T.R.N.C.

NEAR EAST UNIVERSITY

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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 **:** JUDE JOSHUA CALEB

Signature **:**

Date **:** 05 June 2017

ACKNOWLEDGEMENTS

A study of this caliber can never be successful with my effort alone, so I want to take this time to appreciate everybody that has helped me to complete this challenging, yet fruitful journey.

Let me begin by thanking my supervisor in person of Assist. Prof. Dr. Usama Alshana who was a part and a parcel of every step in this research, being patient with me despite my shortcomings and developing my confidence by giving me the opportunity to express myself.

I would also like to thank my co-supervisor Prof. Dr. İhsan Çalış who despite his overwhelming schedule always responded promptly to my needs without hesitation; I am amazed by his humility.

I am grateful to my lecturers Assoc. Prof. Dr. Hayati Çelik and Assist. Prof. Dr. Banu Keşanlı who were helpful in getting me some journal articles and encouraging me.

I am grateful to the Jury members for agreeing to read and contribute to this thesis through their valuable comments despite their busy agenda.

I also want to thank Azmi Hanoğlu, Duygu Yiğit Hanoğlu and Fehmi Burak Alkaş for the assistance they rendered me in the laboratory.

I would also like to thank Prof. Dr. Ali Hakan Göker from the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ankara University, for analyzing the isolated capsaicinoid standards by MS and NMR.

Without the scholarship from my State Government, Kaduna State, I would not have had the opportunity to pursue a Master degree in the first place, so I will forever remain grateful.

Several people have also helped me indirectly. Though they were not in the battlefield with me, their contribution through various forms was significant. This goes to my family back in Nigeria; My mother Mrs. Dinatu Caleb who is always praying for me, my siblings; Patience, Cephas and Halita who are always concerned with my progress, and my best friend Huldah Goje.

Not forgetting my housemates for their moral support; Solomon, Fidelis, Jerry, Deacon Ezekiel and my colleague and constant companion in the laboratory Manasseh and FIF Cyprus led by Pst. Hillary Mwale.

Finally, I give glory to God Almighty for giving me the strength and ability to complete this study.

In memory of my late father Joshua Caleb who taught me how to be a man not in an obvious way, and trusted me to carry on by passing away on my 18th birthday

ABSTRACT

Caleb, J. J. Dispersive liquid-liquid microextraction of some capsaicinoids from different cultivars of *Capsicum annuum* prior to their determination by HPLC. Near East University, Institute of Health Sciences, Analytical Chemistry Program, Master of Science Thesis, Nicosia, 2017.

Dispersive liquid-liquid microextraction (DLLME) was used prior to high-performance liquid chromatography (HPLC) for the extraction of three major capsaicinoids in pepper (i.e., capsaicin, dihydrocapsaicin and nordihydrocapsaicin). Optimum extraction conditions were: 100 μL chloroform (extraction solvent), 1.25 mL acetonitrile (disperser solvent) and 30 s extraction time. The analytes were back-extracted into 300 μL of 50 mM sodium hydroxide in methanol 45/55% (v/v) solution within 15 s for injection into HPLC. A reversed-phase column (Agilent Zorbax SB-Aq, 4.6 x 150 mm, 5 μm) was used for separating the analytes using a mobile phase consisting of 55/45% (v/v) methanol/0.5% (v/v) acetic acid at 25 ^{0}C and a flow rate of 1.2 mL/min, an injection volume of 5 μL. The analytes were monitored using a diode array detector (DAD) at 280 nm. Average enrichment factors were in the range of 4.4 to 10.2 and limits of detection ranged from 8.7 to 18.5 mg/kg. Calibration graphs showed good linearity with coefficient of determination (R^2) higher than 0.9930 and relative standard deviation (%RSD) lower than 6.9 and 7.8% for intraday and interday precision, respectively. Standards of the three capsaicinoids were isolated using reversed-phase medium pressure liquid chromatography (MPLC) and were characterized by LC-MS and 1D-(^{1}H- and ^{13}C-NMR) and 2D-NMR (COSY, HSQC and HMBC). DLLME-HPLC was applied to six capsicum samples with an average recovery of 48.7%. The proposed method was proven to be simple, rapid and efficient for the isolation and preconcentration of capsaicinoids from different cultivars of *Capsicum annuum*.

Keywords: Capsaicin, Capsaicinoid, Dihydrocapsaicin, Dispersive liquid-liquid microextraction, HPLC, Nordihydrocapsaicin.

ÖZET

Caleb, M. T. Kapsaisinoidlerin HPLC ile tayini öncesi farklı *Capsicum annuum* kültürlerinden dispersif sıvı-sıvı mikroekstraksiyonu. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Analitik Kimya Programı, Yüksek Lisans Tezi, Lefkoşa, 2017.

Kapsikum numunelerinden majör kapsaisinoidlerin (kapsaisin, dihidrokapsaisin, nordihidrokapsaisin) yüksek performanslı sıvı kromatografisi (HPLC) ile tayin öncesi estraksiyonu için dispersif svı-sıvı mikroekstraksiyon (DLLME) yöntemi kullanıldı. Optimum ekstraksiyon koşulları aşağıdaki gibidir: 100 µL kloroform (ekstraksiyon çözücüsü), 1.25 mL asetonitril (dispersiyon çözücüsü) ve ekstraksiyon süresi 30 s. Analitler 300 µL hacminde metanol: sodium hidroksit (50 mM) 55:45 (h/h) karışımı ile 15 s süreyle geri ekstrakte edilip doğrudan HPLC'ye enjekte edildi. Analitlerin ayrılmasında ters faz kromatografi kolonu (Agilent Zorbax SB-Aq, 4.6 × 150 mm, 5 µm) ve metanol: asetik asit [%0,5 (h/h)], 55:45 (h/h) içeren hareketli faz, 1,2 mL d^{-1} akış hızı, 5 µL enjeksiyon hacmi ve 25°C'de çalışıldı. Analitler diyot serili dedektör ile 280 nm dalga boyunda izlendi. Zengileştirme faktörleri 3,3-20,8, teşhis sınırları (LOD) 8,7-18,5 mg kg^{-1} arasındadır. Kalibrasyon grafikleri, determinasyon katsayıları (R^2) 0.9930'den büyük olacak şekilde doğrusallık göstermektedir. Gün içi ve günler arası kesinlik bağıl standart sapma cinsinden (%RSD) sırasıyla 6,9 ve 7,8'den küçüktür. Üç kapsaisinoidin standartları yeşil biber turşusundan ters-faz orta basınçlı sıvı kromatografisi (MPLC) ile izole edilerek LC-MS, 1D- (^{1}H ve ^{13}C NMR) ve 2D-NMR (COSY, HSQC ve HMBC) ile karakterize edildi. Önerilen DLLME-HPLC yöntemi altı farklı biber çeşidine uygulanarak kapsaisinoidlerin kapsikum matriksinden arındırılması, izolasyonu ve zenginleştirilmesi için basit, hızlı ve etkili bir yöntem olduğu kanıtlandı.

