine	1:1	2.28	11.0
Biphasic	Tripropylamine	1:1	2.83	10.7
Biphasic	N″-Hexyl-N,N,N′,N′-tetrabutylguanidine	2:1	7.91	13.6
Biphasic	Trioctylamine	1:1	9.45	10.9

It can be seen in **Table 1.1** that some of guanidines form monophasic systems with water (e.g., N,N,N′,N′-Tetramethylguanidine), while others irreversibly form monophasic systems with water after adding CO_2 and cannot return back to biphasic under every known condition because they are highly basic (e.g., N''-hexyl-N,N,N',N

′ -tetramethylguanidine). Other solvents such as tripropylamine remain as biphasic with water. It can be concluded From **Table 1.1** that some solvents such as triethylamine and *N*,*N*-dimethylcyclohexylamine can form switchable systems with water at 1:1 (v:v) ratio.

It was reported that secondary amines are faster than tertiary amines in terms of the formation of monophasic but they require higher energy and temperature to remove CO_2 to switch to biphasic which was thought to be due to the formation of ammonium carbamate in the solution [42]. On the other hand, tertiary amines require much less energy to remove CO_2 from ammonium bicarbonate solution. Hence, using secondary amine as SPS is more energy exhaustive than tertiary amines making them less favorable for this purpose.

By determining the log of octanol/water ($logP_{o/w}$) partition coefficient and their log of ionization constant (pK_a), the choice of the solvent will be easier. Amines should have ($logP_{o/w}$) between 1.5-2.5 because with the lower their ($logP_{o/w}$), the most polar they are and the easier they would form monophasic with water. On the contrary, the higher ($logP_{o/w}$), the lower the polarity of the solvent and the more difficult, it would form biphasic with water even with the addition of CO_2 to the mixture. In addition, amines should have (pK_a) higher than 9.5 because below this value, the basicity is not enough to react with carbonated water in order to switch its form.

The separation of the SPS from a homogenous mixture does not need any special equipment. However, there are many physical and chemical methods that have been also tried for phase separation as shown in **Figure 1.11**. Most of these methods are not favorable due to low recovery and possible loss of the analyte(s) [42]. The addition of sodium hydroxide has been reported to provide good and rapid phase separation and high recovery of the SPS [43].

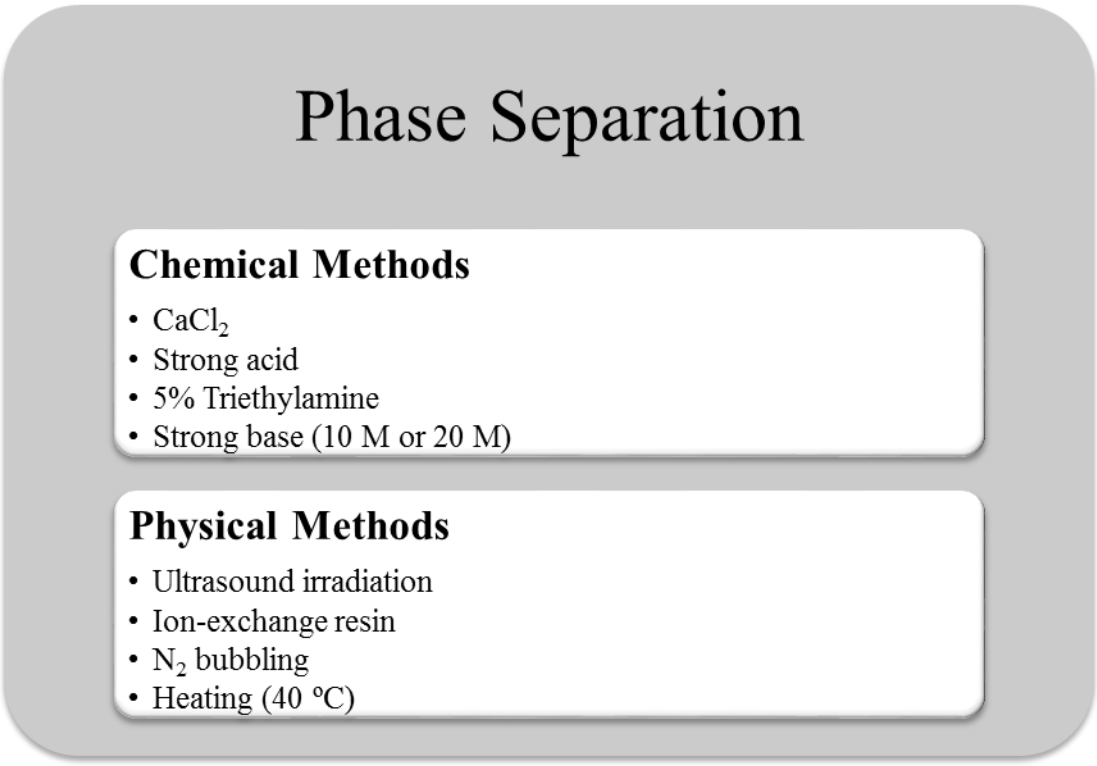


Figure 1.11: Chemical and physical methods for phase separation.

The ability of using CO_2 to switch the properties of a medium provides an extra important advantage of the method, the design of eco-efficient approach. The low cost and non-toxic nature of CO_2 make it a perfect transition phase in extraction techniques. Another fascinating advantage of using SPS is their ability to provide infinite contact between the extraction phase and the analyte due to its complete homogeneity with the sample solution. Hence, there is no need for disperser solvents which are one of the drawback of DLLME. Moreover, since switching the SPS is instantaneous upon the addition of a strong base, phase separation can be obtained without the need for centrifugation. Such solvents used for SPS are themselves green solvents [44,45]. Based on such superior advantages, it is so obvious that the application of SPS in microextraction context has a high potential and would gain high interest among analytical chemists and in other fields as well.

In large-scale applications, SPS have been used to extract bitumen from oil sands [46], soy oil from crushed soybeans [47], phenols as a mixture from the lignin microwave-pyrolysis oil [39], polycyclic aromatic structures-enriched portion from direct coal liquefaction residue [48], and Lipids from algal biomass [49,50].

Few publications have suggested the used of SPS for microextraction. Some applications include the extraction of aluminum from biological sample and its determination by flame atomic absorption spectrometry (FAAS) [51], cadmium ions in baby food by FAAS [52], uranium in water, sediment, soil and rock samples by UV-vis spectrophotometry [53], lead in fresh and waste water samples by FAAS [54], cadmium in water, vegetable, fruit and cigarette samples by FAAS [43], mercury in water and hair samples by UV-vis spectrophotometry [55], and copper in water, food and hair samples by FAAS [56].

To the best of our knowledge, this is the first report on the applications of SPS-LPME prior to HPLC for the determination of molecular analysis.

1.5 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography started in the early 20th century. The basic study started in the early 1940s by Martin and Synge. But, progress in this field did not take place until the 1960s after scientists found out that better separation could be obtained by reducing the inner diameter, decreasing the size of the packing materials, afterward increasing the flow rate of the mobile phase which led up to increase in the pressure. Then, the separation time decreased at the same time with corresponding increase in resolution, leading to the necessity to use high-performance (pressure) liquid chromatography. HPLC instrument is shown in **Figure 1.12**.

Figure 1.12: HPLC instrument.

HPLC is a separation technique using a liquid phase that pass through a porous stationary phase. The liquid and the stationary phases should be compatible with the sample components so that the sample can equilibrate between them and spend a considerable residence time in both. Distribution of the analyte between these two phases can be considered as distribution coefficient (*K*) as shown in **Equation 1.1**:

$$K = \frac{\text{Concentration of the analyte in the stationary phase}}{\text{Concentration of the analyte in the mobile phase}}$$ **Equation 1.1**

HPLC is an active analytical method, an important preparative separation technique with high recognition due to high reproducibility, high accuracy, applicability to a wide range of substances, appropriateness with many detectors like UV-VIS, fluorescence, electrochemical, mass spectrometry, and others. HPLC has rapidly developed into an innovative, fast and highly selective analytical tool with widely distributed application in

biology, chemical engineering, food, pharmaceuticals, petrochemical industries and environment protection.

The porous stationary phase is the heart of HPLC because the mobile phase passing through the stationary phase is a fundamental merit of the chromatographic process. Mass transfer of the solute molecules should swap between the mobile phase and the stationary phase fast and frequently, to obtain equilibrium of solutes in the chromatographic system and to gain a high column efficiency. Also, there are many properties that should be considered in the stationary phase to provide fast mass transfer kinetics. First, the diffusion distances in the stationary phase must be small. Secondly, the surface area between the stationary phase and the mobile phase must be large.

HPLC requires continuous evolution of its part including injectors, pumps, detection units, processors, data recorders, mobile phases and stationary phases. In addition, the development of HPLC separation modes which include normal-phase (NP), reversed-phase (RP), ion-exchange (IE) and size-exclusion (SE).

The choice of the mode of liquid chromatography depends on the molecular weight of the analyte and its polarity according to the schematic diagram shown in **Figure 1.13**.

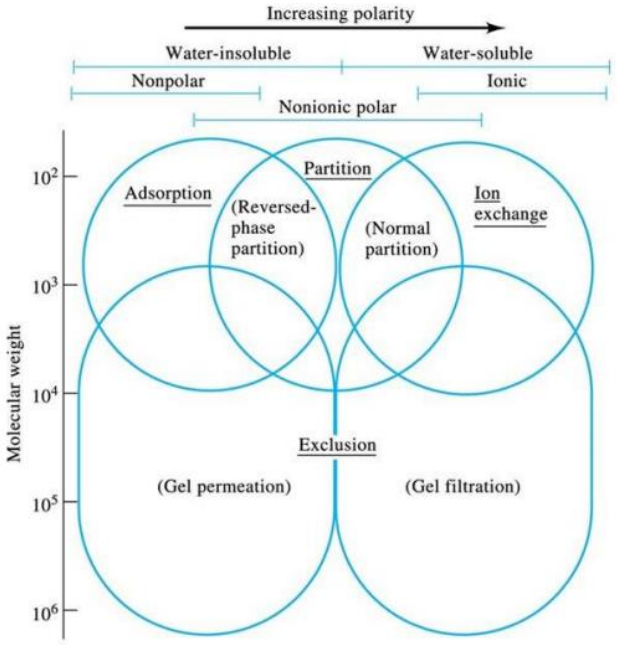


Figure 1.13: Schematic diagram of liquid chromatography modes.

There are three packing materials in HPLC, inorganic, organic and inorganic-organic hybrid with different particles size and inner diameter, as shown in **Figure 1.14**.

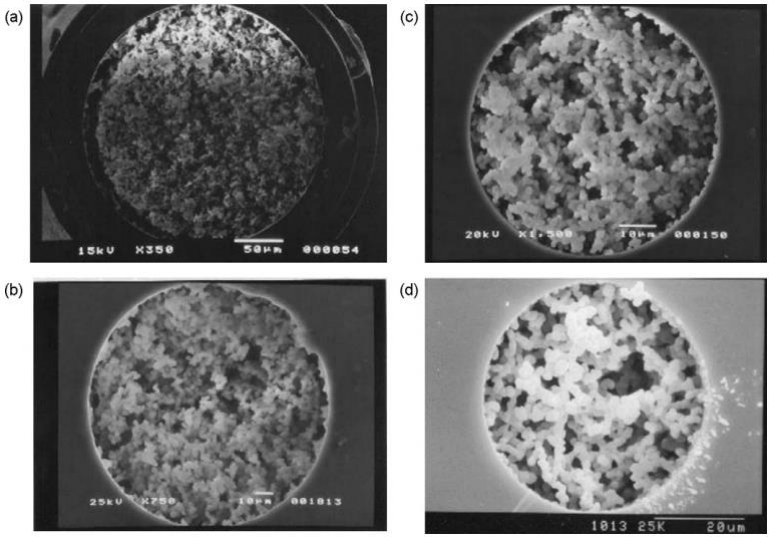


Figure 1.14: Different particles size and inner diameter of the column chromatography prepared (a) 250 µm; (b) 100 µm; (c) 75 µm; and 50 µm fused silica.

The most widely used modes of HPLC are NP and RP. In NP, the stationary phase is polar such as silica gel, or silica cores bonded to functional groups like cyano, amino and diol. Mobile phases used in NP are non-polar solvents such as hexane, cyclohexane, heptane etc. In RP, the stationary phase is non-polar like alkyl group chains bonded to silica cores called octadecyl silica gel (ODS) with different length C8 or C18 and mobile phases are polar like water, methanol, ethanol, acetonitrile, acetone, tetrahydrofuran, etc. Choosing the mode depends on the polarity of the analyte. By knowing partition coefficient ($logP$) of the analyte, a chromatographer can decide on the correct choice of the mode.

$logP$ is the ratio of the concentration of the analyte between two immiscible liquids, generally octanol and water, between which an equilibrium for the analyte takes place, The logarithm of this ratio is referred to as $logP$ or $logP_{o/w}$ (**Equation 1.2**).

$$logP_{o/w} = log\frac{[analyte]Octanol}{[analyte]Water} \qquad \textbf{Equation 1.2}$$

Silica is the most widely used inorganic packing material for stationary phase in HPLC. Due to its monodispersing property, which means that it contains a uniform particles size, modifiable surface, surface area and pore diameter, controllable porosity, high mechanical strength and good chromatographic efficiency. Limitations of using silica, nevertheless, exist like instability at low pH below 2 where silica starts to dissolve and at higher pH than 8, where it starts to crack and lose its bonded phase [57].

By using organic packing materials, which can resist severe pH, but suffer from extreme swelling, mechanical stability can be low and disappointing chromatographic performance might be observed. Inorganic-organic hybrid materials have both inorganic and organic composition characteristics. They possess improved thermal and chemical stability but they are vulnerable to hydrolysis and dissolution of the silyl-ether bond in an alkaline mobile phase [58].

RP is the most commonly used stationary phases because of their stability and high chromatographic efficiency that can be obtained due to their fast mass transfer effects [59]. In addition to their applicability to a wide range of analytes and samples, less or non-toxicity of the mobile phases are applied in this mode as compared to mobile phases are used in NP.

Moreover, the hydraulic resistance of the stationary phase to the flow of mobile phase should be average, because the length of the column will be limited that can be used with a given pumping system due to high hydraulic resistance.

Mikhail Tswett used a column packed with fine particles. He understood the idea of using fine particles to obtain better separation. But in return, decreasing the particle size resulted in slower mobile phase velocity, which was a serious disadvantage, especially, due to the absence of a pump in that era; the flow of the mobile phase was forced by gravity [60].

For about a century after this invention, all the chromatographic packing materials used were fine particles. Until the late 1960s, column chromatography was carried out by passing the mobile phase through the stationary phase under gravitational force. But, this progress prevented the accomplishment of neither fast separations nor high efficiency because the particles size of the stationary phase could not get smaller and the length of the column was limited. The characteristics that have been successfully developed in the late 1950s were gas chromatography, control of flow rate, on-line detection, pressurized mobile phase and expanded to column chromatography and HPLC. This is due to the availability of the dependable pumps that could deliver any solvent used as the mobile phase at a constant flow rate, under pressures up to 400-500 bar. This type of pumps did not cause any problems, their mechanism was straightforward and their maintenance was easy and not costly. Difficulties started with high-pressure pumps (above 500 bar) which were complicated and more expensive.

When fast analysis is required, there are some other possible choices, one of which is the column temperature. Temperature is an essential factor that clearly affects the viscosity of the mobile phase. By increasing the temperature, the viscosity of the mobile phase decreases, which increase the velocity. Column efficiency is hardly affected, so the same resolution will be obtained with faster separation [61].

The most important basic concepts in HPLC used to characterize and determine the chromatographic behavior include these four main terms: retention (or capacity factor), selectivity, efficiency and resolution. The capacity and selectivity can be controlled by the column manufacturer. However, efficiency and resolution can be controlled by the chromatographer. To minimize band broadening, the chromatographic system should be optimized.

1.5.1 Retention factor (k')

To obtain an efficient separation, the column should be able to retain the analyte and separate the other components of the sample. The capacity factor (k') can be defined as in **Equation 1.3**:

$$k' = \frac{t_R - t_M}{t_M} \qquad \textbf{Equation 1.3}$$

where,

t_R is the retention time of the analyte (i.e., the time consumed by the sample to reach the detector) and t_M is the retention time of an unretained species.

The larger the retention factor, the higher the ability of the column to retain analytes which will improve the resolution of the separation because it is given longer analysis time that should be optimized with the resolution. An ideal value for k' would fall between 5 and 7, illustrating a good balance between analysis time and resolution.

However, a value for k' would falling between 3 and 10 is, for many separations, acceptable. k' can be controlled by changing the mobile phase composition, pH, buffer concentration or column temperature.

1.5.2 Selectivity (∝)

The selectivity factor (∝) of a column is defined as the degree of separation between successive peaks (generally called as critical pair). For the two species A and B, α can be defined as in **Equation 1.4:**

$$\propto = \frac{k'_B}{k'_A} = \frac{(t_R)_B - t_M}{(t_R)_A - t_M} \qquad \textbf{Equation 1.4}$$

where,

k'_A and k'_B are the retention factors of A and B, respectively.

When $\alpha = 1$, the first peak appeared in the dead volume, or the retention time of the two compounds, A and B, are equal [i.e., $(t_R)_A = (t_R)_B$], resulting in a complete overlap of the critical pair. Selectivity can be controlled by changing the mobile phase identity or changing the column.

1.5.3 Efficiency (*N*)

Theoretical plate number (N) is an index that indicates column efficiency. It describes the number of plates as defined according to plate theory, and can be used to determine column efficiency based on calculation in which the larger the theoretical plate number, the sharper the peaks. The theoretical plate number (N) is included as a numerical value

in column instruction manuals and inspection reports. The theoretical plate number is represented by **Equation 1.5**.

$$N = \frac{L}{H} \qquad \textbf{Equation 1.5}$$

where,
L is the column length (usually in centimeters) and H is the plate height.

Efficiency can be practically calculated from a chromatogram using **Equation 1.6**:

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad \textbf{Equation 1.6}$$

N can be controlled by optimizing the flow rate.