Anahtar sözcükler: Dihidrokapsaisin, Dispersif sıvı-sıvı mikroekstrakiyon, HPLC, Kapsaisin, Kapsaisinoid, Nordihidrokapsaisin.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ACN	Acetonitrile
BE	Back extraction
BES	Back-extraction solution
BRP	Big red pepper
CAP	Capsaicin
CF	Chloroform
COSY	Homonuclear correlation spectroscopy
DAD	Diode array detector
DCM	Dichloromethane
DHC	Dihydrocapsaicin
DLLME	Dispersive liquid-liquid microextraction
EF	Enrichment Factor
ESI	Electrospray ionization
ETD	Evaporation to dryness
FR	Flow rate
FT-NMR	Furrier Transform- Nuclear Magnetic Resonance
GC	Gas chromatography
GPP	Green pepper pickle
HMBC	Heteronuclear multiple bond correlation
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear multiple quantum correlation
IL	Ionic liquid
IR	Infrared
LC-MS	Liquid chromatography-mass spectrometry
LGP	Light green pepper
LRP	Long red pepper
MPLC	Medium-pressure liquid chromatography
NDHC	Nordihydrocapsaicin
n-Hex	n-Hexane

NP	Normal phase
RF	Radio frequency
RP	Reversed-phase
SGC	Small green chili
SOE	Salting-out extraction
TL	Toluene
TMS	Tetramethylsilane
TRNC	Turkish Republic of Northern Cyprus
UV	Ultra violet
YP	Yellow pepper

CHAPTER 1

INTRODUCTION

1.1 Capsaicinoids

Peppers originated from the Americas and are popularly known for spicy and pungent taste that is caused by a group of compounds known as capsaicinoids from the genus capsicum, making them a popular spice in food around the world [1]. Although over twenty capsaicinoids have been found in various species of pepper [2], there are two major capsaicinoids responsible for up to 90% of the pungency of pepper, which are capsaicin (CAP) and dihydrocapsaicin (DHC) [3]. Other compounds include nordihydrocapsaicin (NDHC), homodihydrocapsaicin, and homocapsaicin, etc. [4]. Genetic and environmental factors such as specie, agro-climatic conditions, cultivator and ripening stage of the fruit has been reported to influence capsaicinoid accumulation and pungency of peppers [5]. The chemical structures of the most common capsaicinoids are given in **Figure 1.1**.

As it can be seen from **Figure 1.1**, the chemical structures of capsaicinoids are very similar and they differ only in double bond or length of the aliphatic chain.

1.1.1 Applications of Capsaicinoids

Capsaicinoids are mostly used in the food industry either as coloring or flavoring agents [6]. Aside from being used as a spice, capsaicinoids find a wide range of application in the pharmaceutical industry especially capsaicin. Studies on anticancer and antitumor revealed that capsaicin can suppress carcinogenesis in the breast, prostrate, colon, lungs and human bladder [7]. Capsaicin is used for topical application in analgesic therapy for some neuropathic and osteoarthritic pain states [8]. Recently, capsaicin is used for clinical purpose in topical creams and gels to relieve intractable neuropathic pain, uremic pruritus, and

rheumatoid arthritis. Capsaicin has also been proven valuable in non-allergic (vasomotor) rhinitis, migraine, cluster headache, herpes zoster, and bladder over activity [9]. Capsaicin has also shown great promise in the control of obesity. Epidemiological studies gave evidence associates consumption of capsaicinoid-containing foods and lowering obesity, this is due to the widely accepted notion that increasing energy expenditure and reducing energy intake form the basis for weight management. Consumption of a right dosage of capsaicin an hour before low intensity exercise improves lipolysis, which might be a valuable supplement of treating people with hyperlipidemia and obesity [10]. Capsaicinoids have also been reported to possess antimicrobial effect against disease-causing bacterial and aquatic microorganisms that coat submerged surfaces of ships [11].

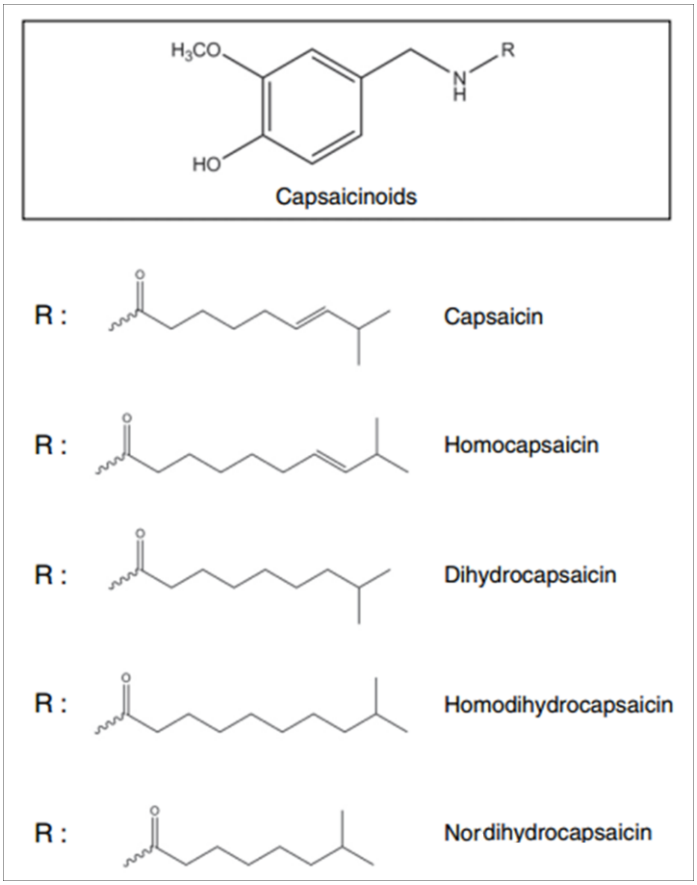


Figure 1.1: Chemical structure of some major capsaicinoids [4].

1.1.2 Side Effects of Capsaicinoids

Even with the important pharmacological and clinical uses of capsaicin, a major draw-back in its application is when high doses (above 100 mg capsaicin per body weight) are administered for a long period of time, this might cause peptic ulcers, increases the chances of developing liver, duodenal, stomach and prostate cancer together with the enhancement of breast cancer metastasis [12].

1.1.3 Literature Review on Capsaicinoids

Capsaicin was isolated for the first time in 1816 by P.A. Bucholz [13]. In commercial capsicums, capsaicin generally comprises 33-59%, dihydrocapsaicin accounts for 30-51%, nordihydrocapsaicin is 7-15%, and the remainder is less than 5% of the capsaicinoids [14].

The content of capsaicin in pepper is one of the major parameters that determine its commercial quality [6]. Several methods have been reported in the literature for extracting capsaicinoids with different solvent systems, temperature and extraction time. Capsaicinoids are relatively hydrophobic making the use of relatively non-polar organic solvents necessary for their successful extraction. A study was conducted comparing three solvents [i.e., methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN)] for the extraction of capsaicinoids from Naga king chili, which is believed to be the world's hottest pepper. It was discovered that MeOH provided the highest recovery of capsaicinoids followed by EtOH and finally ACN. The optimum extraction time was 5 h [15]. Another study made use of EtOH as the extracting solvent, achieving an extraction time of approximately 50 min [1].

The need for high throughput and reduced cost of analysis has placed a high demand for high speed and low cost of analysis in areas where high-performance liquid chromatography (HPLC) is applied for pharmaceutical and food analysis [16]. It is challenging to achieve rapid and high efficient separation due to the complexity of the sample matrix [17].

The rapidity and reliability of HPLC has made it the method of choice for analyzing capsaicinoids. HPLC methods with ultraviolet (UV) [18], fluorescence [19] and electrochemical [20] detectors have been assayed for determining capsaicinoids. Mass spectrometry (MS) detectors have also been widely used [21, 22].

Argentation solid-phase microextraction was applied for the purification of commercial capsaicin and dihydrocapsaicin standards, achieving high extraction purity of 99.6% and 96% for capsaicin and dihydrocapsaicin, respectively [23]. Pressurized-liquid extraction has also been reported for the extraction of capsaicinoids from pepper [24]. The main advantage of this method is that it reduces the amount of organic solvents that need to be used for analysis compared to traditional methods of extraction. Ultrasound-assisted extraction of capsaicin from pepper was shown to reduce the quantity of organic solvent, time and temperature [25].

1.2 Dispersive Liquid-Liquid Microextraction

Even with the exponential growth in analytical techniques for the past few decades due to the design and application of sophisticated techniques such as chromatography, spectroscopy, electrochemistry and microscopy, the state of the current instrumentation is still not enough to get all information from a sample directly without some sample pre-treatment steps, known as sample preparation. In an analytical procedure, sample preparation involves an extraction process with the aim of isolation and enrichment of analyte from the sample matrix [26].

The drawbacks of conventional sample preparation methods are well known and documented in the literature. Some worth mentioning are the tedious and large consumption of toxic organic solvents involved in liquid-liquid extraction (LLE), which are harmful to the researcher, living organisms and to the environment. Solid-phase extraction (SPE) uses less volume of organic solvents but is still considered significant. In addition, SPE cartridges are expensive and disposable, generating waste which is harmful to the environment [27].

Recently, the focus is shifted towards the development of efficient, economic and miniaturized sample preparation techniques. Assadi and his team in 2006 developed a novel liquid-phase microextraction (LPME) technique, which they called dispersive liquid-liquid microextraction (DLLME) [26]. This new technique has since then gained a wide acceptance, recognition and popularity among analytical chemists and in other fields due to its high rapidity of extraction, simplicity, environmental friendliness, high extraction efficiency and affordability [28].

DLLME consists of a ternary solvent system; namely, a disperser solvent, an extraction solvent and an aqueous sample. The extraction and the disperser solvents are rapidly injected into an aqueous sample in a conical test tube to form a cloudy solution containing micro droplets of extraction solvent, which are dispersed fully in the aqueous solution. Equilibrium is achieved instantaneously due to the infinitely large surface area of contact between the acceptor and the donor phase making extraction time to be very fast which is one of the major advantages of this method. A centrifugation step is necessary to collect the extraction phase at the bottom of the conical tube. The choice of conical tube is for easy collection of the extraction phase [29].

The choice of extraction solvent is based on the ability of the solvent to extract the analyte from the sample matrix and immiscibility with the aqueous phase, while the disperser solvent has to be miscible with both the extraction solvent and the aqueous solution [27]. The extraction solvent can be denser than water such as chlorinated solvents which include chloroform and dichloromethane, tetrachloromethane or less dense than water such as 1-undecanol, 1-dodecanol, 2-dodecanol, hexadecane in which case solidification of the floating organic drop can be applied for these solvents which solidify at room temperature [30].

For lower density solvents that do not solidify at room temperature, special devices can be used for collecting the extraction solvent at the top of the aqueous sample, low density based solvent de-emulsification, adjustment of the solvent's mixture density and sequential injection-DLLME [31]. Some of these methods also eliminate the need of the centrifugation

step that is considered the most time consuming step of this method [32]. The disperser solvent is selected on the bases of miscibility in the extraction solvent and aqueous sample. Common disperser solvents used include acetonitrile, acetone, methanol and ethanol [29].

Gas Chromatography (GC) was the first instrument to be used for DLLME [26] in which case the extract could be injected directly into the instrument due to the compatibility of the organic extraction solvent with the instrument. Other instruments such as capillary electrophoresis (CE) [33] and atomic absorption spectrometry (AAS) [34] were also reported in the literature. HPLC is now the most widely used instrument for DLLME [29].

Recent advances in DLLME are geared towards the use of less toxic solvents due to the high toxicity of chlorinated solvents [27]. Ionic liquids are considered as "green solvents" capable of replacing toxic organic solvents used in DLLME. They are a group of non-molecular organic salts with meting point below 100 $^{\circ}$C which causes them to remain in the liquid form at room temperature, hence the name room-temperature ionic liquid (RTIL) [35]. A review by Trujillo-Rodríguez et al. [36] gives a detailed explanation of the various modes of ionic liquid-based dispersive liquid-liquid microextraction (IL-DLLME). The use of nanoparticles for enhancement of DLLME is a recent development in which the unique characteristics of nanoparticles such as increased surface area, optical, electrical, magnetic, catalytic properties and their ability to retain different functional groups to their surface have made them applicable in solid-liquid sorption processes applicable to DLLME [37].