1.5.4 Resolution (R_s)

Resolution (R_s) is used to express the degree of separation between two critical peaks. It is affected by the retention factor (k'), selectivity (α) and efficiency (N) of the column as shown in **Figure 1.15.**

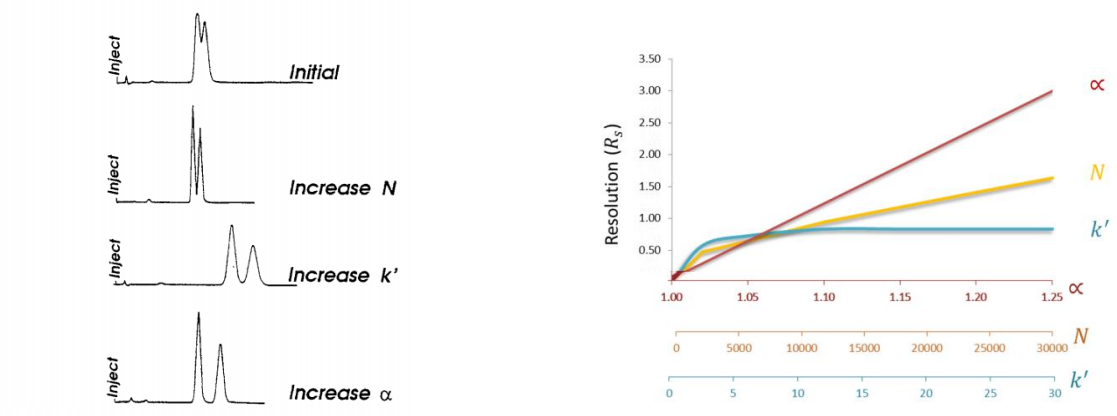


Figure 1.15: Effect of efficiency, retention factor and selectivity on resolution.

The relationship between the resolution and these separation factors can be described by **Equation 1.7**:

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{k'}{k'+1}\right)\left(\frac{\propto -1}{\propto}\right) \qquad \textbf{Equation 1.7}$$

(i) (ii) (iii)

The value of R_s should be larger than 1.5 (baseline resolution) to obtain a precise and accurate quantification of two peaks with minimum peak overlapping. Increasing the selectivity has the highest effect on resolution. On the other hand, increasing the retention factor up to 10 has a significant effect on resolution, beyond which less effect is observed. Increasing efficiency would always improve resolution (**Figure 1.15**). Optimum resolution can be obtained within the shortest time using a systematic approach such as the one summarized in **Figure 1.16**.

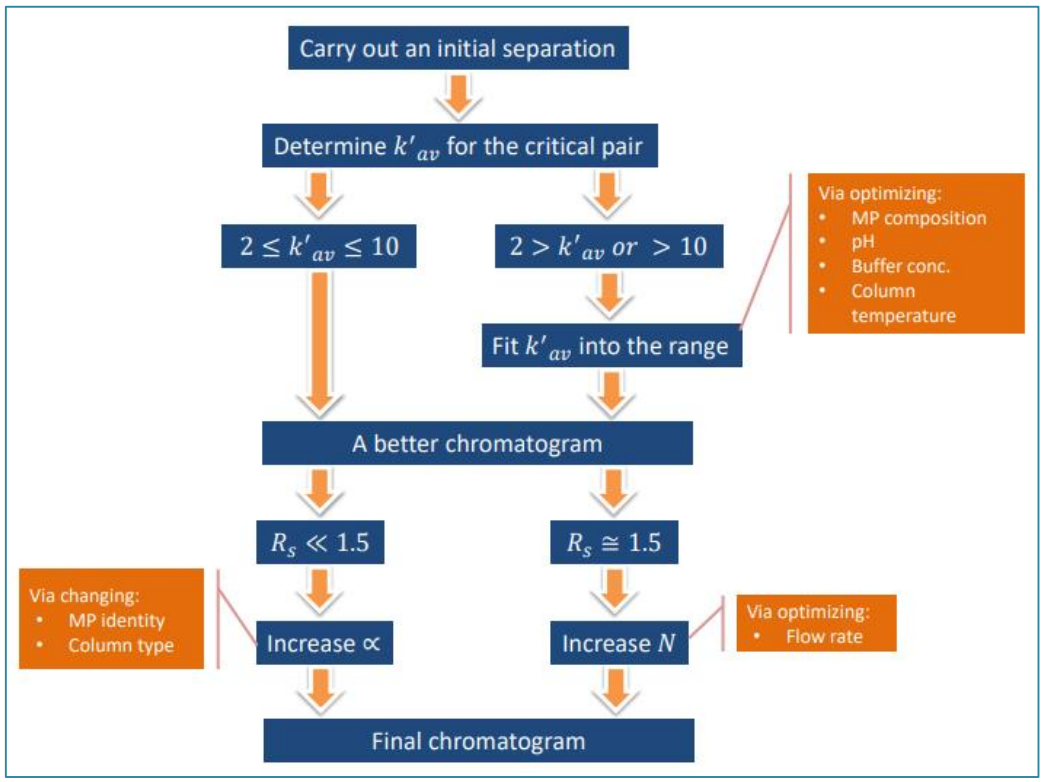


Figure 1.16: Systematic approach to HPLC separations.

A strategy or an approach to the design of this HPLC assay can be broken down into the following six steps:

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

Equation 1.7 is a fundamental relationship in HPLC, which allows a chromatographer to control resolution (R_s) by varying k'_{av}, N and $\propto$, where k'_{av} is the average of retention factors of a critical pair, N is the number of theoretical plates (efficiency) and $\propto$ is the

selectivity factor. The three terms (i), (ii) and (iii) of the equation are essentially independent, so that one term can be optimized first then another.

1.5.5 Mode of elution in HPLC

After separation of the sample components due to different adsorption to the stationary phase and different solubilities in the mobile phase, elution of separate bands of the analytes is the next step. There are two modes of elution in chromatography depending on the composition of the mobile phase. Isocratic elution is used when the sample is injected and eluted from the column without changing the mobile phase composition during the chromatographic run. It is mostly used when the sample components are relatively similar to the stationary phase and can be eluted one by one quickly. The other mode, called gradient elution, involves a gradual change of the mobile phase composition during the run until the separation of sample components is accomplished. It is mostly used in RP chromatography and also used with compounds which are different from stationary phase. The gradient elution can be binary gradient which is made of two different solvents or ternary that is made of three different solvents and quaternary which contain four different solvents. The effect of gradient elution in solving the "general elution problem" namely, long analysis time and poor resolution, is shown in **Figure 1.17**.

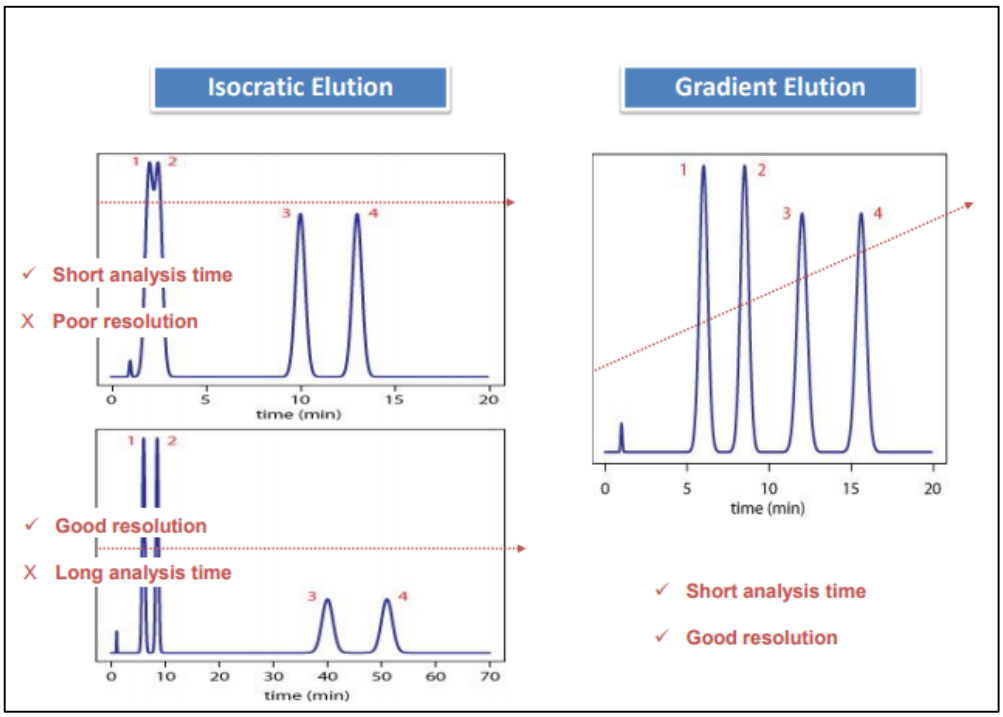


Figure 1.17: Isocratic and gradient elution.

The main instrumental components of HPLC are:

1. Solvent reservoirs that contain the mobile phase which is also connected to the degassing system. The gradient system has a minimum of two solvent reservoirs, while isocratic has just one solvent reservoir.
2. A pump to deliver the mobile phase and the sample through the instrument.
3. Injector which is used to introduce the sample to the instrument. An auto-sampler with variable volumes (0.1-100) µL can operate unattended, or manual injector with fixed volume with different loop volumes can be used. It means that when the volume needs to be changed the loop should be changed also with the desired volume.
4. A column that is used to separate the analyte. There are two types of columns: guard and analytical columns.

5. Another HPLC accessory is column oven which is used to adjust the temperature (generally between 4 and 80 °C).
6. A detector to visualize the separated sample components. Some common detectors include diode array detectors (DAD), refractive index, fluorescence detectors and mass spectrometers, etc.
7. A waste bottle is needed which contains the mobile phase and the eluted analytes.
8. Data processors are used to store and analyze the results [62].

1.6 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance (NMR) is used for determining the structure of organic compounds. It depends on the interaction between the magnetic moments of the nuclei of different atoms and the magnetic fields. The magnetic moment of a nuclear related to the nuclear spin relies on the nucleus spin number and its properties. The nuclei with an even number of protons and neutrons will have zero nuclear spin and magnetic moment. While the nuclei with an odd number of protons and neutrons will have non-zero spin and magnetic moment. Some of the nuclei that have odd number of protons and neutrons are ^{1}H, ^{13}C, ^{15}N and ^{31}P having the spin number of $^{1}/_{2}$, which is used in many applications of magnetic resonance [61,63].

Electronegativity of the surrounding atoms affects the shielding of the magnetic field which in turn affects the absorption. The signal of NMR is also affected by many factors as follows: the number of signals that presents how many different kinds of protons are there, the location illustrates the shielded and de-shielded protons, the intensity shows the number of protons of the same type, and splitting which shows the number of protons on adjacent atoms.

1.7 Column Chromatography

There are two types of columns in chromatography: ready-to-use-pre-packed columns which are filled with the bulk from the manufacturer and columns that are packed by the user. Pre-packed columns can be of disposable and non-disposable designs; disposable columns should be simple, easy-to-manufacturer and should be made of economical materials like polypropylene. This type cannot be re-packed again when column period is over, it should be discarded. Non-disposable columns are made of glass walls and high-grade materials can be re-packed with different stationary phase by the user [64].

1.8 Literature Review

In the literature there are many extraction and analytical methods that have been used to extract and analyze piperine from different pepper berries, which include; UV spectroscopy [65], capillary electrochromatography [66], NMR [9,67], microliquid chromatography [68], liquid chromatography/mass spectrometry (LC/MS) [69], among other techniques.

Friedman et al. used 2 mL of 80% ethanol as the extraction solvent for piperine and the extraction time was 60 min using sonication and 10 min for centrifugation at 12000 rpm at 5ºC. Then, 20 µL from the supernatant was injected into HPLC. For the separation, the authors used a gradient system with a mobile phase of ACN/H_2O acidified with 0.5% formic acid. The gradient started from 31% to 55% ACN for 70 min at a flow rate of 1 mL min^{-1} [70].

Kozukue et al. used isocratic elution for the separation of piperine by HPLC with (30/70%) ACN/H_2O acidified with 0.5% formic acid at a flow rate of 0.8 mL min^{-1} [71].

Piperine has also been extracted with Soxhlet extractor using diethyl ether for 20 h. HPLC was used for the separation; the piperine extract was dissolved in 20 mL

methanol and filtered before being injected into HPLC with different mobile phase compositions starting with doubly-distilled water, (50/50) methanol-water, (80/20) methanol-water, and (50/50) methanol-water [72].

1.9 Aim of This Study

The aim of this study is to develop an extraction method which is simple, fast, efficient and green to be combined with HPLC for the determination of piperine in black and white pepper in addition to other important requirements: preconcentration, minimizing the use of toxic solvent, sample clean-up and an efficient separation of the piperine without and interference from the other matrix components.

To the best of our knowledge, this is the first report on development of SPS-LLME prior to HPLC for the determination of piperine in black and white pepper and the first use of scaled-up DLLME for the isolation of piperine standard.

1.10 Future Work

In the near future, the developed SPS-LLME will be applied to other types of analytes of analytical, pharmaceutical, environmental and industrial interest in related matrices. Experimental parameters affecting the extraction method which are thought to enhance the preconcentration and extraction efficiency will be further studied and understood.

CHAPTER 2
EXPERIMENTAL

2.1 Instrumentation

Chromatographic separations were carried out by an Agilent 1200 series HPLC system (USA) equipped with quaternary pump, vacuum degasser, autosampler, column oven, DAD detector and Agilent ChemStation for LC 3D Systems (Rev. B.03.01) software. The column used in the separation was a reversed-phase (Agilent Eclipse XDB-C18. 4.6 mm ID x 150 mm, 5 μm). An electronic balance (Mettler Toledo) was used for accurate weighing of solid samples and piperine standard.

Isolation of piperine was carried out by using column chromatography packed with (100 g) silica gel. TLC Silica gel 60 F_{254} $(20 \times 20\ cm)$ from Merck. TLC chamber from CAMAG was used for monitoring the spots on the plate.

Structural determination of piperine was done with FT-NMR (^{1}H 400 MHz, ^{13}C 100 MHz) from Varian Mercury Agilent (USA) at Prince Sattam bin Abdulaziz University by Prof. Dr. Hasan Abou Gazar (Yusufoğlu), who is acknowledged for this support. Tetramethylsilane (TMS) was used as the internal standard. $CDCl_3$ was used as the solvent for dissolving the crystals of piperine.

2.2 Reagents and Solutions

HPLC-grade acetonitrile, sodium chloride, trimethylamine, dimethylcyclohaxylamine, sodium hydroxide and vanillin were acquired from Sigma-Aldrich (Germany). Toluene, ethyl acetate and acetic acid were acquired from Riedel- de haën (Germany). Sulfuric acid was acquired from Fluka (USA).

2.3 Apparatus

Heater and UV lamp from CAMAG, and a rotary evaporator from BÜCHI (rotavapor R-210) was used for evaporating the fractions to dryness. Hettich Eba 20 centrifuge (Germany), Binder oven (USA), Eppendorf micropipette of different volumes from Sigma-Aldrich (USA) and tips from ISOLAB laborgeräte GmbH were used. A solvent filtration system (BORU CAM 1000 mL) and Whatman filters (0.45 μm and 0.2 μm), were used for filtering deionized water (DI). A coffee grinder from Sinbo (model SCM 2927, P.R.C) was used for blending black and white pepper samples. Ultrasound and water bath were from ISOLAB laborgeräte GmbH (Germany). Vortex machine was from Heidolph Reax. A refrigerator from Blomberg was used for preserving samples and standards until analysis.

2.4 Sampling and Sample Pre-treatment

Seven samples of black pepper from different origins (i.e., Brazil, India, Pakistan, Sri Lanka, UK and Vietnam) and one sample of white pepper (Jamaica) were purchased from local markets in Cyprus, Kuwait, Saudi Arabia and Pakistan.

2.4.1 Sampling Blending

Using the grinder, each sample was blended and homogenized into fine powder and stored in a well-sealed glass bottles till analysis.

2.4.2 Solid-Liquid Extraction

A portion of the fine powder (0.1 ± 0.01 g) was transferred into a 15-mL falcon tube and 5.0 mL of 50/50 (%v/v) ACN/H_2O were added. This mixture was vortexed for 1 min, centrifuged for 2 min and filtered through a cotton wool.

2.4.3 Salting-out extraction (SOE)

The filtered solution was transferred into another falcon tube and 3.0 mL of saturated NaCl were added. The mixture was vortexed for 1 min and centrifuged for 2 min at 6000 rpm. Approximately, 500 µL of ACN salted out, 50.0 µL of which were used for SPS-LLME.

2.4.4 SPS-LLME

50 µL of the extract were transferred into a falcon tube and made up to 4.0 mL with DI water, 1.5 mL of the SPS were added to the solution and the mixture was vortexed for 10 s. Then, 1.0 mL of 20 M NaOH was added and vortexed for 10 s. Approximately, 600 µL of SPS floated at the surface, all of which were transferred into a microvial and completed to 1.0 mL with the mobile phase. The mixture was vortexed for 10 s before being injected into HPLC. A schematic diagram of the extraction procedure is shown in **Figure 2.1**.

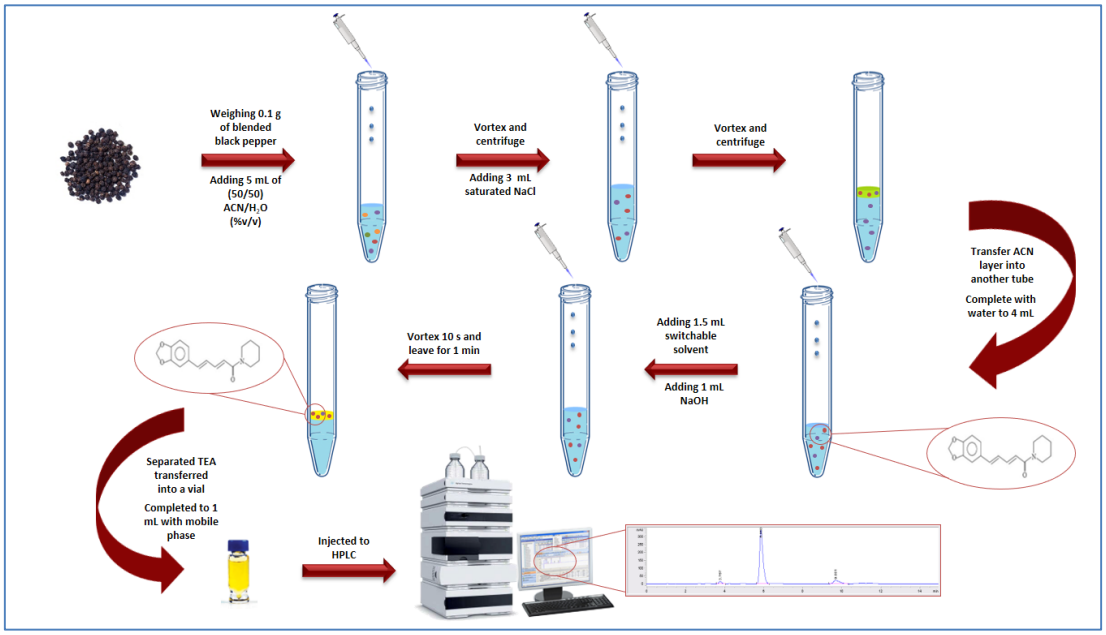


Figure 2.1: SPS-LLME procedure.