1.3 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) belongs to a class of liquid chromatography techniques in which the mobile phase is a liquid. The development of HPLC came as a result of the need to provide more efficient separation that would be achieved by using more refined packing material in a reduced analysis time. This would be achieved by delivering the mobile phase by pump which would cause high pressure that only a special instrument can withstand, hence the birth of HPLC [38].

The principle of liquid chromatography can be divided into four parts; namely, partition chromatography, adsorption chromatography, ion-exchange chromatography and size-exclusion chromatography.

Partition chromatography is the most widely used among these techniques. It can be further subdivided based on the polarity of the mobile phase being used. For polar mobile phase such as ACN, MeOH, and tetrahydrofuran (THF) eluted on a low polarity stationary phase such as octadecyl (C-18) group-bonded silica gel (ODS), the mode of partition chromatography is known as reversed-phase (RP), while for non-polar mobile phase such as n-hexane (n-Hex) and chloroform eluted on a polar stationary phase like silica gel, the mode is known as normal-phase (NP) because it was the first principle that was applied for chromatographic separation.

RP-HPLC has become the most widely used mode of partition chromatography for different kinds of analytes, and high efficiency of separation because of the use of relatively less toxic mobile phases than in NP.

The partition coefficient (P) is an important parameter that determines the mode of partition chromatography applied for the separation of a given analyte in a sample matrix. It is defined as the ratio of the concentration of the solute between two immiscible solvents. The logarithm of this ratio is known as $logP$ as defined in **Equation (1.1)**. A common biphasic system of n-octanol and water is generally used for such calculation.

$$\log P_{O/W} = log \frac{[analyte]_{octanol}}{[analyte]_{water}} \quad \textbf{(1.1)}$$

The implication of $logP$ is that an analyte with a low $logP$ value is considered polar, while an analyte with a high $logP$ value is non-polar.

1.3.1 Modes of Elution in HPLC

There are two modes of elution in HPLC known as isocratic elution done by delivering a mobile phase with constant composition during analysis and gradient elution, which is done by varying the composition of the mobile phase during analysis.

The isocratic elution is the simpler mode of elution and the most preferred one because it is available in all HPLC instruments unlike the gradient elution which requires a specialized instrument and it is easier to understand the impact of factors affecting the separation, but the advantage of the gradient elution over isocratic elution is that it can frequently solve the "general elution problem" in chromatography which is poor resolution and long analysis time. Gradient elution can also be used to improve the resolution between the peaks and shorten analysis time and can be more powerful in separating structurally very closely related substances.

To determine the mode of separation that is suitable for a given set of analytes, preliminary test using "gradient scanning" can be carried out accompanied by some calculations to determine if isocratic elution is possible and what composition of the mobile phase would be required for the isocratic elution. **Figure 1.2** is a graphical illustration and an equation used to determine the elution mode suitable for a given analyte.

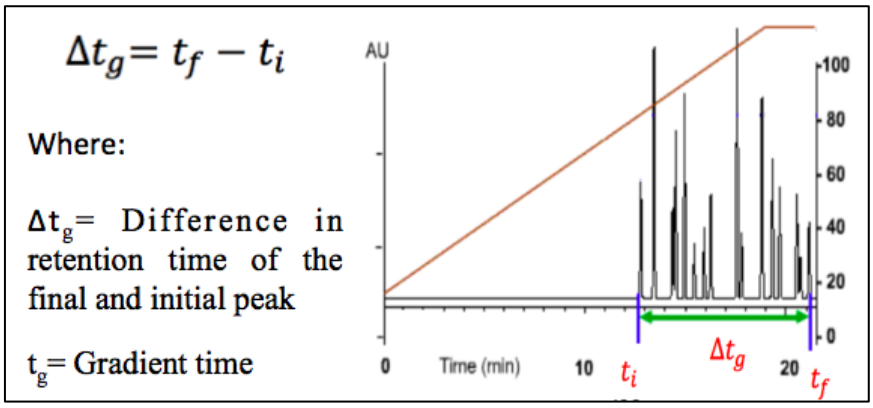


Figure 1.2: Equations and graphical illustration of determining the suitable mode of elution.

After obtaining the values from the chromatogram, the final decision is made based on estimations as given in **Figure 1.3**. If isocratic elution is possible, deciding on the composition of the mobile phase is based on dividing Δt_g by 2 and extrapolating the composition of the mobile phase corresponding to the retention at that point.

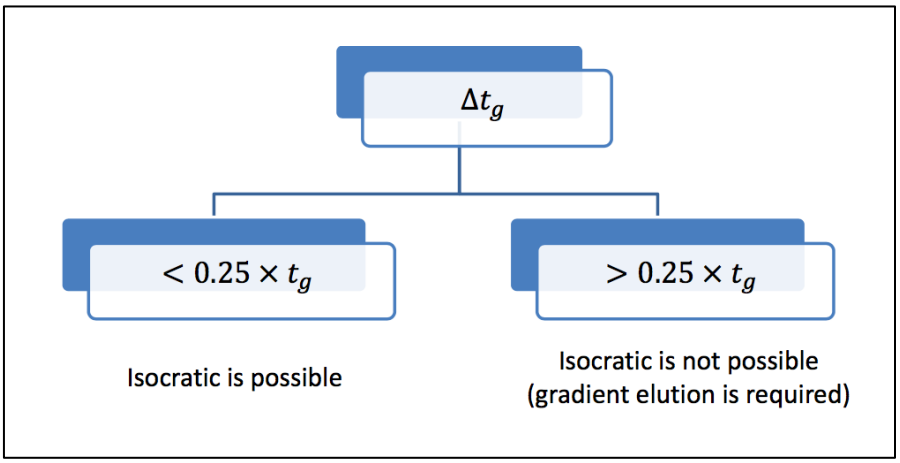


Figure 1.3: Deciding on the elution mode.

1.3.2 Optimization of HPLC Conditions

For Optimization of the HPLC condition, a systematic approach is always recommended. The opposite of the systematic approach is the "Random walk" which is performing experiment in a "random" or in an uncoordinated way. Even though acceptable separation might be achieved by using the "random walk", understanding of the interaction between parameters might not be possible and insight about the sensitivity of the modification of conditions (robustness) might not be feasible leading to higher number of experiments than required.

1.3.3 Equations Describing the Factors Affecting Resolution

The factors affecting the resolution of a chromatogram are taken into consideration in a systematic way. These factors include the retention (or capacity) factor (k'), number of

theoretical plate (efficiency) (N), selectivity factor ($\propto$) and resolution (R_s). Mathematical equations describing these terms are given in **Table 1.1**.

Table 1.1: Equations describing separation in chromatography.

Equation	Term	Meaning	Equation
$k' = \frac{t_R - t_M}{t_M}$	k' t_R t_M	Retention (capacity) factor Retention time Dead time	**(1.2)**
$N = 16\left(\frac{t_R}{W}\right)^2$	N W	Number of theoretical plate (efficiency) Peak width	**(1.3)**
$\propto = \frac{k'_B}{k'_A} = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$	$\propto$	Selectivity factor	**(1.4)**
$R_s = \frac{\sqrt{N_{av}}}{4} \times \frac{k'_{av}}{k'_{av}+1} \times \frac{\propto -1}{\propto}$	R_s N_{av} k'_{av}	Resolution Average N of two adjacent peaks Average k' of two adjacent peaks	**(1.5)**

1.3.4 Changing k'

This parameter can be improved by changing the mobile phase composition (e.g., MeOH/H_2O, 70/30 to 50/50, v/v), the mobile phase pH (e.g., 2.0-9.0), temperature of the column (e.g., 8-60 $^{\circ}$C), by adding a buffer or changing the concentration of that buffer (e.g., 10.0-50.0 mM).

1.3.5 Changing α

This parameter can be improved by changing the column type (e.g., reversed phase, normal phase, ion exchange, etc.), or the mobile phase identity (e.g., THF/H_2O, MeOH/H_2O, ACN/H_2O etc.).

1.3.6 Changing *N*

This parameter can be improved by changing the column length (e.g., 40-200 mm), using a column with a different internal diameter (i.d.) (e.g., 3.2, 4.6, 5.4 mm, etc.), or particle size (e.g., 2.0-10.0 μm), or by changing the flow rate (e.g., 0.5-2 $mL\ min^{-1}$).

1.3.7 Effect of k', α and N on Resolution

As can be seen from **Figure 1.4,** increasing both α and N will drastically improve R_s, while increasing k' up to 10 will increase R_s after which there will be no significant effect.

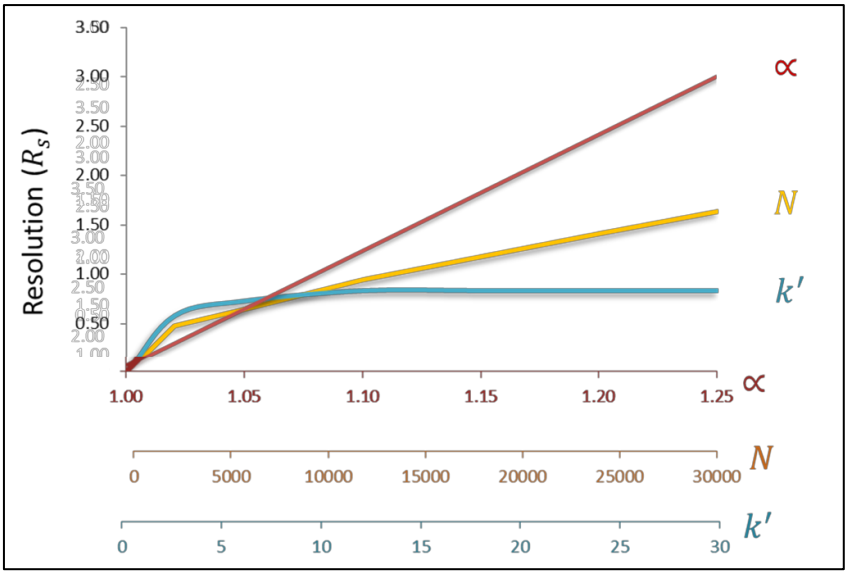


Figure 1.4: Effect of k', α and N on resolution.

A successful use of HPLC for separating the target analytes depends on the choice of the right combination of operating conditions: the type of column packing, column dimensions, particle size, flow rate of the mobile phase, the mobile phase composition and identity, pH of the mobile phase, concentration of the buffer used for adjusting the pH, type and concentration of the mobile phase modifier and column temperature. Therefore, in order to minimize the number of experiments, a good understanding of the various factors that

control HPLC separations is required. 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 (1.5) is a fundamental relationship in liquid chromatography, which allows a chromatographer to control resolution (R_s) by varying k'_{av}, N and $\propto$. The three terms of the equation (i.e., $\frac{\sqrt{N_{av}}}{4}$, $\frac{k'_{av}}{k'_{av}+1}$ and $\frac{\propto-1}{\propto}$) are essentially independent, so that one term can be optimized first then another. Separation efficiency as measured by N can be varied by changing the column length or mobile phase flow rate. k'_{av} can be varied by changing the solvent strength, the ability of the mobile phase to provide large or small k'_{av} values. Separation selectivity as measured by $\propto$ can mainly be varied by changing the identity of the mobile and/or the stationary phase.

Each of these three terms can be varied to improve resolution (R_s). After an initial separation is carried out, the chromatogram is evaluated. If R_s is poor and k'_{av} is small, it should be first increased to fit into the optimum range of $2 \leq k'_{av} \leq 10$. No other change in separations would give as large an increase in R_s for as little effort. When k'_{av} is already within the optimum range, and resolution is still marginal, the best solution is usually an increase in N.

Normally, this means an increase in separation time. However, the necessary change in experimental conditions is easily predicted, and little effort would be spent to achieve the

required increase in N and R_s. If k'_{av} is within the optimum range but with a very small resolution between the two adjacent peaks, here, the necessary increase in N would probably require a very long separation time, and it might even be impossible to achieve (e.g. when $\propto = 1$). In this case, what is needed is an increase in $\propto$.

An increase in $\propto$ results in a displacement of one band center, relative to the other, and an increase in R_s. The time of separation and the heights of the two bands are not much changed for moderate changes in $\propto$. However, predicting the right conditions for the necessary change in $\propto$ is seldom a straightforward procedure, and it often involves much effort. Thus, an increase in $\propto$ can provide the shortest possible separation times, but the effort required to discover the right experimental conditions may represent a greater investment than one would care to make. Therefore, a change in $\propto$ may well be preferable when a large number of such separations are involved. Adding to this is the fact that as N is increased, so is the analysis time; band heights rapidly decrease, which is not favorable for later quantitative analysis. **Figure 1.5** summarizes a systematic approach toward separation of target analytes in HPLC.