2.5 Extraction and Isolation of Piperine from Black Pepper

Black pepper (70 g) were blended into fine powder using the coffee blender. The powder was transferred into a 1000 mL volumetric flask and 700 mL of 50:50 ACN/H_2O were added. The mixture was placed in the ultrasound bath for 10 min, then it was filtered and centrifuged for 2 min. The supernatant was used for SOE.

2.5.1 Salting-out extraction (SOE)

The solution was transferred into a separatory funnel as shown in **Figure 2.2**. Saturated NaCl (420 mL) was added and the mixture was shaken for few seconds. A small amount of NaCl (approx. 5 g) was added to the mixture and it was shaken again until it became saturated with NaCl. Then, 3 mL of 20 M NaOH were added and allowed to stand on a retort stand until phase separation between aqueous and ACN layers occur. The ACN layer was transferred into a beaker and was ready to be used as the disperser solvent in the scaled-up DLLME.

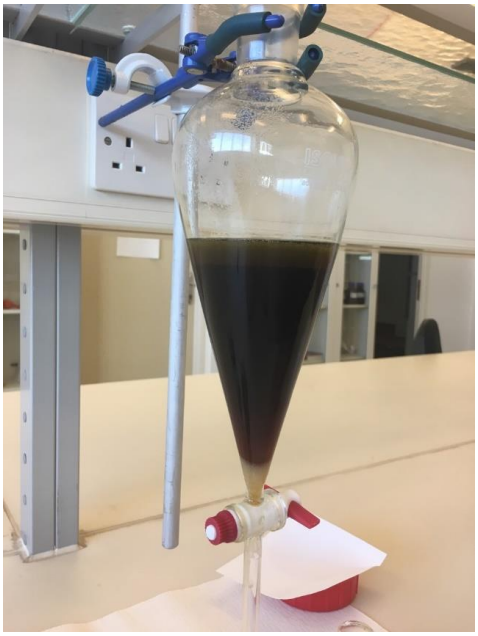


Figure 2.2: Salting-out extraction

2.5.2 Scaled-up DLLME

ACN (140 mL) was transferred into a 1000 mL measuring cylinder and 23 mL of chloroform (CF) were added; 583 mL with DI water and 59 mL of 1.0 M NaOH were added to the solution. The mixture was transferred into a beaker, placed in the ultrasound bath for 10 min and centrifuged in portions for 2 min at 6000 rpm. CF which sedimented at the bottom was collected (approx. 15 mL) (**Figure 2.3)**, and was analyzed using HPLC before continuing with the isolation procedure.

Figure 2.3: Collected CF from scaled-up DLLME.

2.5.3 Preparation of piperine for isolation by column chromatography

The final CF extract from DLLME was evaporated by rotary evaporator at 43 ºC, starting with 443 mbar and reducing the pressure until all the CF evaporated after about an hour as shown in **Figure 2.4**. The collected solid residue was 2.76 g **(Figure 2.5)**, some of it was re-dissolved in a small volume of 7:3 (%v/v) toluene:EtOAC which was checked with TLC (**Figure 2.6**) and used as a reference sample as all the procedure is shown in **Figure 2.7**.

Figure 2.4: Evaporation of CF by rotary evaporator.

Figure 2.5: The collected crude solid residue.

Figure 2.6: TLC with toluene: ethyl acetate 70/30 (%v/v).

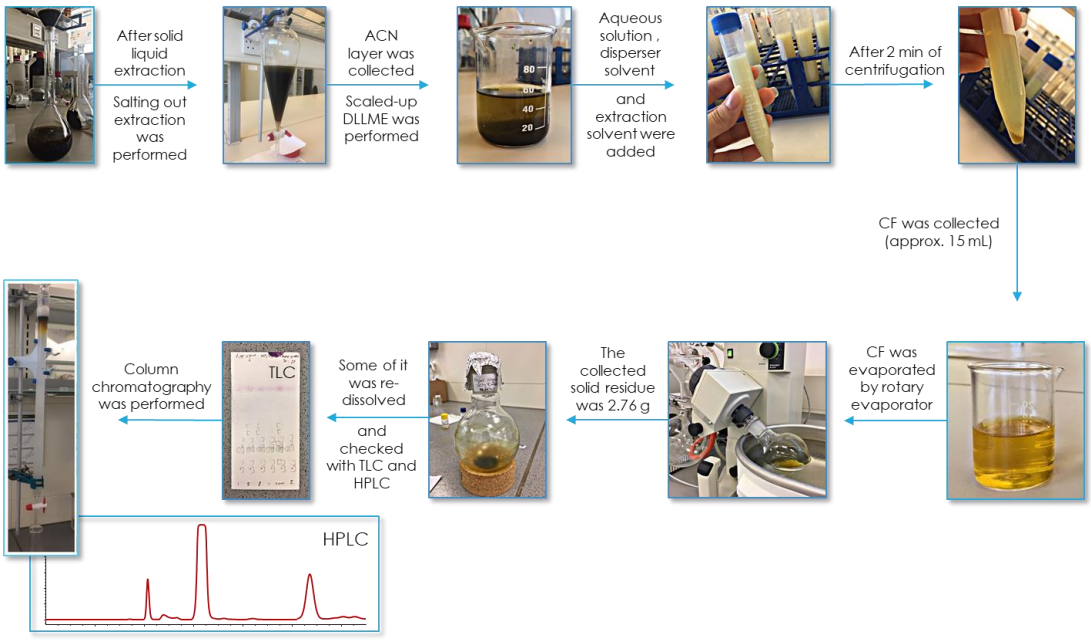


Figure 2.7: Extraction and Isolation of Piperine from Black Pepper.

2.5.4 Column chromatography

The column had been prepared by dissolving 100 g of silica gel in 100 mL toluene:ethyl acetate (90:10), packed into the column and covered up with cotton wool to obtain fixed sample zone. After making sure that there were no air bubbles trapped inside the column during the packing step, the solid residue, that had been dissolved in an appropriate amount of the mobile phase toluene:ethyl acetate (90:10), was loaded into the column. Gradient elution started from 90:10 until 60:40 toluene:ethyl acetate was performed and 130 fractions were collected.

TLC and HPLC were used to analyze the fractions. The fractions which were similar (46-51) and (52-63) that were thought to contain piperine were collected separately, each in a pre-weighed round bottom flask and the solvent was evaporated (toluene:ethyl acetate) by rotary evaporator. The weight of the final extract was 800 mg for the 46-51 fractions and 999.6 mg for 52-63 fractions. After a while, this extract started to crystalize by itself probably because of high concentration of piperine as shown in **Figure 2.8**. An appropriate amount of these crystals was sent for structural characterization by NMR.

Figure 2.8: Crystallization of piperine.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Selection of Maximum Absorption Wavelength (λ_{max})

Choosing λ_{max} is an essential and an important step which helps to eliminate the errors and to improve the selectivity and sensitivity of the determination. The first choice of λ_{max} can be based on literature, where it was mentioned that maximum absorption of piperine is 343 nm [4]. However, from the absorption profile of piperine obtained in our experiment (**Figure 3.1**), and with the help of 3D plot (**Figure 3.2**), 346 nm was chosen as optimum.

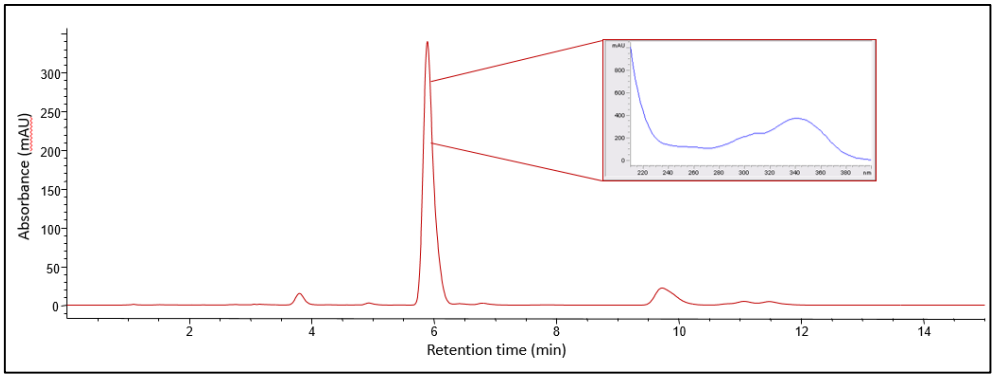


Figure 3.1: Absorption profile of piperine.

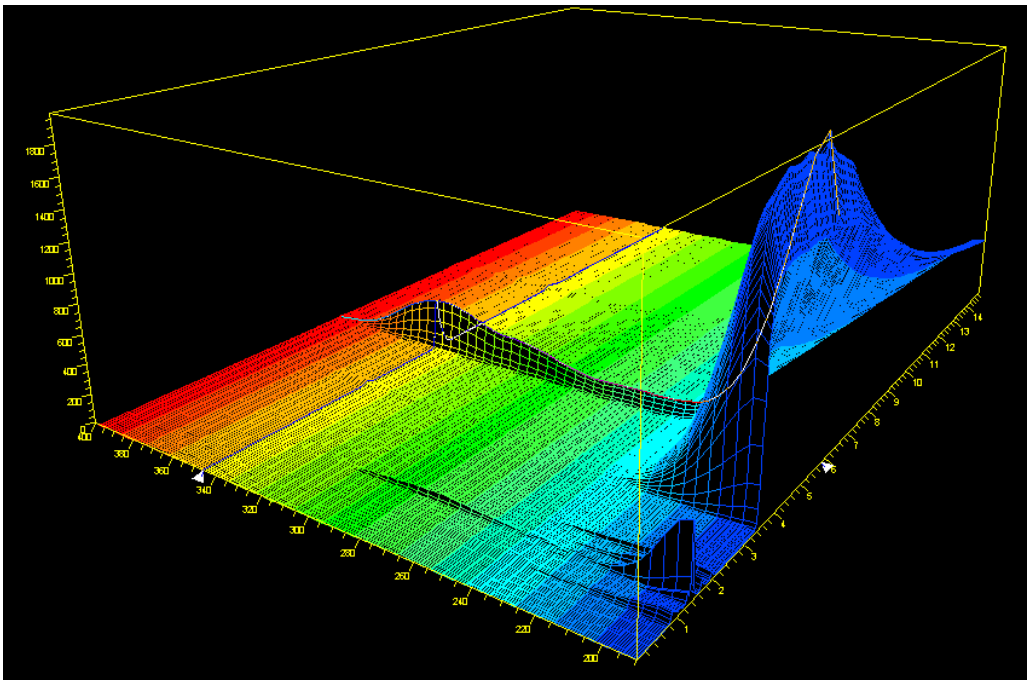
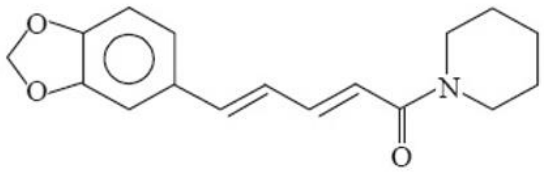


Figure 3.2: 3D Plot of piperine standard.

3.2 Optimization of Extraction Methods

3.2.1 Optimization of extraction parameters

In the solid-liquid extraction of piperine from black pepper, ACN was used as the extraction solvent due to its intermediate polarity. This choice was applied after checking the polarity of piperine and it turned out that piperine had an intermediate polarity, as also indicated by its log *P* (**Figure 3.3**).



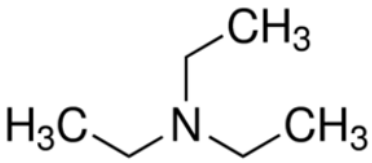
$\log P = 2.78$

Figure 3.3: log *P* of piperine.

Accordingly, ACN/H_2O (50/50%) was used for extracting piperine from the solid sample with the help of vortex mixing. Upon centrifugation, phase separation was made possible through SOE.

3.2.2 Switchable-polarity-solvent liquid-liquid microextraction (SPS-LLME)

SPS based on TEA have an intermediate polarity (**Figure 3.4**). Hence, they are suitable for extraction of intermediate-polarity analytes.



$\log P = 1.26$

Figure 3.4: log *P* of triethylamine.

Apart from the polarity of piperine, it is important to examine the different forms it has at different pH ranges. Piperine is present in its neutral (non-ionized) form within a wide range of pH (i.e., from 4.8 and above). It can be ionized in very acidic media at pH values below 4.8 (**Figure 3.5**). Therefore, since the apparent pH of the sample solution

obtained after SOE is about 7, piperine is present in its neutral form, changing the pH of the solution was not necessary**.**

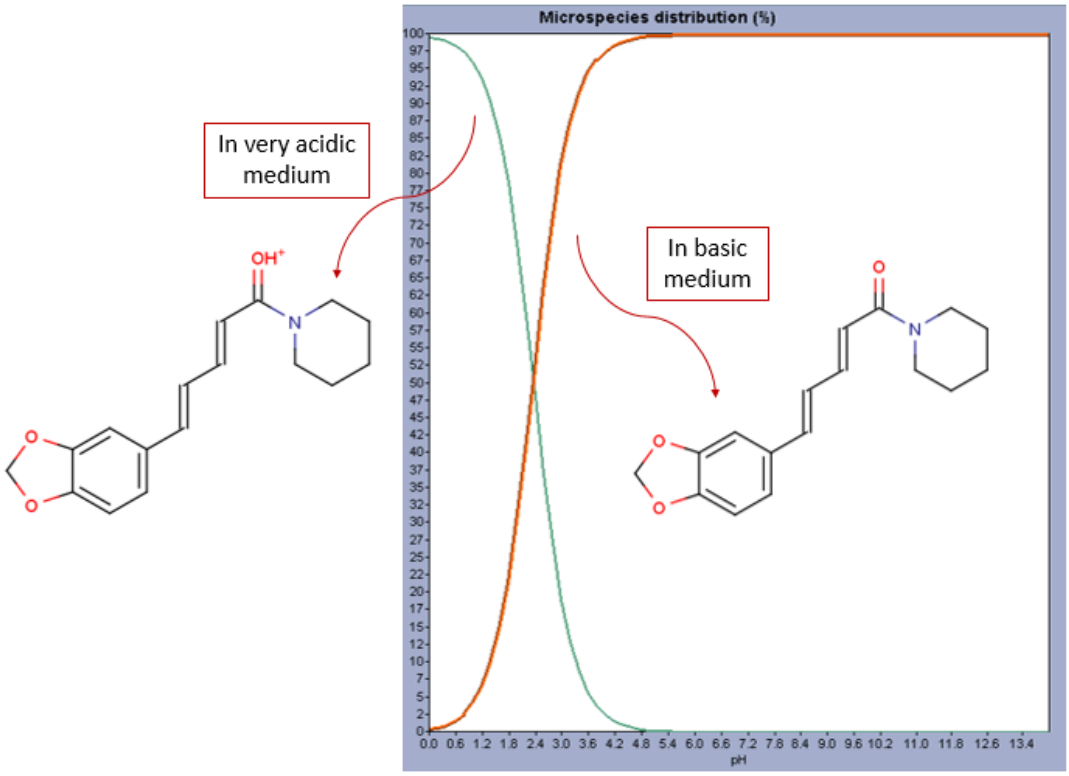


Figure 3.5: Microspecies distribution of piperine.

Phase separation was achieved by adding 20 M NaOH to the solution. By doing so, the SPS was made immiscible with the aqueous sample solution and it floated on the surface.

3.3 Optimization of HPLC Conditions

Chromatographic system was optimized in order to obtain high selectivity, sensitivity, efficiency and resolution in addition to minimizing broad broadening and/or overlap with other sample components.

3.3.1 Type of the mobile phase

The mobile phase composition is a very important parameter in HPLC. There were three possible options for organic solvents in the mobile phase, MeOH, ACN and THF. From the Snyder Polarity Index of the three solvent as shown in **Figure 3.6**. ACN has a polarity of 6.2, which is intermediate compared to the two other polarities of MeOH ($P = 6.6$) and THF ($P = 4.2$). Hence, ACN/H_2O was chosen as a starting point, considering that the analyte has intermediate polarity too. Moreover, ACN has low back pressure as shown in **Figure 3.7** due to its low viscosity and UV cut off as compared to MeOH and THF, which would result into lower noise in the UV region as shown in **Figure 3.8**. Hence, ACN was the solvent of choice for the mobile phase.

Snyder Solvent Polarity Index

Solvent	Polarity index (Snyder L. R., 1974)
Cyclohexane	0
n-hexane	0
n-decane	0.3
i-octane	0.4
Carbon tetrachloride	1.7
i-propyl ether	2.2
Toluene	2.3
Benzene	3.0
Dichloromethane	3.4
Methylene chloride	3.4
Chloroform	3.4-4.4
Ethylene dichloride	3.7
Tetrahydrofuran	**4.2**
Ethyl acetate	4.3
Propanol, 2-	4.3
Methyl acetate	4.4
Methyl ethyl ketone (MEK)	4.5
Ethanol	5.2
Pyridine	5.3
Acetone	5.4
Methoxyethanol, 2-	5.7
Acetic acid	6.2
Acetonitrile	**6.2**
Dimethyl sulfoxide	6.5
Methanol	**6.6**
Water	**9.0**

Figure 3.6: Snyder solvent polarity index.

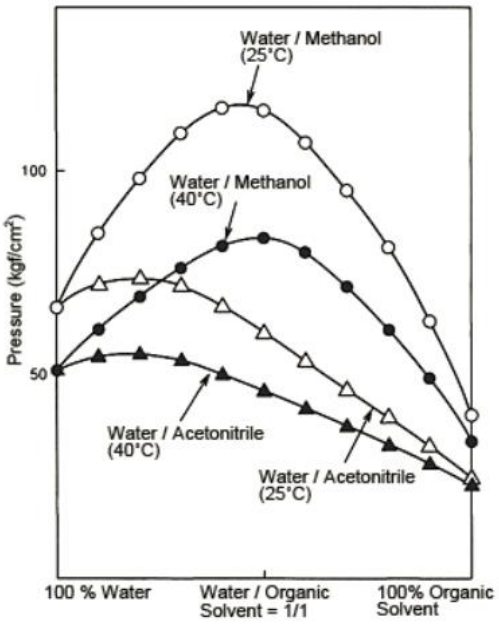


Figure 3.7: Relationship between organic solvent mixture ratio and back pressure of the column.

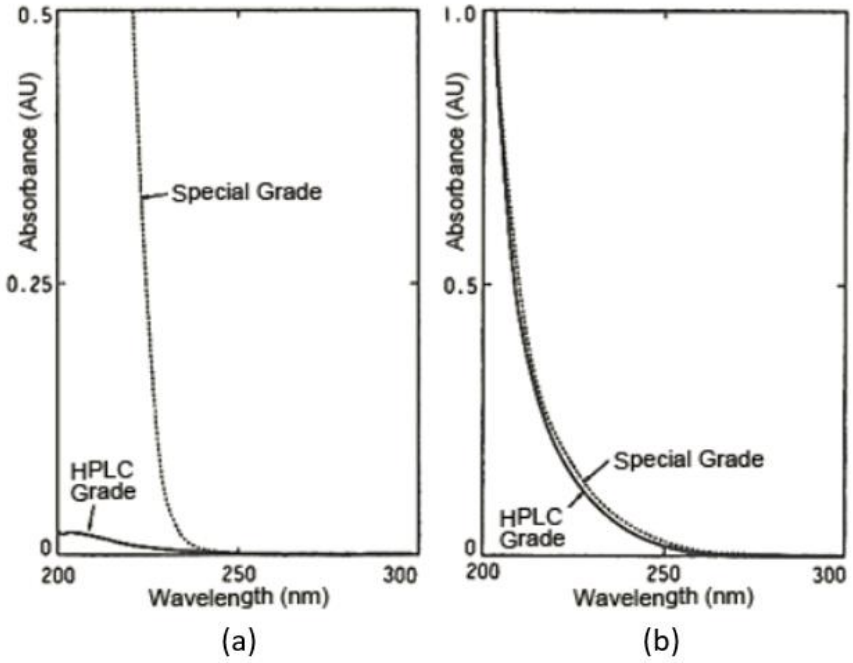


Figure 3.8: UV absorption spectrum of: (a) acetonitrile and (b) methanol reagents.

3.3.2 Optimization of the mobile phase composition

Mobile phase compositions that were considered for examining the effect of mobile phase composition included; 40, 42.5, 45, 47.5 and 50% ACN in water $(\%, v/v)$. By increasing the volume of ACN in the mobile phase, the retention time of piperine decreased, which was due to decrease in the polarity of the mobile phase. However, 45% ACN was chosen for this work **(Figure 3.9)** because k'_{av} for the three peaks, including piperine, was within the ideal range of 5 to 7, and the retention time was also acceptable (within 10 min).

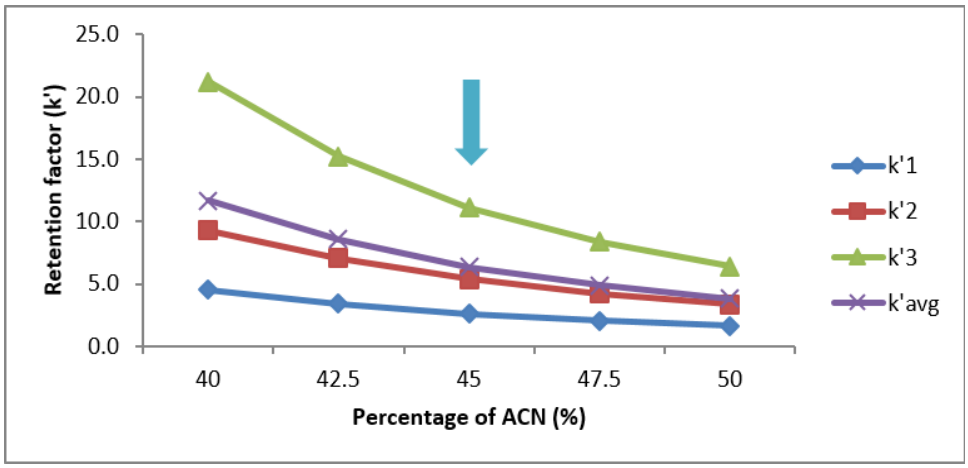


Figure 3.9: Optimization of the mobile phase composition.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 10 s.

3.3.3 Effect of adding acetic acid as a modifier in the mobile phase

Effect of adding different concentrations of acetic acid to the mobile phase starting from 0 to 1.0%, was examined through concentration intervals of 0.25%, going no further than 1% because high concentration of the acid in the mobile phase was thought to damage the column by dissolving the silica core. As shown in **Figure 3.10**, the addition of the acid to the mobile phase did not have any significant improvement. Hence, 0%

was chosen; piperine was separated without any overlap with the other compounds that were present in the sample.

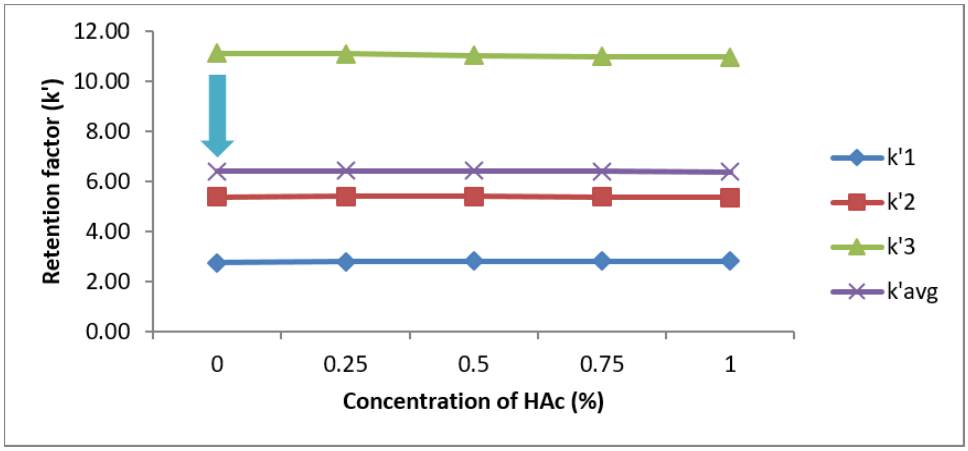


Figure 3.10: Effect of adding acetic acid as a mobile phase modifier.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 10 s.

3.3.4 Optimization of the flow rate

The aim of optimizing the flow rate was to obtain a good separation efficiency that would ultimately result into good resolution and to reduce the retention time without overlapping of the analyte peak with those of other components. Optimization started from 0.8 to 1.4 mL min^{-1} through 0.1 mL min^{-1} intervals. It is well known that increasing the flow rate decreases the retention time and peak areas. Therefore, using corrected peak area, which is obtained by dividing the peak area over the retention time and plotting the ratio against the flow rate, was though to better account for the effect of flow rate. As shown in **Figure 3.11**, the flow rate had little effect of the corrected peak area. However, considering the retention time and the column back-pressure, a flow rate of 1.1 mL min^{-1} was selected as optimum.

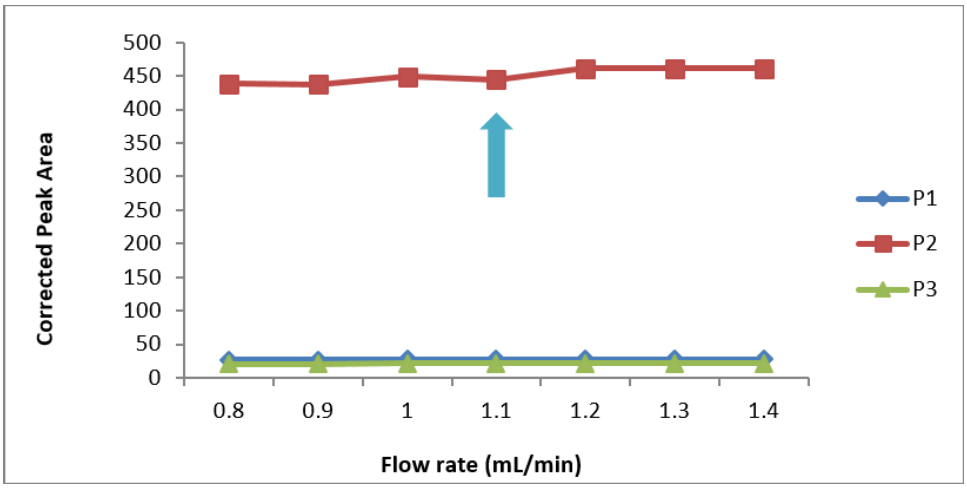


Figure 3.11: Optimization of the flow rate.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 10 s.

3.4 Optimum HPLC Conditions

Optimum conditions of HPLC are summarized in **Table 3.1**.

Table 3.1: Optimum HPLC conditions.

Physical parameters	Column	Agilent Eclipse XDB-C18. 4.6 mm ID x 150 mm (5 µm)
	Flow Rate	1.1 mL min^{-1}
	Temperature	Ambient (20 °C)
	Detector/wavelength	DAD 346 nm (BW 16). Reference: none
	Injection volume	5.0 µL
Chemical parameters	Mobile phase	ACN:H_2O 45:55 ($\%v/v$)

3.5 Switchable-Polarity Solvent Liquid-Liquid Microextraction (SPS-LLME)

Optimizing extraction conditions can improve many aspects of the extraction method such as robustness, extraction efficiency, sensitivity and selectivity. Influential parameters on SPS-LLME were studied in details and optimized.

3.5.1 Optimization of the type of extraction solvent for SPS-LLME

As discussed earlier (**Section 1.4**), tertiary amines are preferred as SPS-based extractions. Dimethylcyclohexylamine (DMCHA) and triethylamine (TEA) were considered for this purpose. Solvent recovery in both cases was similar. However, when DMCHA was used splitting or shouldering of the analyte peak occurred, the reason of which was not well understood. However, using TEA was more adequate and provided better peak shape and symmetry as well as a higher efficiency as shown in **Figure 3.12**. In addition, it gave a higher extraction efficiency **(Figure 3.13)** and was eventually chosen as the optimum extraction solvent.

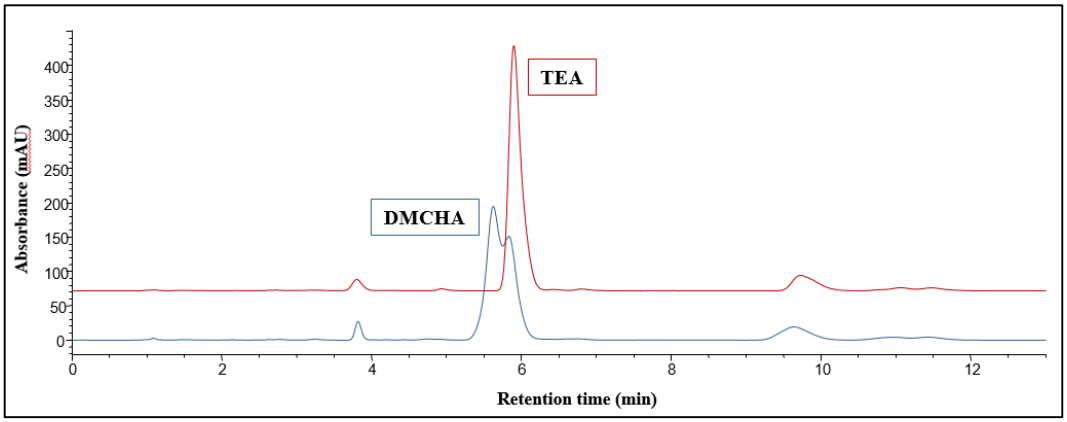


Figure 3.12: Comparison between DMCHA and TEA.

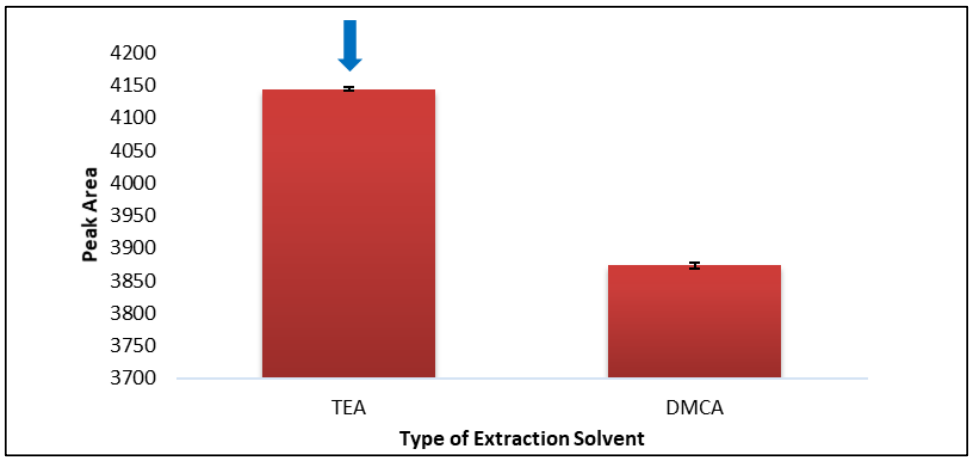


Figure 3.13: Selection of extraction solvent.

Conditions: Volume of extraction solvent: 1.0 mL; Volume of DI water: 5.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 1 min; Centrifugation time: 3 min; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.2 Optimization of the volume of TEA

Starting from 500 µL to 2000 µL and increasing the volume by 250 µL, the effect of volume of the extraction solvent on the extraction efficiency and the enrichment factor (EF) was examined. Using large volumes of the extraction solvent provided high recovery of piperine up to some point (i.e., 1500 µL). However, increasing the volume further started to decrease the EF which was thought to be due to dilution of the analyte in the solvent.

100 µL as the volume of the extraction solvent was also checked, but the recovery of the extraction solvent was very low and it was difficult to collect the floated TEA layer. 1500 µL was chosen as the optimum volume of the extraction solvent (**Figure 3.14)**. It is noteworthy to mention here that this volume of the SPS contained 750 µL of TEA.

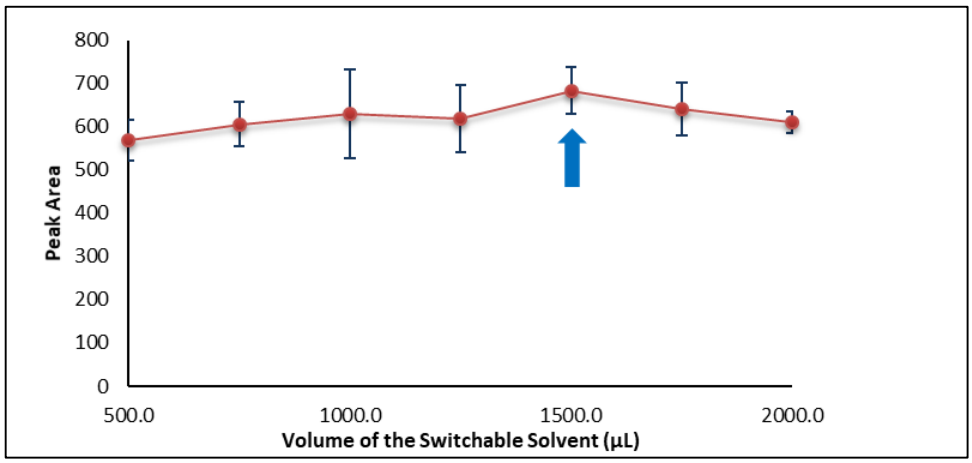


Figure 3.14: Optimization of the volume of the SPS.

Conditions: Extraction solvent: TEA; Volume of DI water: 5.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 1 min; Centrifugation time: 3 min; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.3 Optimization of the volume of the deionized water

In LLE or LPME techniques using immiscible liquids, increasing the volume of DI water (aqueous phase) provides higher surface area between the extraction solvent and the aqueous layer. However, in SPS-LLME, the surface area between the sample solution and the extraction solvent is infinite due to the complete miscibility of the solvent with the aqueous solution. Therefore, increasing the volume of the DI water did not have effect on the recovery of the piperine (**Figure 3.15**). In addition, the collected volume of the extraction solvent after phase separation was constant (i.e., 300 µL).

The volume of DI water was increased starting from 1.0 to 6.0 mL within 1.0 mL intervals. Hence, 4.0 mL was chosen as an intermediate reasonable volume for the DI water (**Figure 3.15**).

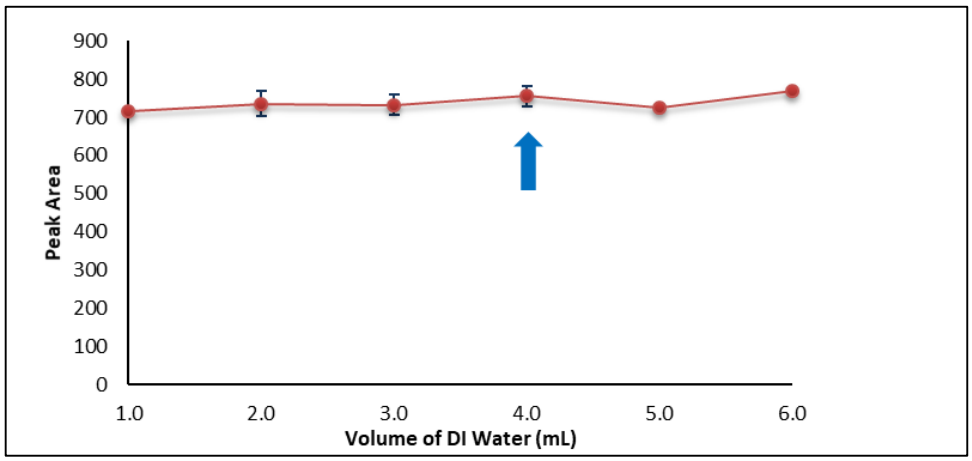


Figure 3.15: Optimization the volume of DI water.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of NaOH: 1.0 mL; Extraction time: 1 min; Centrifugation time: 3 min; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.4 Optimization the volume of the organic modifier

The effect of adding ACN as a modifier in the sample solution was also studied. ACN is the most commonly used disperser solvent in DLLME. It increases extraction efficiency by dispersing the organic immiscible solvent into the sample solution, increasing the surface area of contact and thereby extraction efficiency. Although it had been expected that the addition of ACN as an organic modifier in SPS-LLME would not have any effect or might even have a negative effect on extraction efficiency, its effect was studied by adding 0, 500, 1000, 1500, and 2000 µL of ACN to the sample solution within 500 µL intervals. Expectedly, peak area decreased by increasing the volume of ACN. The addition of ACN resulted into decreasing the polarity of the donor solution. Therefore, the partition equilibrium of the analyte between TEA and the aqueous solution favored the latter. The optimum recovery for piperine was obtained with no addition of ACN **(Figure 3.16)**.