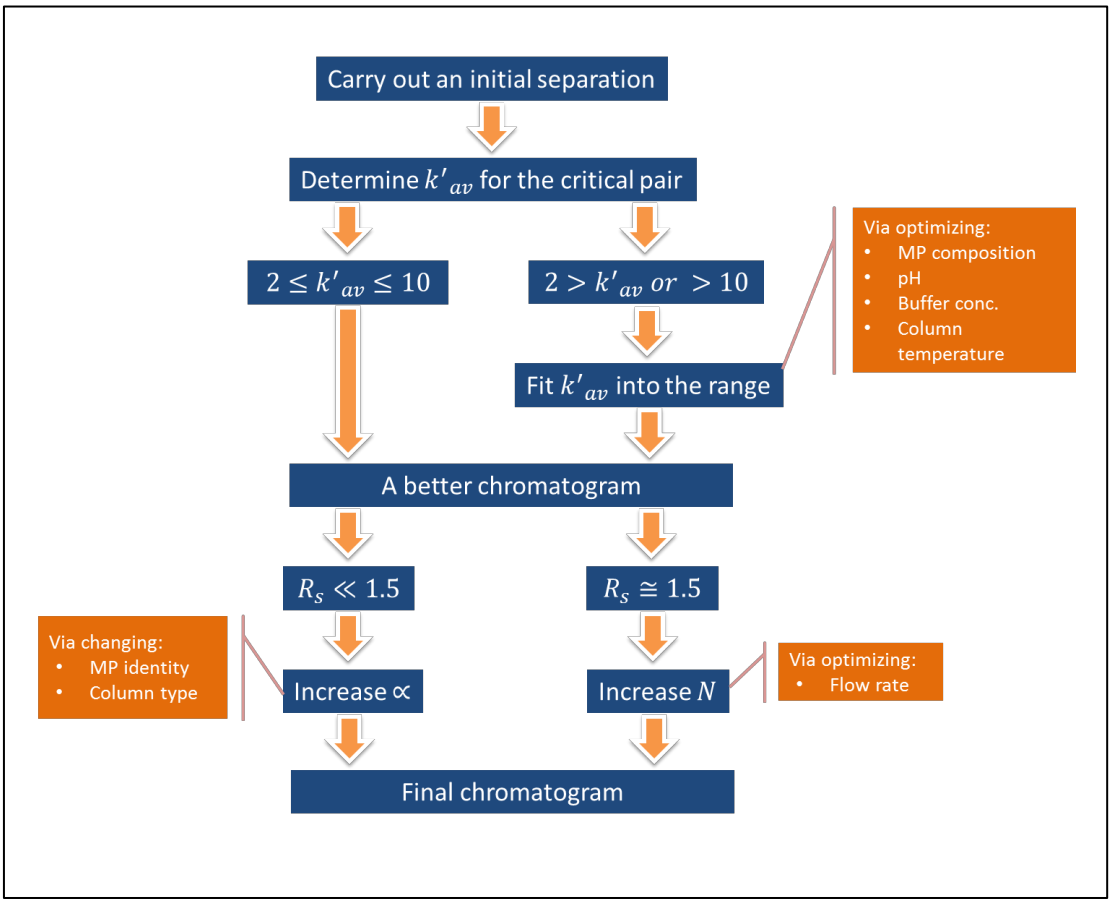


Figure 1.5: Systematic approach to HPLC optimization.

1.4 Medium-Pressure Liquid Chromatography (MPLC)

Medium-pressure liquid chromatography (MPLC) is a technique that is applied for preparative chromatography mainly in pharmaceutical, food and chemical industries [39]. This technique relies on the use of longer columns with larger internal diameters that can be easily filled and refilled and requires higher pressure compared to low-pressure liquid chromatography to be able to sustain high flow rates. MPLC is generally equipped with a compressed air simple pumping set-up or a reciprocating pump to be able to fulfill the requirement for a simple complementary or supplementary method to open-column chromatography with the advantage of higher resolution and shorter separation time.

1.4.1 Factors Affecting Efficiency of Separation in MPLC

There are several factors that affect the efficiency of separation in MPLC such as pulse damping, column dimension, sample introduction, column filling and sample size. In the case of column filling, the analyst is responsible for filling the column with particle size ranging from 25 to 200 μm. The mode of packing is a very essential requirement for a good separation; the modes are slurry packing and dry packing using vacuum and nitrogen over-pressure. In the case of column dimension, it was observed by experiment that with long column and small internal diameter better resolution was obtained than with shorter columns with larger internal diameter, both containing the same stationary phase.

1.4.2 Determination of the Solvent System for MPLC

Determination of the solvent system can be done efficiently using HPLC and transferring the method to MPLC. Thin-layer chromatography (TLC) can also be used as a tool to find the optimal MPLC conditions. The drawback of using TLC is that it is a non-equilibrated system, so transfer of the method directly to MPLC can be challenging and often requires an intermediate step.

1.4.3 Columns Used in MPLC and Sample Injection

The inner core of the chromatographic column is made up of transparent glass protected by a plastic protective coating. Separation can sometimes be visualized with the eye. The column size can range from 130 to 1880 mL. Coupling of the column can be done to increase the resolving power. A Teflon ring is used to seal the joint between the columns. Sample injection can be done directly into the column by means of a septum or through a sample loop. Direct injection is preferred in many cases because the sample can be lost inside the sample loop especially when working with very small sample volumes and because the purpose is preparative, making every volume of the sample significant.

1.5 Mass Spectrometry (MS)

This is probably one of the most informative analytical tools available owing to the wide range of data that can be derived from this instrument. Some applications include:

- Elemental analysis of samples,
- Determination of the structures of inorganic, organic and biological samples,
- Determination of qualitative and quantitative composition of complex mixtures,
- Determination of the structure and composition of solid surfaces,
- Determination of isotopic ratios of atoms in samples.

MS instruments can be classified into two based on the analyte. These are: (1) atomic MS instruments which, are used for identification of elements present in a sample and their concentration and (2) molecular MS which are used for identification and/or quantitation of molecules present in a sample. Molecular MS will be discussed further for the purpose of this study.