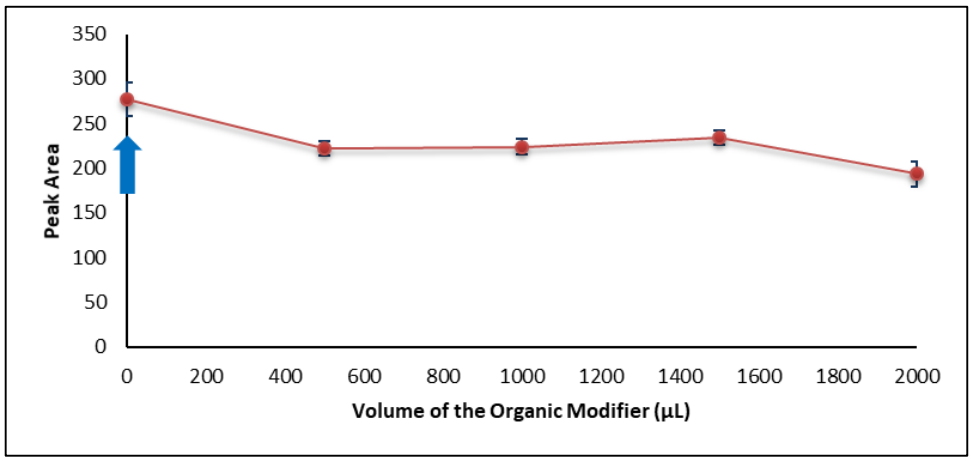


Figure 3.16: Optimization the volume of the organic modifier.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 1 min; Centrifugation time: 3 min; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.5 Optimization of the ionic strength

Generally, increasing the ionic strength the donor aqueous solution via addition of a salt increases the polarity of the solution and results in higher recovery of nonpolar analytes throughout salting-out. In other words, the solubility of hydrophobic analytes would be reduced in the aqueous solution. In some cases, however, no effect or even converse effect has been observed do to other physicochemical properties such as surface tension.

The concentration of NaCl in the donor solution was increased from 0 to 5 (% w/v) within 1% intervals through the addition of increasing volumes of a stock NaCl solution. No effect was observed, probably because addition of small concentrations of the salt compared to the high concentration of 20 M NaOH added for phase separation had negligible effect on the ionic strength of the sample solution. Hence, no NaCl was added in subsequent experiments **(Figure 3.17)**.

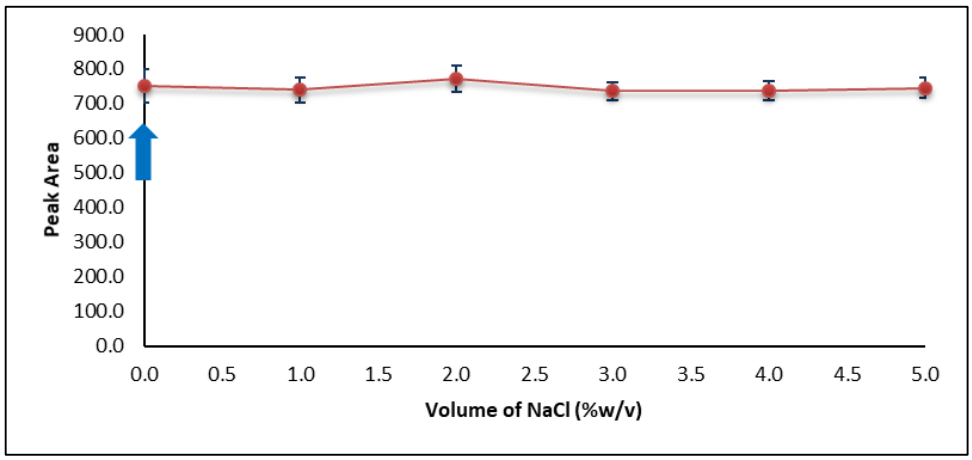


Figure 3.17: Optimization of the ionic strength.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 1 min; Centrifugation time: 3 min; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.6 Optimization of extraction time

Extraction time in SPS-LLME may be defined as the time interval between the addition of the SPS to the donor solution and the addition of NaOH to cause phase separation. Extraction time was studied starting from 0 s to 150 s and there was no effect, which was thought of as being the result of complete miscibility of the extraction solvent was with the sample solution (**Figure 3.18**). However, 10 s of extraction time through vortex mixing was used as an optimum extraction time to ensure better robustness and higher reproducibility of the method.

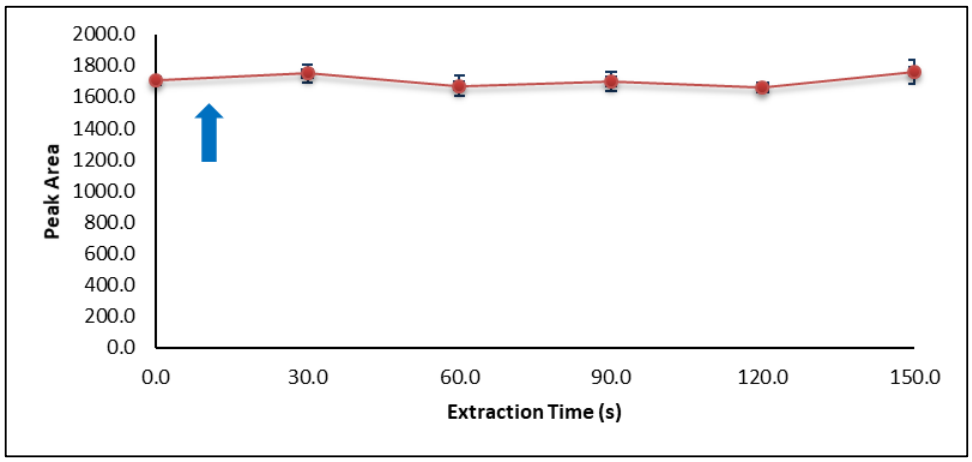


Figure 3.18: Optimization of the extraction time.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Centrifugation time: 3 min; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.7 Optimization of centrifugation time

Centrifugation time used for separating the organic extraction solvent that contain the analyte from the aqueous solution was increased from 0 min to 4 min and no effect was observed because adding 20 M of NaOH cause instantaneous phase separation with constant volume of the SPS via switching off the polarity of the SPS; CO_2 was freed from the structure of the ionic liquid and TEA became completely immiscible again with the aqueous phase and immediately floated on the surface of the sample solution. No centrifugation was necessary in further experiments (**Figure 3.19**).

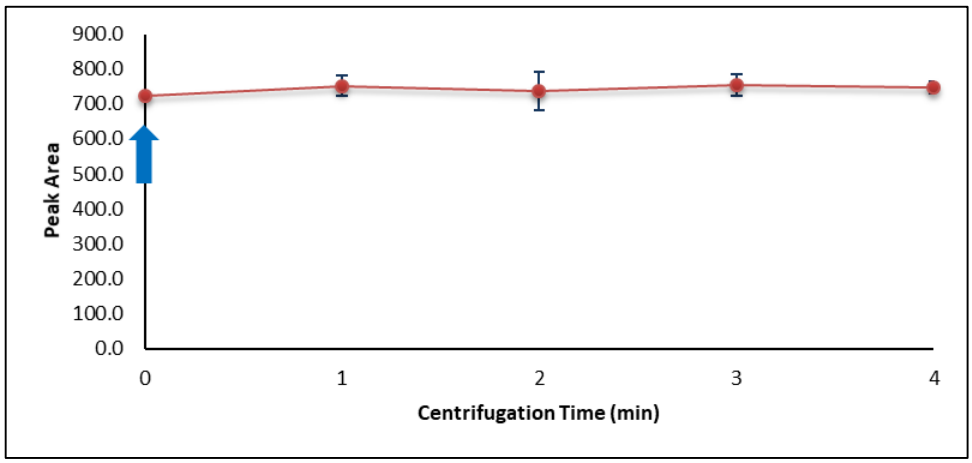


Figure 3.19: Optimization of centrifugation time.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 4.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 10 s; Sample introduction: Direct injection. HPLC conditions: as mentioned in **Table 3.1.**

3.5.8 Optimization of sample introduction of the final extract into HPLC

Sample introduction to the HPLC is an important step to maintain the life time of the column, because the solvent in the final extract should be compatible with both the column and the mobile phase. RP column was used with polar mobile phase, so the final extract should be miscible with the aqueous mobile phase. Three sample introduction methods of the final extract into the instrument were considered, namely; back-extraction, evaporation-to-dryness and direct injection.

In the back-extraction method, 50 mM of acetic acid (HAc) was used as the acceptor phase. According to the microspecies distribution of piperine, pK_a is equal to 2.4, implying that it can be ionized in an acidic medium (**Figure 3.20**).

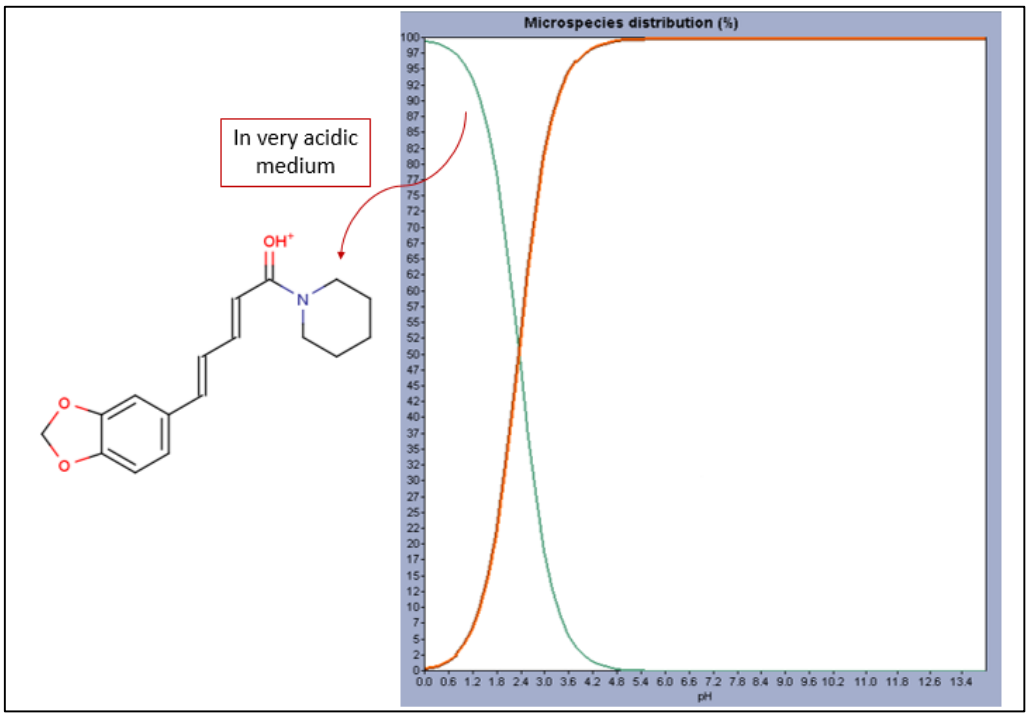


Figure 3.20: Microspecies distribution form of piperine in the acidic medium.

However, the recovery was very poor, with small peak obtained for piperine. This was thought to be due to inability of 50 mM HAc to neutralize TEA, a basic solvent itself. Using high concentration of the acid was considered but was avoided because higher concentrations would damage the column by dissolving silica gel, so back-extraction was not suitable.

In the evaporation-to-dryness method, after evaporating the solvent and reconstituting the analyte in the mobile phase ACN:H_2O 45:55 ($\%v/v$), good recovery with "clean" baseline was obtained. But, the main disadvantages of this method were time consumption, loss of the analyte during the evaporation and relatively high cost due to high consumption of nitrogen gas.

The third method was to introduce the final extract into HPLC with direct injection of the final extract, because TEA was miscible with the mobile phase ACN:H_2O [45:55 ($\%v/v$]. It is easy, fast and high extraction efficiency of piperine was obtained.

Representative chromatograms obtained with of the three sample introduction methods are given in **Figure 3.21**. Direct injection was chosen for the rest of the experiments **(Figure 3.22)**.

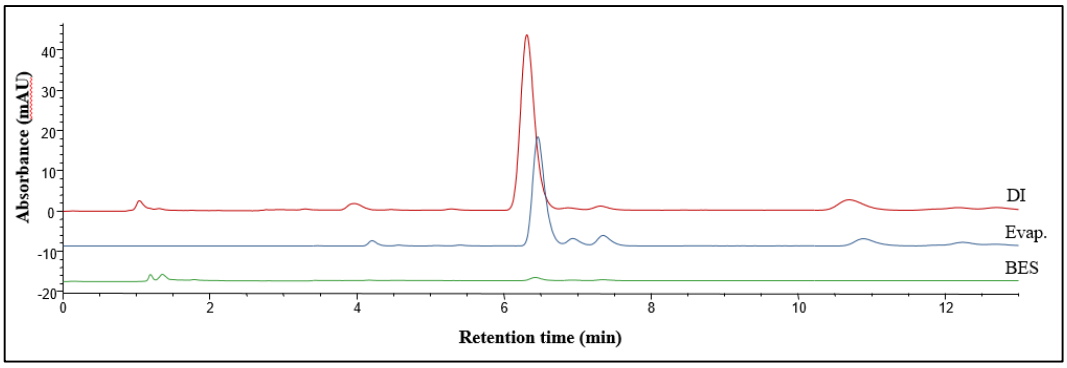


Figure 3.21: Representative chromatograms obtained with of the three sample introduction methods.

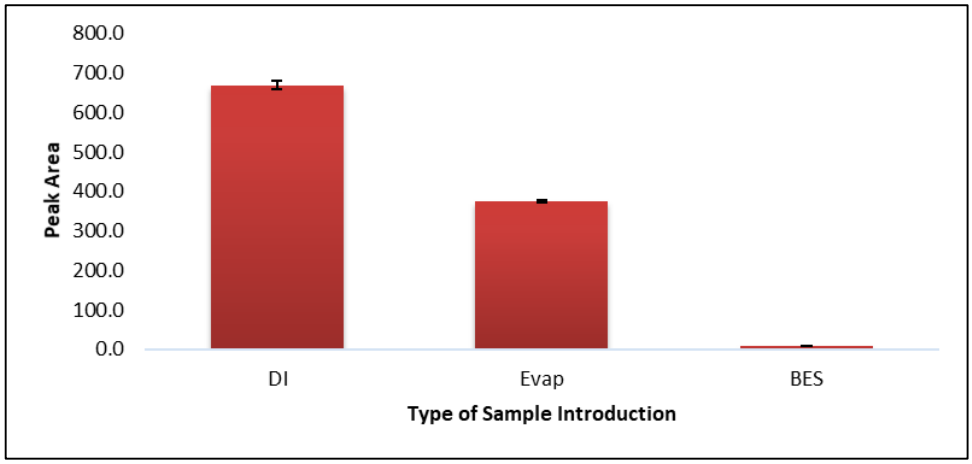


Figure 3.22: Mode of sample introduction.

Conditions: Extraction solvent: TEA; Volume of extraction solvent: 1500 µL; Volume of DI water: 5.0 mL; Volume of NaOH: 1.0 mL; Extraction time: 10 s. HPLC conditions: as mentioned in **Table 3.1.**

3.6 Optimum SPS-LLME Conditions

The optimum conditions of the SPS-LLME method are summarized in **Table 3.2**.

Table 3.2: Optimum SPS-LLME conditions.

Extraction solvent	Triethylamine (TEA)
Volume of the sample solution	4 mL
Volume of the extraction solvent	1500 µL
Volume of the NaOH	1 mL
Extraction time	10 s
Sample introduction	Direct injection

3.7 Calibration, Quantitation and Figures of Merit

The piperine standard isolated at our laboratory was used under optimum RP-HPLC conditions to plot an aqueous calibration curve. Standards were prepared in the mobile phase ACN:H_2O 45:55 ($\%v/v$), at the concentrations of 100, 250, 375, 400, 500, and 625 mg L^{-1}. As shown in **Figure 3.23**, coefficient of determination (R^2) was 0.9999 within a linear dynamic range between 100 and 625 mg L^{-1}, indicating a good linearity.

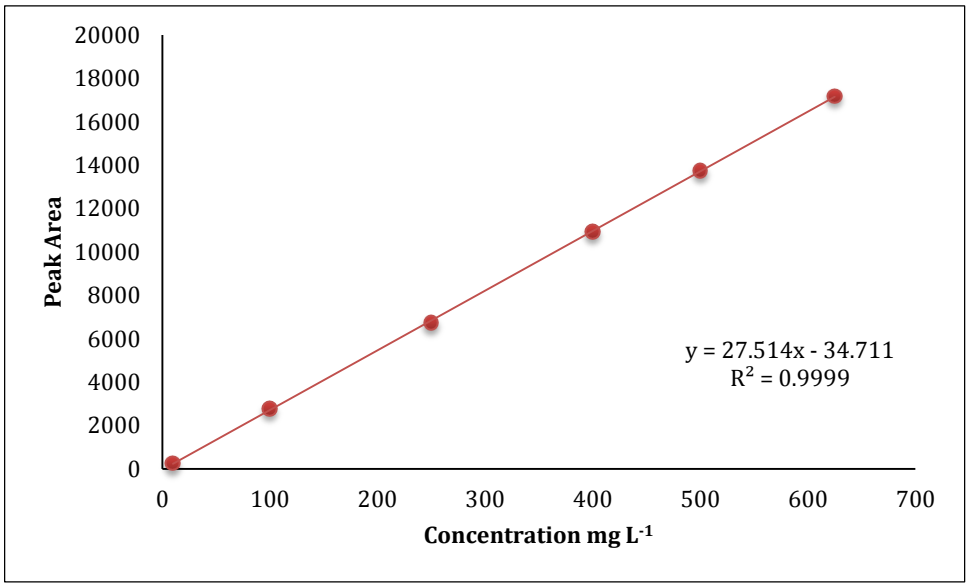


Figure 3.23: External aqueous calibration graph for piperine

To check for any matrix effect, calculate the concentration and recovery, the developed method was applied for the extraction of piperine from eight unspiked samples of black pepper and one white pepper. The samples were also spiked at five levels, 100, 250, 375, 500 and 675 mg L^{-1} of piperine and standard addition calibration graphs were plotted for each sample. The slope was different for each sample indicating the presence of matrix effect. Limits of detection (LOD), based on $3S_b/m$, where S_b is the standard deviation of the blank signal and m is the slope of the calibration graph, ranged from 0.4 to 1.2 mg g^{-1} and limits of quantitation (LOQ), based on $10S_b/m$, ranged from 1.3 to 3.9 mg g^{-1} of Reproducibility of the method, expressed as percentage relative standard deviation (%RSD), ranged from 1.2 to 4.7% for intraday and 2.1 to 10.0% for interday precision. R^2 ranged between 0.9950 and 0.9990, whereas, linear dynamic range (LDR) was obtained between LOQ and 35.0 mg g^{-1}. Figures of merit are summarized in **Table 3.3**.

Table 3.3: Analytical performance of SPS-LLME-HPLC.

Sample/ origin		Regression equation[(b)]	R^2	LOD[(c)] (mg g^{-1})	LOQ[(d)] (mg g^{-1})	LDR[(e)] (mg g^{-1})	%RSD[(f)]	
							Intraday	Interday
Black	Pooled[(a)]	$y = 5.0(\pm 0.05)x - 7356.4(\pm 19.8)$	0.9990	0.6	1.9	1.9-35.0	3.1	4.9
	Brazil	$y = 0.45(\pm 0.003)x - 481.1(\pm 1.2)$	0.9990	0.4	1.3	1.3-35.0	1.5	2.6
	India	$y = 0.69(\pm 0.01)x - 738.7(\pm 5.3)$	0.9960	1.1	3.7	3.7-35.0	2.6	4.0
	India 2	$y = 0.62(\pm 0.008)x - 460.4(\pm 3.16)$	0.9980	0.7	2.5	2.5-35.0	4.0	8.1
	Pakistan	$y = 0.77(\pm 0.01)x - 358.9(\pm 4.3)$	0.9970	0.8	2.7	2.7-35.0	2.1	3.6
	Sri Lanka	$y = 0.8(\pm 0.01)x - 792.9(\pm 4.9)$	0.9975	0.9	3.0	3.0-35.0	4.7	10.0
	UK	$y = 1.68(\pm 0.02)x - 1953.2(\pm 10.9)$	0.997	0.9	3.1	3.1-35.0	2.7	4.0
	Vietnam	$y = 10.1(\pm 0.14)x - 5152.8(\pm 56.3)$	0.9979	0.8	2.7	2.7-35.0	4.5	9.1
White	Jamaica	$y = 0.74(\pm 0.01)x - 444.3(\pm 5.9)$	0.9950	1.2	3.9	3.9-35.0	1.2	2.1

[a]A pooled sample prepared by mixing equal masses of the seven black pepper samples.

[b]$Peak\ area = slope(\pm SD) \times concentration(mg\ g^{-1}) + intercept(\pm SD)$

[c]Limit of detection

[d]Limit of quantitation

[e]Linear dynamic range

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

Regression equations shown in **Table 3.3** were used to calculate the original concentration (in mg g^{-1} and as %, g g^{-1}) in the unspiked samples. Percentage relative recoveries (%RR) were calculated by spiking the samples at two levels, 250 mg L^{-1} (12.5 mg g^{-1}) and 500 mg L^{-1} (25.0 mg g^{-1}) and were found to range between 95.6 and 104.7%. These recovery values assisted us to correct for the actual concentration of piperine in the original sample. The concentrations of piperine originally present in black pepper samples ranged from 23.3 mg g^{-1} (2.3%, g g^{-1}, Pakistan) to 58.1 mg g^{-1} (5.8%, g g^{-1}, UK) and that found in the white pepper sample was 30.2 mg g^{-1} (3.0%, g g^{-1}, Jamaica) (**Table 3.4**).

Table 3.4: Relative recoveries of piperine from black and white pepper samples.

Sample	Added (mg g^{-1})	Found		%RR
		mg g^{-1}	%, g g^{-1}	
Pooled	-	74.2	7.4	-
	12.5	12.2	1.2	97.5
	25.0	24.5	2.4	97.9
Brazil	-	53.5	5.3	-
	12.5	12.3	1.2	98.8
	25.0	25.4	2.5	101.6
India	-	53.5	5.4	-
	12.5	12.4	1.2	99.3
	25.0	24.2	2.4	96.6
India 2	-	37.4	3.7	-
	12.5	12.8	1.3	102.7
	25.0	25.6	2.6	102.3
Pakistan	-	23.3	2.3	-
	12.5	13.1	1.3	104.7
	25.0	25.6	2.6	102.4
Siri Lanka	-	50.1	5.0	-
	12.5	13.0	1.3	104.1
	25.0	25.0	2.5	100.0
UK	-	58.1	5.8	-
	12.5	12.0	1.2	95.8
	25.0	24.0	2.4	96.0
Vietnam	-	25.6	2.6	-
	12.5	12.0	1.2	95.6
	25.0	25.8	2.6	103.3
White/ Jamaica	-	30.2	3.0	-
	12.5	12.5	1.2	99.8
	25.0	25.6	2.6	102.6

3.8 Isolation of Piperine

3.8.1 Scaled-up DLLME

The chromatogram for the extract obtained after the scaled-up DLLME procedure (**Section 2.5.2**) is shown in **Figure 3.24.** It can be observed from the chromatogram that the extract contained three major compounds that absorbed at 346 nm.

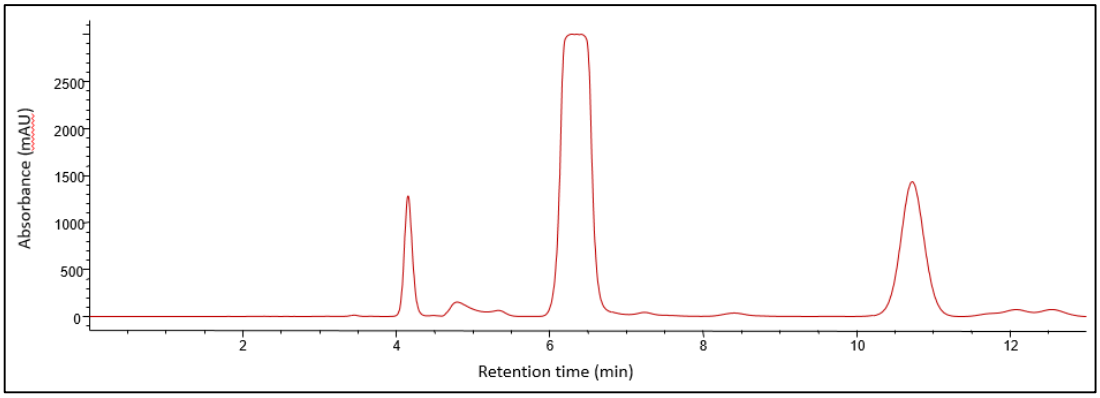


Figure 3.24: Chromatogram of the extract obtained after the scaled-up DLLME procedure.

3.8.2 Column chromatography

After packing silica gel to the column, choosing the right mobile phase composition was an important step. Gradient elution was chosen and the fractions were tested with TLC to check whether they contained the analyte or not. HPLC was also used to check the identity of the analyte through its UV spectra. Eluents in column chromatography were in the order of: 300 mL, 90:10 (%, v/v) toluene:EtOAC, 200 mL, 85:15 toluene:EtOAC, 200 mL, 80:20 toluene:EtOAC, 100 mL, 75:25 toluene:EtOAC, 200 mL, 70:30 toluene:EtOAC and 600 mL, 60:40 toluene:EtOAC until all the compounds in the extract were eluted from the column.

A yellow zone was obtained after loading the sample into the column as shown **Figure 3.25**, which is a characteristic color of piperine. Piperine was not eluted at the beginning. It was not before forty fractions when it was observed. Black pepper is rich with polar and non-polar compounds that interfered with piperine.

Figure 3.25: Isolation of piperine by column chromatography.

TLC was performed by applying replicate spots (10 fractions on a TLC plate) in addition to the reference sample from the crude extract to compare the retardation factor (R_f) of piperine using a mobile phase of 7:3 (%v/v) toluene:EtOAC.

After the plate was dried, a UV lamp was used to check the spots at 254 and 366 nm as shown in **Figure 3.26**. The plate was also sprayed with 1% vanillin and 5% H_2SO_4 to

visualize the separated compounds as shown in **Figure 3.27**. Piperine started appearing from fraction 46 to 63. These fractions were thus collected and evaporated to dryness using a rotary evaporation after checking the purity of these fractions by HPLC. A sample of the final solid extract was sent for NMR analysis for characterization and structural elucidation of the isolated compound.

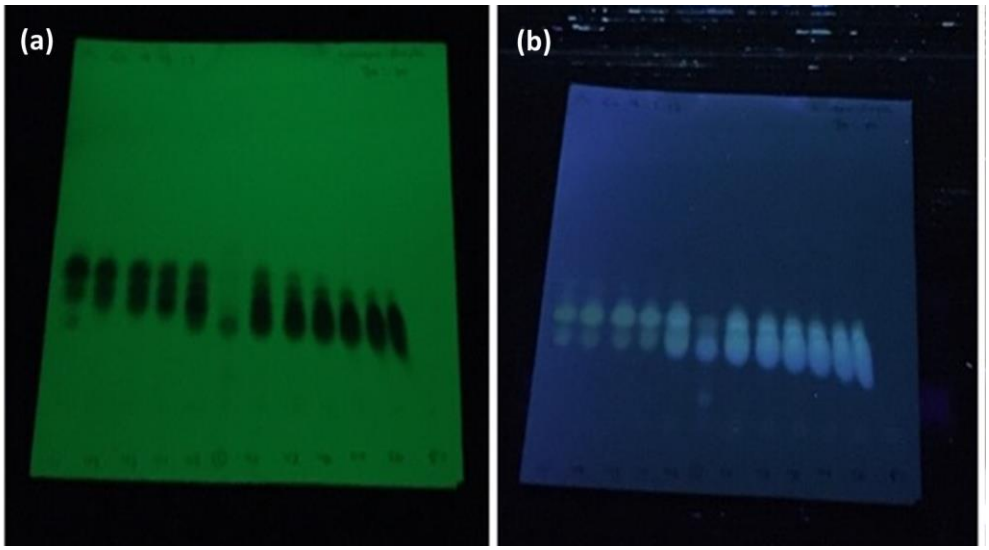


Figure 3.26: TLC plates the under UV light at (a) 254 nm and (b) 366 nm.

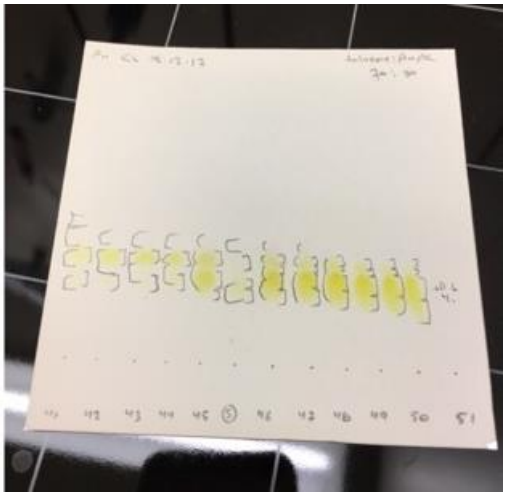


Figure 3.27: TLC plates after being sprayed with 1% vanillin and 5% H_2SO_4.

Chromatograms obtained with the 46-51 fractions (**Figure 3.28)** and 52-63 fractions (**Figure 3.29**) showed high concentration of piperine in those fractions.

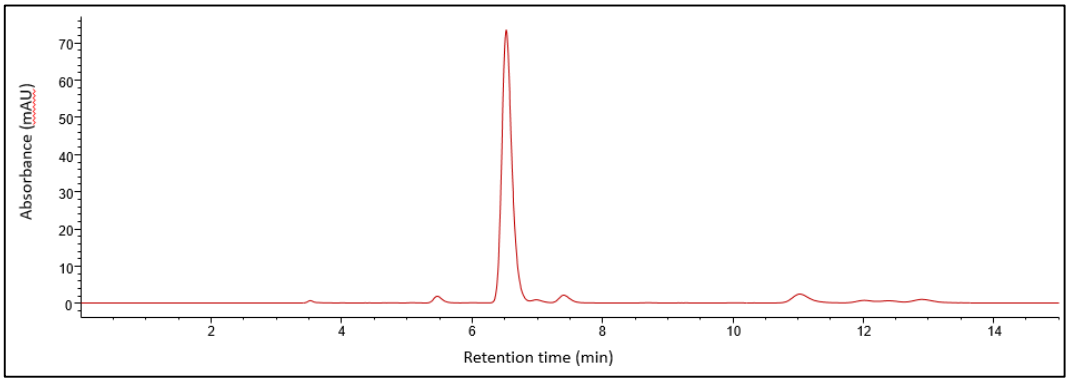


Figure 3.28: A chromatogram obtained with the 46-51 fractions.

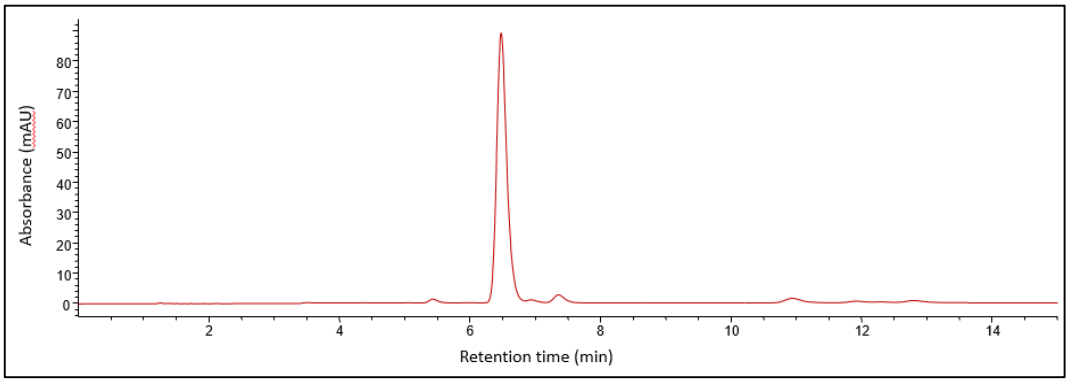


Figure 3.29: A chromatogram obtained with 52-63 fractions.

3.9 Structural Elucidation Using NMR

NMR results obtained for piperine which was isolated from the chloroform extract of *Piper nigri fructus* using scaled-up DLLME and purified using silica gel column chromatography are discussed below. Piperine was obtained as yellow colored crystals as shown in **Figure 3.30**, the purity of which was also checked by HPLC under optimized chromatographic conditions.

Figure 3.30: Yellow crystals of piperine.

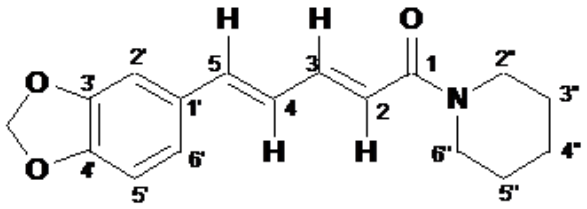


Figure 3.31: Numbered structural formula of piperine.

Table 3.5: ^{1}H and ^{13}C-NMR data of piperine [(*E*-*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoyl-2-piperidine)] ($CDCl_3$: ^{1}H: 500 MHz, ^{13}C: 125 MHz).

C/H Atom	Multiplicity (DEPT-135)	^{13}C-NMR (δppm)	^{1}H-NMR (δppm), *J* (Hz)	HMBC From C to H
2,4-Pentadienoyl				
1	C	165.40	-	H-2, H-3
2	CH	120.07	6.42 d (14.6)	H-3, H-4
3	CH	142.46	7.39 ddd (14.6, 14,6, 2.3)	H-2, H-4, H-5
4	CH	125.35	7.42†	H-2, H-3, H-5
5	CH	138.19	7.42†	H-2, H-3, H-4, H-2', H-6'
3,4-Methylenedioxyphenyl				
1'	C	130.98	-	H-5', H-4, H-5
2'	CH	105.64	6.96 d (1.6)	H-3, H-5', H-6'
3'	C	148.18	-	C$\underline{H_2}$-O-C$\underline{H_2}$, H-5'
4'	C	148.11	-	C$\underline{H_2}$-O-C$\underline{H_2}$, H-6'
5'	CH	108.46	6.75 d (8.2)	-
6'	CH	122.50	6.87 dd (8.2, 1.6)	H-2', H-5
CH_2-O-CH_2	CH_2	101.28	5.95 s	-
Piperidine				
2"	CH_2	43.22	3.62 br s	H-3", H-4", H-5"
3"	CH_2	26.74	1.57 m	-
4"	CH_2	24.65	1.64 m	H-3"
5"	CH_2	25.63	1.57 m	-
6"	CH_2	46.89	3.51 br s	H-3", H-4", H-5"

All assignments are based on 2D-NMR experiments (COSY, HSQC and HMBC)

†Signal pattern is unclear due to overlapping.

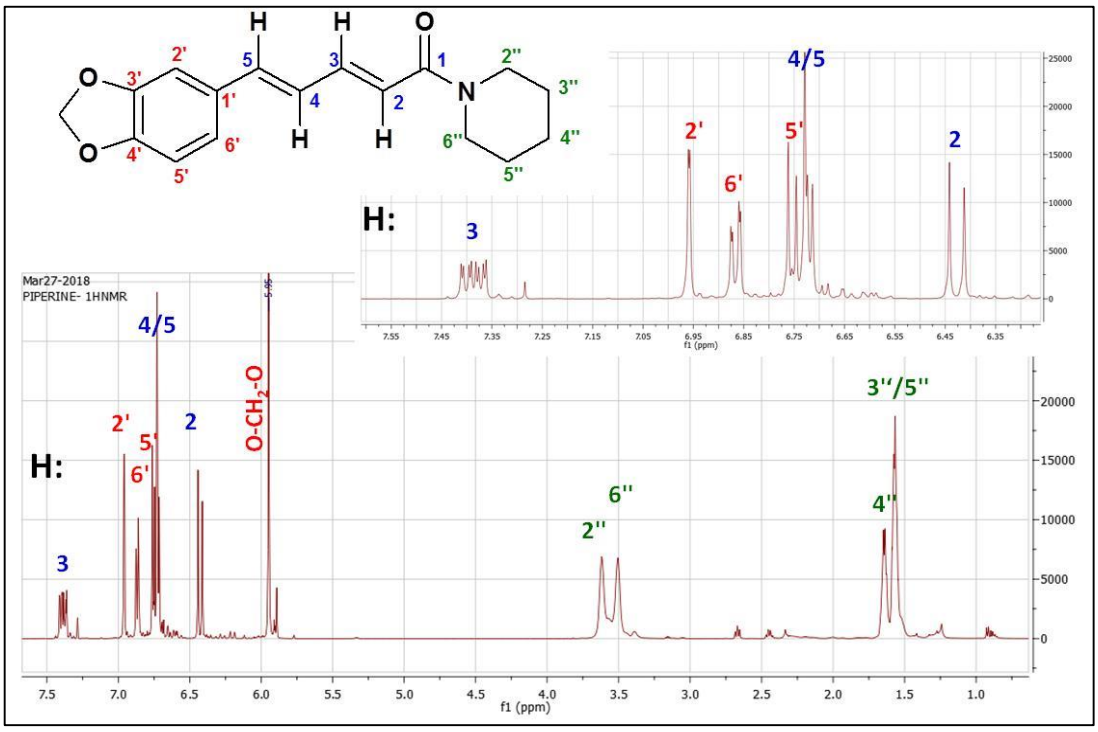


Figure 3.32: ^{1}H-NMR Spectrum of piperine (500 MHz, $CDCl_3$).

The structure of the isolated compound (**Figure 3.31**) has been confirmed by NMR spectroscopical studies. The ^{1}H-NMR spectrum of piperine (**Figure 3.32**) exhibited four spin systems which were established using COSY experiment (**Figure 3.34**). The COSY experiment revealed that the first spin system signals arose from the methylene protons of piperidine moiety at high field of the spectrum. The signals appearing at 3.62 (2H), 3.51 (2H), 1.64 (2H) and 1.57 (4H) were assigned to H_2-2″, H_2-6″, H_2-4″ and H_2-3″ and H_2-5″, respectively. The assignments were based on a COSY experiment (**Figure 3.34**).