1.5.1 Mass Spectrometer

This instrument is used to produce ions and separate them by their mass-to-charge ratios, m/z. Usually, the vast majority of ions produced are singly charged. Therefore, for a practical purpose, the mass number of the ion is used to replace the ratio.

The general principle of molecular MS involves the bombardment of analyte vapor with a stream of electrons leading to a loss of an electron resulting into the formation of the molecular ion M^+ as shown by the reaction below;

The dot indicates that the molecular ion is a radical ion that has the same molecular mass but one less electron as its original molecule.

Molecules are excited due to the energy generated from their collision with energetic electrons. Relaxation now occurs by fragmentation of part of the molecular ions to produce lower masses of ions. The fragmentation pattern is a useful tool used in identifying compounds.

Positive ions produced due to electron impact are sorted according to their mass-to-charge ratios by the slit of a mass spectrometer and displayed in the form of a mass spectrum.

1.5.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

The combination of liquid chromatography and mass spectrometry is a powerful merger that takes advantage of the separation strength of liquid chromatography and the sensitivity and selectivity of mass spectrometry. The major problem of coupling these two techniques is due to the fact that a gaseous sample is needed for mass spectrometry while the output of LC is a solute dissolved in a solvent. As a result, the solvent needs to be vaporized. The vapor produced from the LC solvent is 10-1000 times more than the carrier gas in GC. Majority of the solvent is required to be removed.

The recent approach used for removal of excess solvent makes use of a low flow rate atmospheric pressure ionization technique. The most common ionization sources are electrospray ionization and atmospheric pressure ionization. The LC-MS technique provides fingerprint of a particular eluate without the need to rely on retention time, as is the case in conventional HPLC. The combination also provides information about molecular mass, structural information and accurate quantitative analysis [38].

1.6 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is among the most powerful technique available to chemists for structural elucidation of chemical species. The technique is also applicable to the quantitative determination of absorbing species.

The principle of NMR spectroscopy relies on the measurement of absorption of electromagnetic radiation in the radio-frequency (RF) region of roughly 4 to 900 MHz. Unlike UV, visible and IR absorption that involves outer electrons in the absorption process; nuclei of atoms are involved in the case of NMR spectroscopy. The analyte needs to be placed in an intense magnetic field to cause nuclei to develop the required energy state for absorption to occur.

The chemical environment a given nuclei resides affects the frequency of RF radiation that is absorbed by the nuclei. This effect is known as spin-spin splitting which makes it possible for a wealth of information to be extracted to elucidate chemical structures.

The chemical shift (δ) is used for functional group identification and their structural arrangement of groups. The exact δ values may depend on the nature of solvent and concentration of solute. These effects are coming for protons that exhibit hydrogen bonding. A typical example is a proton of alcohol or amine functionality.

1.7 Aim of the Study

The aim of this study is to develop a fast, robust and efficient extraction method using DLLME combined with HPLC for the preconcentration and determination of three major capsaicinoids (i.e., capsaicin, dihydrocapsaicin and nordihydrocapsaicin) from peppers and scaling up DLLME for the isolation of these capsaicinoids by MPLC followed by characterization their using NMR and MS.

To the best of our knowledge, this is the first report on the application of DLLME with HPLC for the determination of capsaicinoids in peppers and the first attempt to scale up DLLME for preparative purpose.

CHAPTER 2
EXPERIMENTAL

2.1 Instrumentation

Chromatographic separation were performed with an Agilent technologies 1200 series HPLC system (USA) equipped with a diode array detector, a column oven, an autosampler, a quaternary pump and a degasser. The instrument was controlled by Agilent ChemStation for LC 3D systems (Rev. B.03.01) software. A reversed-phase column (Agilent Zorbax SB-Aq $4.6 \times 150\, mm, 5\, \mu m$) was used. Merck TLC Silica gel 60 F_{254} ($20 \times 20\, cm$) was used for TLC. Camag UV lamp, TLC plate heater and development chamber were used for viewing TLC spots, heating and developing the plate, respectively.

For isolation of capsaicinoids, an MPLC system (BÜCHI, Germany) was used which is equipped with a pump manager C-615, pump module C-605, and a fraction collector C-660. The column was packed with a LiChroprep RP-18 (25-40 μm) packing material (Merck, USA). The fractions were evaporated to dryness using a Heidolph Laborota 4001 efficient rotary evaporator equipped with a Huber minichiller. A Mettler Toledo electronic balance was used for weighing, while a CHRIST Alpha 1-4 LD plus Lyophilizer was used for crystallizing the pure standards.

For structural characterization, spectral analysis was performed using a Varian Mercury (Agilent, USA) FT-NMR (^{1}H 400 MHz, ^{13}C 100 MHz). DMSO-d_6 and $CDCl_3$ solvents were used for dissolving the crystals. Tetramethylsilane (TMS) was used as the internal standard. Mass spectra analyses were performed by electrospray ionization (ESI) on a Waters Alliance HPLC and ZQ micromass LC-MS spectrometer.

2.2 Reagents and Solutions

HPLC grade methanol, acetonitrile, tetrahydrofuran and chloroform with purity higher than 99%, sodium hydroxide, sodium chloride, 1-undecanol, 1-dodecanol, dichloromethane, diphenylether, diethylether and vanillin were obtained from Sigma-Aldrich (Germany). Ethyl acetate, toluene and n-hexane and acetic acid were purchased from Riedel-de haën (Germany); sulfuric acid (95-97% purity) was obtained from Fluka (USA).

2.3 Apparatus

Bandelin Sonorex digital ultrasonic bath (Germany) was used for ultrasonication. Centrifugation was performed with Hettich Eba 20 centrifuge (Germany), while vortex was performed on a Heidolph Reax top Vortex. Eppendorf micropipette (Sigma-Aldrich, USA) and tips were used for sample collection and transfer, while a Binder oven (USA) was used for drying the samples. Whatman membrane filters (0.45 μm) and GE infrastructure (0.45 μm) nylon syringe filters were used for filtering the solvents and sample solutions, respectively. A Blomberg refrigerator was used for sample preservation, and Sinbo coffee grinder model SCM 2927 (P.R.C) was used for blending of the dried samples.

2.4 Sampling and Sample Pre-treatment

Five samples of fresh pepper and green pepper pickle were purchased from local markets in Nicosia, TRNC. A representative photograph of the samples analyzed is given in **Figure 2.1** while the names and abbreviation of the samples are given in **Table 2.1**.