The other three spin systems were observed between 7.5 and 5.50 ppm. The singlet signal at δ 5.95 with two proton intensities was assigned to a methylene-dioxy signal of 3,4-methylenedioxyphenyl moiety. The signals observed as an ABX system at δ 6.96 d (J = 1.6 Hz), 6.75 d (J = 8.2 Hz) and 6.87 dd (J = 8.2, 1.6 Hz) were attributed to the aromatic protons of the 3,4-disubstituted-phenyl unit. The rest of the signals observed in the same spin system were clearly assigned to the 2,4-pentadienoyl part of the molecule. The H-2 close to the carbonyl was observed as a well separated doublet at δ 6.42 (J =

14.6 Hz). The coupling constant of 14.6 Hz was consistent a *trans* configuration of the following proton, H-3, which was observed as ddd at δ 7.39 ($J_{3,2}$ = 14.6; $J_{3,4}$= 14,6 and $J_{3,2}$= 2,3 Hz). The H-4 and H-5 protons were observed at 7.42 ppm as overlapped signals.

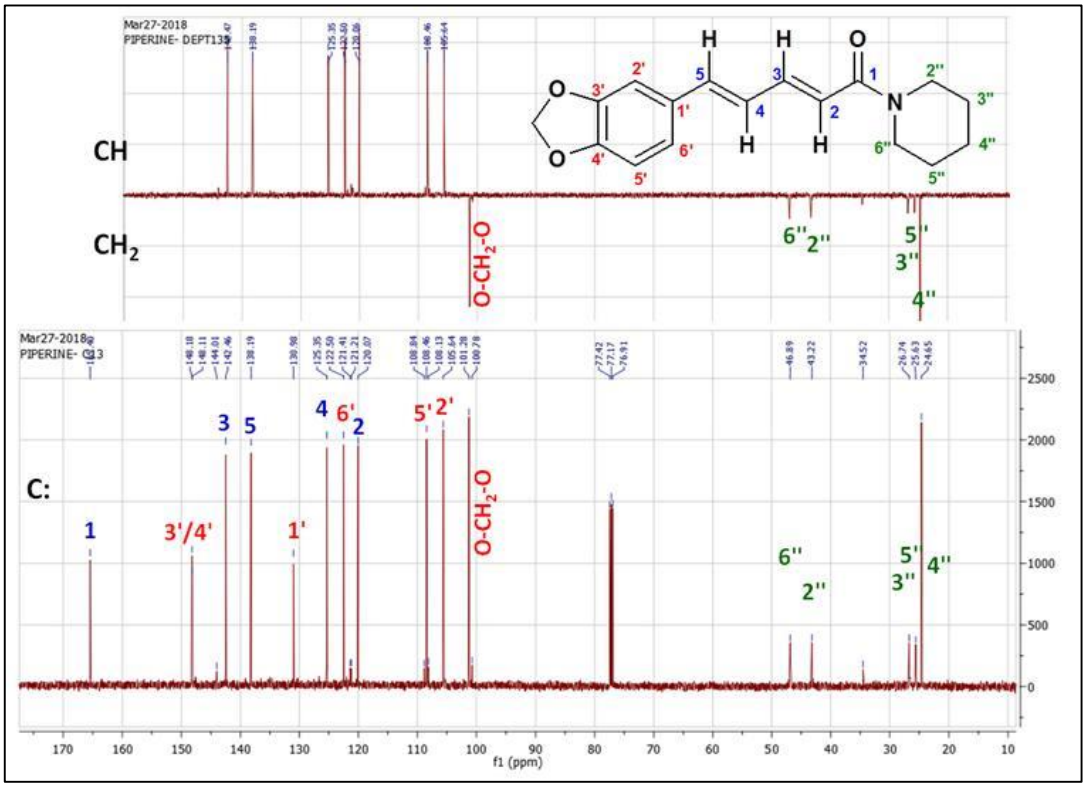


Figure 3.33: ^{13}C-NMR and DEPT Spectra of piperine (125 MHz, $CDCl_3$).

The ^{13}C-NMR spectrum of piperine (**Figure 3.33** and **Table 3.5**) exhibited 17 carbon resonances including 6 methylenes five of which were due to the piperidine unit at δ 43.22, 26.74, 24.65, 25.63 and 46.89 assigned C-2", C-3", C-4", C-5" and C-6", respectively. An additional methylene resonance in DEPT experiment (**Figure 3.33**) was consistent for the presence of a methylenedioxy functionality at 101.28 ppm. The downfield signal at δ 165.40 observed as a quarternary carbon was attributed to the carbonyl carbon of 2,4-pentadienoyl moiety together with the olefinic carbons at δ 120.07, 142.46, 125.35 and 138.19 assigned to H-2, H-3, H-4 and H-5, respectively.

The remaining carbon atoms were assigned to the 3,4-methylenedioxyphenyl moiety **(Table 3.5)**. All assignments were based on a HSQC experiment (**Figure 3.35**).

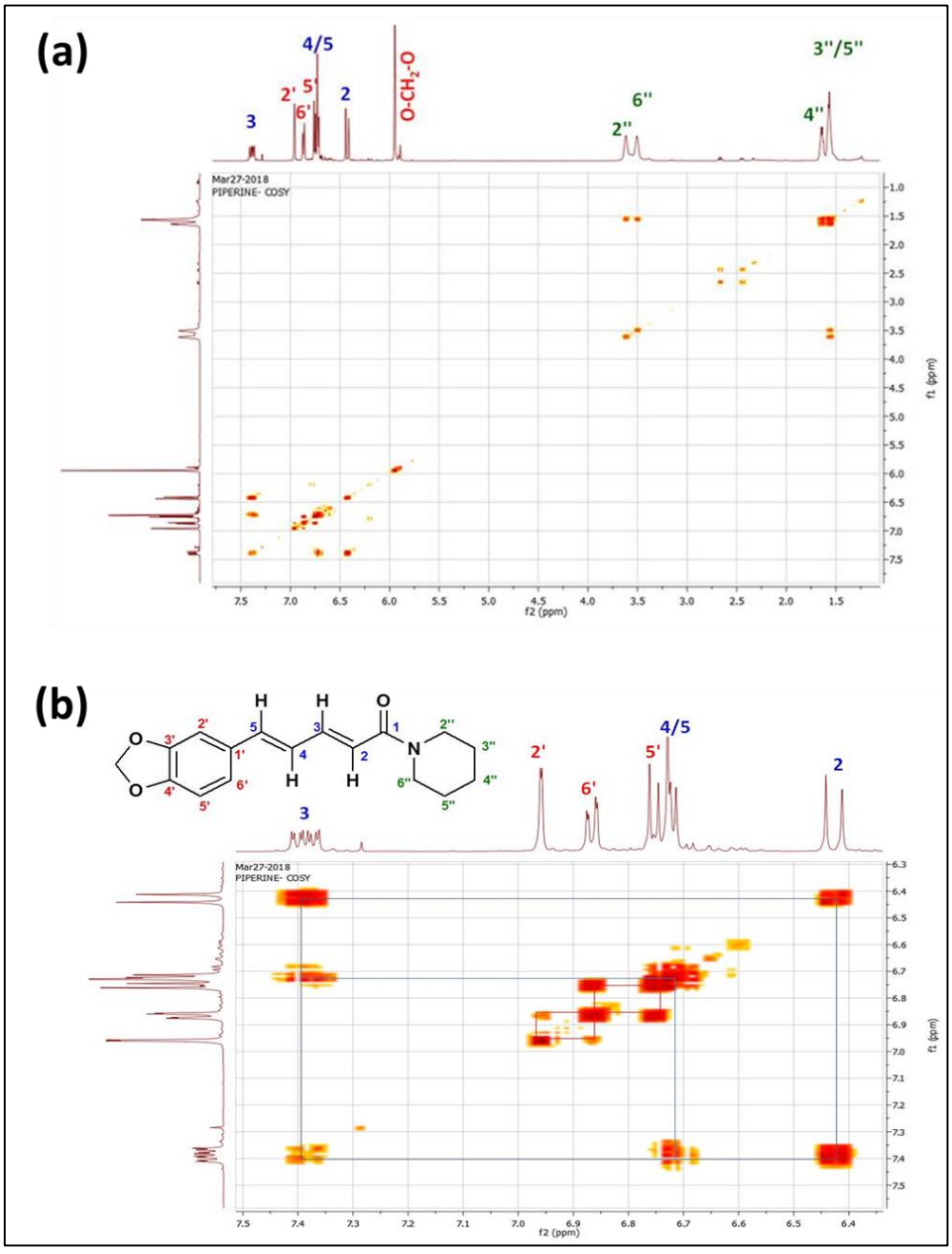


Figure 3.34: Spectrum (a), $^{1}H,^{1}H$-Homonuclear Correlated Spectrum (COSY) of piperine, and spectrum (b), $^{1}H,^{1}H$-Homonuclear Correlated Spectrum (COSY) of piperine.

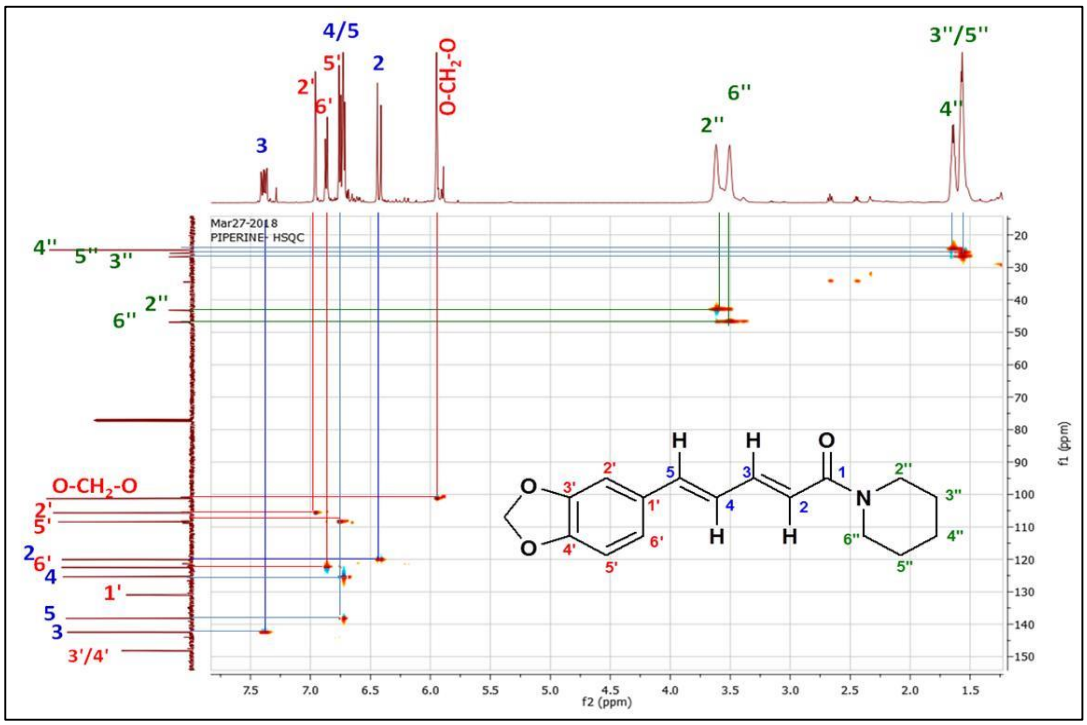


Figure 3.35: $^{1}H,^{13}C$-Heteronuclear Correlated Spectrum (HSQC) of piperine; [(HSQC: Heteronuclear Single-Quantum Correlation) experiment].

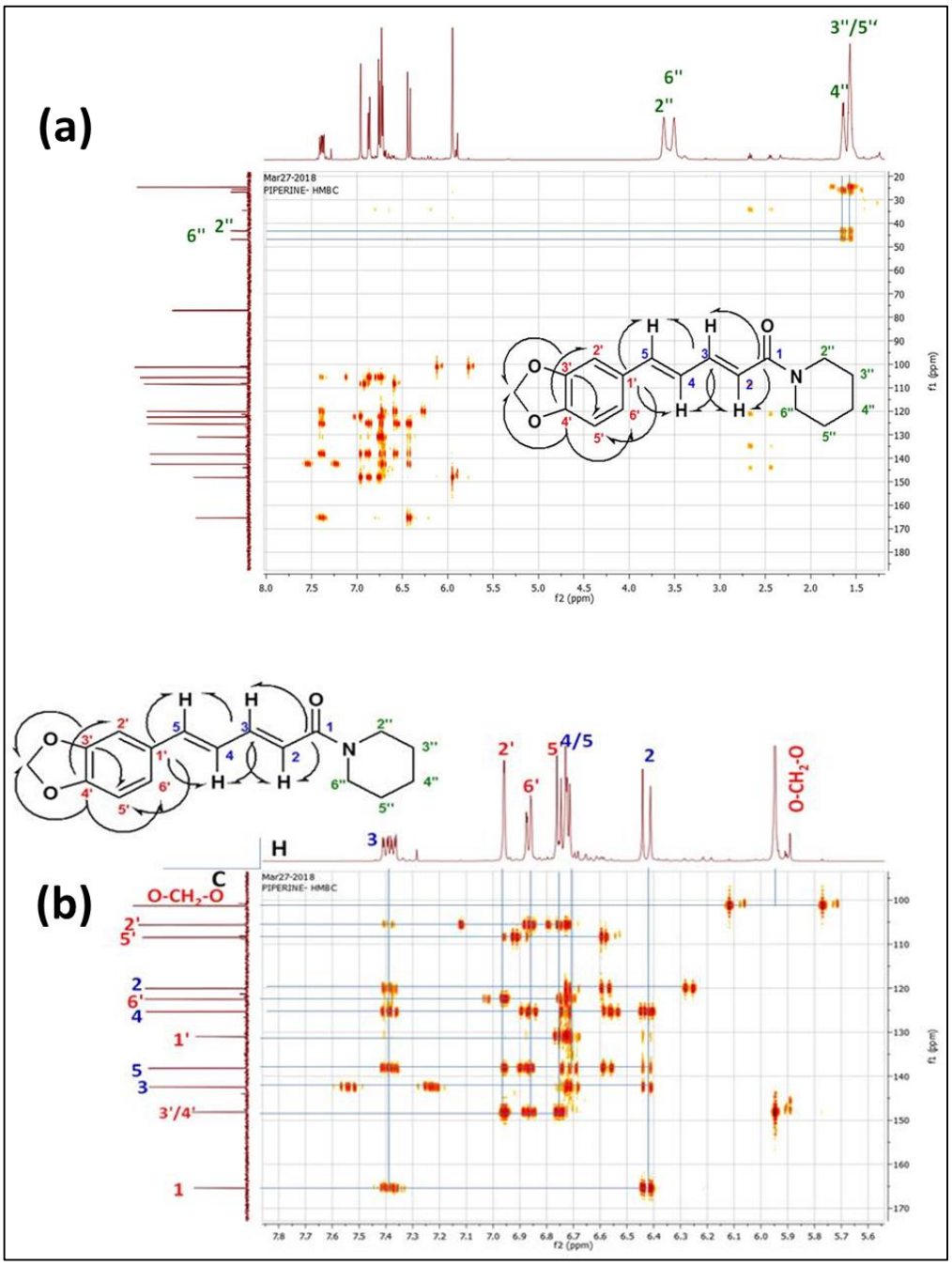


Figure 3.36: Spectrum (a), $^{1}H,^{13}C$-Heteronuclear Multiple Bond Correlated Spectrum (HMBC) of piperine, and spectrum (b),$^{1}H,^{13}C$-Heteronuclear Multiple Bond Correlated Spectrum (HMBC) of piperine.

Intermolecular connectivities were established using HMBC ($^{1}H,^{13}C$-Heteronuclear Multiple Bond Correlated Spectroscopy) experiment [**Figure 3.36 (a and b)]**.

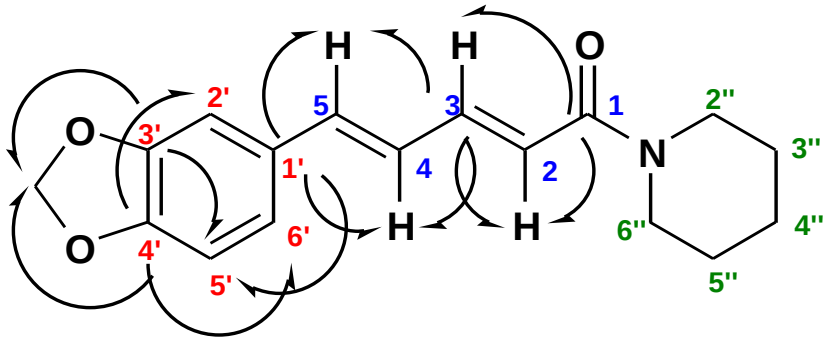


Figure 3.37: $^{1}H,^{13}C$-Heteronuclear Long Range Correlations

Figure 3.37 and **Figure 3.36 (a and b)** show long-range correlations from C atoms to H. The location of methylenedioxy functionality and the connectivity between 1,3,4-trisubstituted-phenyl moiety with the 2,4-pentadienoyl unit were confirmed by the long-range correlations from C-3′ and C-4′ to methylenedioxy protons and from C-1′ to H-5 and H-4. The further significant long-range correlations were observed from carbonyl carbon (C-1) to H-2 and H-3, confirming the structure. Thus, the assignments of proton and carbon resonances were in good aggreement with those in published data [73,74].

3.10 Comparison of SPS-LLME With Other Methods For The Extraction of Piperine from Black and White Pepper

A comparison between our study and other different extraction methods used in the literature for the extraction of piperine from black and white pepper is presented in this section. Pressurized-liquid soxhlet extraction (LP-SOX) was used by Katia S. A. et al.[75]. 5 g of dried sample were weighed and 150 mL of three different solvents: ethyl acetate; ethanol; and hexane were applied for 6 h at the solvent boiling point to extract piperine from the samples.

The same authors[75] also used supercritical fluid extraction (SFE) by placing 20 g of grinded black pepper inside the extractor cell for 4 h under the optimum extraction conditions (i.e., pressure 200 bar and temperture 50 ºC).

The same authors[75] used pressurized liquid extraction (PLE) through using ethanol as an extraction solvent for piperine in about 20 g of blended black pepper after being transferred into the extraction vessel. Extraction time was 60 min at 40 ºC.

Conventional extraction method used for the extraction of piperine started with 1.0 g of sample and mixed with 10 mL of 75% methanol as an extraction solvent boiled for 2 h under the water bath reflux [76].

Friedman et al.[70] used solid-liquid extraction (SLE) to extract piperine from 0.1–0.15 g of black pepper powder. 2 mL of 80% ethanol were used as the extraction solvent using sonication for 60 min and centrifugation at 12000 rpm for 10 min at 5 ºC.

Ultrasound-asisted extraction method (LP-UAE) was used at room temperture with 210 mL of extraction solvent that was added to 7 g of grinded pepper. Extraction was done within 45 min[75].

Ionic liquid based ultrasounic-assisted extraction (IL-UAE) was applied for the extraction of piperine using 1.0 g of dried grinded sample mixed with 10 mL of different ionic liquid solutions. This method consumed 30 min as an extraction time [76].

In our proposed method, switchable polarity solvent liquid-liquid microextraction was performed by weighing 0.1 g of black pepper powder using 750 μL extraction solvent within 10 s as an extraction time without the need for centrifuge or any other equipment for the extraction of piperine as sammurized in **Table 3.6**.

Table 3.6: Comparison of SPS-LLME with other methods for the extraction of piperine from black and white pepper.

Extraction method	Extraction time (h)	Sample size (g)	$V_{org.}$ (mL)	Reference
LP-SOX[a]	6	5	150	[75]
SFE[b]	4	20	-	[75]
SLE[c]	2	1	10	[76]
SLE[c]	1.17	0.1–0.15	2	[70]
PLE[d]	1	20	-	[75]
LP-UAE[e]	0.7	7	210	[75]
IL-UAE[f]	0.5	1	10	[76]
SPS-LLME	0.2	0.1	0.75	This study

[a] Low pressure-based soxhlet extraction.
[b] Supercritical fluid extraction.
[c] Solid-liquid extraction
[d] Pressurized liquid extraction.
[e] Low pressure-based ultrasound-assisted extraction.
[f] Ionic liquid-based ultrasonic-assisted extraction.

CHAPTER 4
CONCLUSION AND RECOMMENDATION

The main purpose of this study was to find a simple, easy, fast, efficient and green extraction method that is compatible with HPLC and can be applied to black and white pepper samples of different origins.

The desired property of the extraction method was efficient separation and sample clean-up of the matrix that interfere with the analyte of interest. At the same time pre-concentration was required especially for the analytes that might be present in small amounts in the sample. In addition, minimizing the amount of toxic organic solvent was desired.

A novel liquid-liquid microextraction method based on the use of switchable-polarity solvents was developed and was termed as SPS-LLME. This method was demonstrated to provide such requirements of green analytical sample preparation. SPS-LLME provided several advantages such as powerful sample clean-up, short extraction time, due to complete miscibility of the switchable solvent with the sample solution, and ease of application without the need for organic modifiers, centrifugation or other special equipment.

For the identification, qualitative and quantitative analysis, RP-HPLC was fast, simple, robust, sensitive with only 5 µL injection volume, high peak areas were obtained. Analysis time was fast making it good for routine analysis and efficient.

Dispersive liquid-liquid microextraction was scaled up for the first time for preparative extraction of piperine. This novel approach, when combined with column chromatography, provided high-purity standard of piperine (more than 97%) as proved by NMR analysis using much smaller volume of organic solvents than conventional

liquid-liquid extraction techniques used for its isolation. The collaboration between the Department of Analytical Chemistry and the Department of Pharmacognosy was fruitful and each department complemented one another.

REFERENCES

1. Szallasi A. Piperine: researchers discover new flavor in an ancient spice. *Trends in Pharmacological Sciences.* 2005;26(9):437-439.
2. Gorgani L, Mohammadi M, Najafpour GD, Nikzad M. Sequential Microwave-Ultrasound-Assisted Extraction for Isolation of Piperine from Black Pepper (Piper nigrum L.). *Food and Bioprocess Technology.* 2017;10(12):2199-2207.
3. Knill CJ, Kennedy JF. Handbook of Herbs and Spices. *International Journal of Biological Macromolecules.* 2007;41(4):488-489.
4. Gorgani L, Mohammadi M, Najafpour GD, Nikzad M. Piperine-The Bioactive Compound of Black Pepper: From Isolation to Medicinal Formulations. *Comprehensive Reviews in Food Science and Food Safety.* 2017;16(1):124-140.
5. Zhang J, Wang Y, Pan DD, et al. Effect of black pepper essential oil on the quality of fresh pork during storage. *Meat Science.* 2016;117:130-136.
6. Ferreira SRS, Meireles MAA. Modeling the supercritical fluid extraction of black pepper (Piper nigrum L.) essential oil. *Journal of Food Engineering.* 2002;54(4):263-269.
7. Darshan S, Doreswamy R. Patented antiinflammatory plant drug development from traditional medicine. *Phytotherapy Research.* 2004;18(5):343-357.
8. Bai YF, Xu H. Protective action of piperine against experimental gastric ulcer. *Acta Pharmacologica Sinica.* 2000;21(4):357-359.
9. Yang YC, Lee SG, Lee HK, Kim MK, Lee SH, Lee HS. A piperidine amide extracted from Piper longum L. fruit shows activity against Aedes aegypti mosquito larvae. *Journal of Agricultural and Food Chemistry.* 2002;50(13):3765-3767.
10. Ebihara S, Kohzuki M, Sumi Y, Ebihara T. Sensory Stimulation to Improve Swallowing Reflex and Prevent Aspiration Pneumonia in Elderly Dysphagic People. *Journal of Pharmacological Sciences.* 2011;115(2):99-104.

11. Srinivasan K. Black pepper and its pungent principle-piperine: A review of diverse physiological effects. *Critical Reviews in Food Science and Nutrition.* 2007;47(8):735-748.
12. Dutta S, Bhattacharjee P. Nanoliposomal encapsulates of piperine-rich black pepper extract obtained by enzyme-assisted supercritical carbon dioxide extraction. *Journal of Food Engineering.* 2017;201:49-56.
13. Ma MH, Cantwell FF. Solvent microextraction with simultaneous back-extraction for sample cleanup and preconcentration: Preconcentration into a single microdrop. *Analytical Chemistry.* 1999;71(2):388-393.
14. Cabaleiro N, de la Calle I, Bendicho C, Lavilla I. Current trends in liquid-liquid and solid-liquid extraction for cosmetic analysis: a review. *Analytical Methods.* 2013;5(2):323-340.
15. Pena-Pereira F, Lavilla I, Bendicho C. Liquid-phase microextraction techniques within the framework of green chemistry. *Trac-Trends in Analytical Chemistry.* 2010;29(7):617-628.
16. Smith RM. Before the injection - modern methods of sample preparation for separation techniques. *Journal of Chromatography A.* 2003;1000(1-2):3-27.
17. Beck IC, Bruhn R, Gandrass J, Ruck W. Liquid chromatography-tandem mass spectrometry analysis of estrogenic compounds in coastal surface water of the Baltic Sea. *Journal of Chromatography A.* 2005;1090(1-2):98-106.
18. Matejicek D, Kuban V. High performance liquid chromatography/ion-trap mass spectrometry for separation and simultaneous determination of ethynylestradiol, gestodene, levonorgestrel, cyproterone acetate and desogestrel. *Analytica Chimica Acta.* 2007;588(2):304-315.
19. Rezaee M, Yamini Y, Faraji M. Evolution of dispersive liquid-liquid microextraction method. *Journal of Chromatography A.* 2010;1217(16):2342-2357.
20. Jeannot MA, Cantwell FF. Solvent microextraction into a single drop. *Anal Chem.* 1996;68(13):2236-2240.

21. Pedersen-Bjergaard S, Rasmussen KE. Liquid-liquid-liquid microextraction for sample preparation of biological fluids prior to capillary electrophoresis. *Anal Chem.* 1999;71(14):2650-2656.
22. Xu L, Basheer C, Lee HK. Solvent-bar microextraction of herbicides combined with non-aqueous field-amplified sample injection capillary electrophoresis. *J Chromatogr A.* 2010;1217(39):6036-6043.
23. Rezaee M, Assadi Y, Hosseinia MRM, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid-liquid microextraction. *Journal of Chromatography A.* 2006;1116(1-2):1-9.
24. Asensio-Ramos M, Ravelo-Perez LM, Gonzalez-Curbelo MA, Hernandez-Borges J. Liquid phase microextraction applications in food analysis. *J Chromatogr A.* 2011;1218(42):7415-7437.
25. Galuszka A, Migaszewski Z, Namiesnik J. The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. *Trac-Trends in Analytical Chemistry.* 2013;50:78-84.
26. Zhu SQ, Zhou J, Jia HF, Zhang HX. Liquid-liquid microextraction of synthetic pigments in beverages using a hydrophobic deep eutectic solvent. *Food Chemistry.* 2018;243:351-356.
27. Mansour FR, Danielson ND. Separation methods for captopril in pharmaceuticals and biological fluids. *Journal of Separation Science.* 2012;35(10-11):1213-1226.
28. Khairy MA, Mansour FR. Simultaneous Determination of Ursodeoxycholic Acid and Chenodeoxycholic Acid in Pharmaceutical Dosage Form by HPLC-UV Detection. *Journal of Aoac International.* 2017;100(1):59-64.
29. Bylda C, Thiele R, Kobold U, Volmer DA. Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. *Analyst.* 2014;139(10):2265-2276.
30. Soltani S, Jouyban A. Biological sample preparation: attempts on productivity increasing in bioanalysis. *Bioanalysis.* 2014;6(12):1691-1710.

31. Alshana U, Ertas N, Goger NG. Determination of parabens in human milk and other food samples by capillary electrophoresis after dispersive liquid-liquid microextraction with back-extraction. *Food Chemistry.* 2015;181:1-8.
32. Us MF, Alshana U, Lubbad I, Goger NG, Ertas N. Dispersive liquid-liquid microextraction based on solidification of floating organic drop combined with field-amplified sample injection in capillary electrophoresis for the determination of beta(2)-agonists in bovine urine. *Electrophoresis.* 2013;34(6):854-861.
33. Zhao EC, Shan WL, Jiang SR, Liu Y, Zhou ZQ. Determination of the chloroacetanilide herbicides in waters using single-drop microextraction and gas chromatography. *Microchemical Journal.* 2006;83(2):105-110.
34. Rasmussen KE, Pedersen-Biergaard S. Developments in hollow fibre-based, liquid-phase microextraction. *Trac-Trends in Analytical Chemistry.* 2004;23(1):1-10.
35. Dadfarnia S, Shabani AMH. Recent development in liquid phase microextraction for determination of trace level concentration of metals-A review. *Analytica Chimica Acta.* 2010;658(2):107-119.
36. Sarafraz-Yazdi A, Amiri A. Liquid-phase microextraction. *Trac-Trends in Analytical Chemistry.* 2010;29(1):1-14.
37. Ho TD, Zhang C, Hantao LW, Anderson JL. Ionic Liquids in Analytical Chemistry: Fundamentals, Advances, and Perspectives. *Analytical Chemistry.* 2014;86(1):262-285.
38. Jessop PG, Heldebrant DJ, Li XW, Eckert CA, Liotta CL. Green chemistry - Reversible nonpolar-to-polar solvent. *Nature.* 2005;436(7054):1102-1102.
39. Fu DB, Farag S, Chaouki J, Jessop PG. Extraction of phenols from lignin microwave-pyrolysis oil using a switchable hydrophilicity solvent. *Bioresource Technology.* 2014;154:101-108.
40. Jessop PG, Phan L, Carrier A, Robinson S, Durr CJ, Harjani JR. A solvent having switchable hydrophilicity. *Green Chemistry.* 2010;12(5):809-814.
41. Jessop PG, Kozycz L, Rahami ZG, et al. Tertiary amine solvents having switchable hydrophilicity. *Green Chemistry.* 2011;13(3):619-623.

42. Zhang JF, Misch R, Tan YD, Agar DW. Novel Thermomorphic Biphasic Amine Solvents for CO2 Absorption and Low-Temperature Extractive Regeneration. *Chemical Engineering & Technology.* 2011;34(9):1481-1489.
43. Yilmaz E, Soylak M. Switchable polarity solvent for liquid phase microextraction of Cd(II) as pyrrolidinedithiocarbamate chelates from environmental samples. *Analytica Chimica Acta.* 2015;886:75-82.
44. Jessop PG, Mercer SM, Heldebrant DJ. CO2-triggered switchable solvents, surfactants, and other materials. *Energy & Environmental Science.* 2012;5(6):7240-7253.
45. Vanderveen JR, Durelle J, Jessop PG. Design and evaluation of switchable-hydrophilicity solvents. *Green Chemistry.* 2014;16(3):1187-1197.
46. Holland A, Wechsler D, Patel A, Molloy BM, Boyd AR, Jessop PG. Separation of bitumen from oil sands using a switchable hydrophilicity solvent. *Canadian Journal of Chemistry-Revue Canadienne De Chimie.* 2012;90(10):805-810.
47. Phan L, Brown H, White J, Hodgson A, Jessop PG. Soybean oil extraction and separation using switchable or expanded solvents. *Green Chemistry.* 2009;11(1):53-59.
48. Wang YG, Niu ZS, Shen J, et al. Extraction of direct coal liquefaction residue using dipropylamine as a CO2-triggered switchable solvent. *Fuel Processing Technology.* 2017;159:27-30.
49. Du Y, Schuur B, Samori C, Tagliavini E, Brilman DWF. Secondary amines as switchable solvents for lipid extraction from non-broken microalgae. *Bioresource Technology.* 2013;149:253-260.
50. Samori C, Torri C, Samori G, et al. Extraction of hydrocarbons from microalga Botryococcus braunii with switchable solvents. *Bioresource Technology.* 2010;101(9):3274-3279.
51. Arain MS, Kazi TG, Afridi HI, Khan N, Ali J. A innovative switchable polarity solvent, based on 1,8-diazabicyclo- 5.4.0 - undec-7-ene and decanol was prepared for enrichment of aluminum in biological sample prior to analysis by

flame atomic absorption spectrometry. *Applied Organometallic Chemistry.* 2018;32(3).

52. Vessally E, Ghorbani-Kalhor E, Hosseinzadeh-Khanmiri R, et al. Application of switchable solvent-based liquid phase microextraction for preconcentration and trace detection of cadmium ions in baby food samples. *Journal of the Iranian Chemical Society.* 2018;15(2):491-498.
53. Soylak M, Khan M, Yilmaz E. Switchable solvent based liquid phase microextraction of uranium in environmental samples: a green approach. *Analytical Methods.* 2016;8(5):979-986.
54. Naeemullah, Shah F, Kazi TG, et al. Switchable dispersive liquid-liquid microextraction for lead enrichment: a green alternative to classical extraction techniques. *Analytical Methods.* 2016;8(4):904-911.
55. Khan M, Soylak M. Switchable solvent based liquid phase microextraction of mercury from environmental samples: a green aspect. *Rsc Advances.* 2016;6(30):24968-24975.
56. Yilmaz E, Soylak M. Switchable solvent-based liquid phase microextraction of copper(II): optimization and application to environmental samples. *Journal of Analytical Atomic Spectrometry.* 2015;30(7):1629-1635.
57. Zhang ML, Qiu HD. Progress in stationary phases modified with carbonaceous nanomaterials for high-performance liquid chromatography. *Trac-Trends in Analytical Chemistry.* 2015;65:107-121.
58. Davies NH, Euerby MR, McCalley DV. Analysis of basic compounds by reversed-phase high-performance liquid chromatography using hybrid inorganic/organic phases at high pH. *Journal of Chromatography A.* 2008;1178(1-2):71-78.
59. Sander LC, Wise SA. Recent advances in bonded phases for liquid-chromatography. *Crc Critical Reviews in Analytical Chemistry.* 1987;18(4):299-415.
60. Guiochon G. Monolithic columns in high-performance liquid chromatography. *Journal of Chromatography A.* 2007;1168(1-2):101-168.

61. Skoog DA, Holler FJ, Crouch SR. *Principles of Instrumental Analysis.* 6th Edition ed: Brooks/Cole Publishing Co.; 2007.
62. Weston A, Brown PR. *HPLC and CE Principles and Practice.* CA, USA: Academic Press; 1997.
63. Mlynarik V. Introduction to nuclear magnetic resonance. *Analytical Biochemistry.* 2017;529:4-9.
64. Schweiger S, Hinterberger S, Jungbauer A. Column-to-column packing variation of disposable pre-packed columns for protein chromatography. *Journal of Chromatography A.* 2017;1527:70-79.
65. Lupina T, Cripps H. UV Spectrophotometric determination of piperine in pepper preparations - collaborative study. *Journal of the Association of Official Analytical Chemists.* 1987;70(1):112-113.
66. Musenga A, Mandrioli R, Ferranti A, D'Orazio G, Fanali S, Raggi MA. Analysis of aromatic and terpenic constituents of pepper extracts by capillary electrochromatography. *Journal of Separation Science.* 2007;30(4):612-619.
67. Catchpole OJ, Grey JB, Perry NB, Burgess EJ, Redmond WA, Porter NG. Extraction of chill, black pepper, and ginger with near-critical CO2, propane, and dimethyl ether: Analysis of the extracts by quantitative nuclear magnetic resonance. *Journal of Agricultural and Food Chemistry.* 2003;51(17):4853-4860.
68. Verzele M, Vandamme F, Schuddinck G, Vyncke P. Quantitative microscale liquid-chromatography of piperine in pepper and pepper extracts. *Journal of Chromatography.* 1989;471:335-346.
69. Scott IM, Puniani E, Jensen H, et al. Analysis of Piperaceae germplasm by HPLC and LCMS: A method for isolating and identifying unsaturated amides from Piper spp extracts. *Journal of Agricultural and Food Chemistry.* 2005;53(6):1907-1913.
70. Friedman M, Levin CE, Lee SU, Lee JS, Ohnisi-Kameyama M, Kozukue N. Analysis by HPLC and LC/MS of pungent piperamides in commercial black, white, green, and red whole and ground peppercorns. *Journal of Agricultural and Food Chemistry.* 2008;56(9):3028-3036.

71. Kozukue N, Park MS, Choi SH, et al. Kinetics of light-induced cis-trans isomerization of four piperines and their levels in ground black peppers as determined by HPLC and LC/MS. *Journal of Agricultural and Food Chemistry.* 2007;55(17):7131-7139.
72. Rathnawathie M, Buckle KA. Determination of piperine in pepper (piper-nigrum) using high-performance liquid-chromatography. *Journal of Chromatography.* 1983;264(2):316-320.
73. Islam MS, Roy AC, Haque MM, Hossain MS, Abu Sayeed M. Phytochemical studies on piper chaba hunter. *International Journal of Pharmaceutical Sciences and Research.* 2015;6(6):2525-2531.
74. Ternes W, Krause EL. Characterization and determination of piperine and piperine isomers in eggs. *Analytical and Bioanalytical Chemistry.* 2002;374(1):155-160.
75. Andrade KS, Trivellin G, Ferreira SRS. Piperine-rich extracts obtained by high pressure methods. *Journal of Supercritical Fluids.* 2017;128:370-377.
76. Cao XJ, Ye XM, Lu YB, Yu Y, Mo WM. Ionic liquid-based ultrasonic-assisted extraction of piperine from white pepper. *Analytica Chimica Acta.* 2009;640(1-2):47-51.