Figure 2.1: Analyzed pepper samples.

Table 2.1: Names of peppers and their abbreviations.

Name of pepper	**Abbreviation**
Big Red Pepper	BRP
Green Pepper Pickle	GPP
Light Green Pepper	LGP
Long Red Pepper	LRP
Small Green Chili	SGC
Yellow Pepper	YP

2.4.1 Drying of Samples

The fresh samples were washed with deionized (DI) water and cut into small pieces with a stainless steel knife after removing the seeds inside. Then, they were dried in the oven at 40°C, for 24 h, after which the temperature was adjusted to 60°C because of the high moisture content of the pepper, to speed up the drying process and to prevent mold growth. The samples were completely dry after another 24 h making the total drying time 48 h.

2.4.2 Blending of Samples

The samples were blended using the grinder to a very fine powder and preserved in well-sealed glass bottles until analysis.

2.4.3 Solid-Liquid Extraction

A sample of 1.0 g of the dried pepper was weighed and extracted with 50 mL 50/50 $(\%, v/v)$ ACN/H_2O for 30 min in an ultrasonic bath at room temperature. The mixture was filtered through a cotton wool, and then using a 0.45 µL filter paper. The solution was transferred into a 50-mL volumetric flask and completed to the mark with 50/50 (%, v/v) ACN/H_2O (hereafter referred to as sample solution).

2.4.4 Salting-Out Extraction (SOE)

5.0 mL of the sample solution were transferred into a test tube and 2.0 mL of saturated NaCl solution were added. The mixture was vortexed for 1 min before it was centrifuged for 3 min at 6000 rpm. Approximately, 1.2 mL of ACN salted out, 1.0 mL of which was used for DLLME.

2.4.5 DLLME

1.0 mL of ACN from the salting-out extraction was diluted to 10 mL with DI water in a closed cap conical tube after adding an extra 250 µL of ACN. This ACN volume acted as the disperser solvent in DLLME. 100 µL each of chloroform and acetic acid were added and the mixture was vortexed for 30 s and centrifuged for 3 min at 6000 rpm.

2.4.6 Back-Extraction

The chloroform layer that settled at the bottom of the tube was transferred completely into a microvial and back-extracted into 300 µL of back-extraction solution (BES) composed of MeOH/50 mM NaOH $(55/40\%, v/v)$, a composition that is similar to the HPLC mobile phase. The mixture was vortexed for 1 min and centrifuged for 3 min at 6000 rpm. 5 µL of the aqueous extract were injected into HPLC. A schematic presentation of the general DLLME procedure with evaporation-to-dryness or back-extraction is given in **Figure 2.2**.

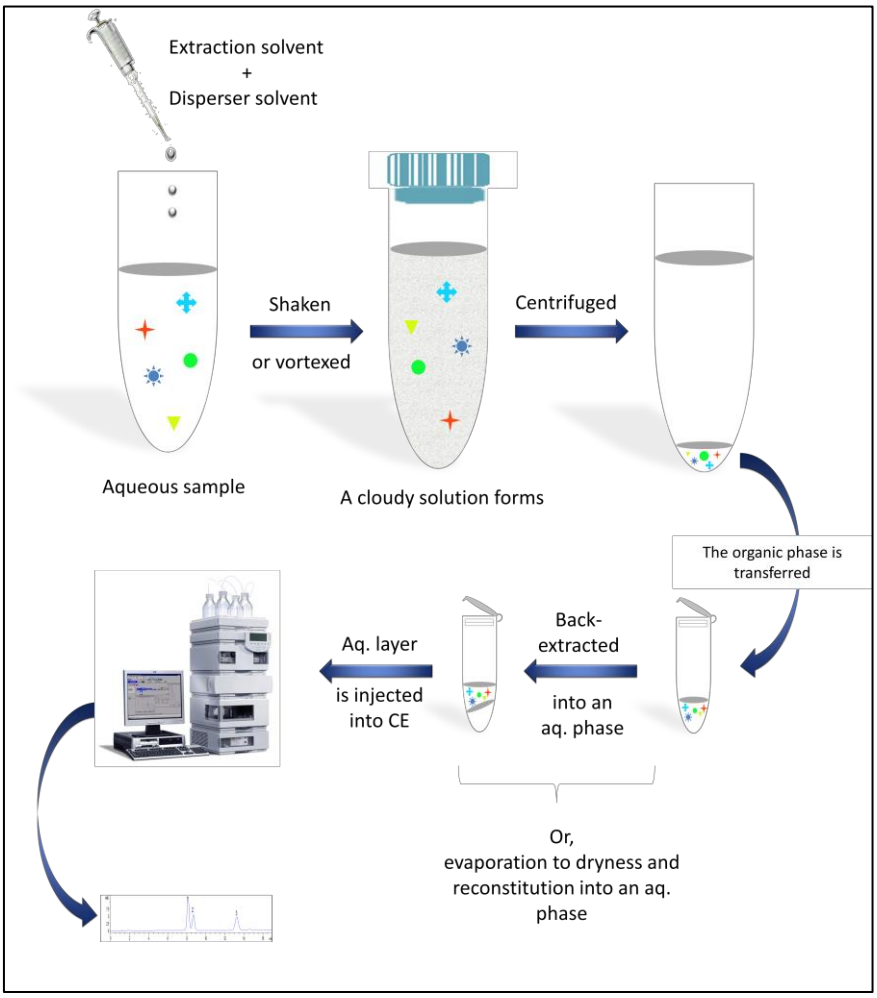


Figure 2.2: General DLLME procedure.

2.5 Sample Preparation for Extraction and Isolation of Capsaicinoid Standards

25 g of the dried pepper were transferred into a 500 mL volumetric flask and 50/ 50% (v/v) ACN/H_2O were added to the mark and ultrasonicated for 1h in an ultrasonic bath at room temperature. The extract was then filtered through a cotton wool and then through a 0.45 µL filter paper and the filtrate was taken for salting-out extraction. The procedure was repeated in batches six times making the total mass of 150 g of dried pepper used for extraction.

2.5.1 Salting-Out Extraction (SOE)

400 mL of the extract was collected into a 500 mL volumetric flask, NaCl was added and the mixture was shaken vigorously until the solution was saturated with approximately 3.0 g of NaCl. It was noticed that the addition of salt resulted in salting-out of the ACN to form a supernatant layer (**Figure 2.3**). The solution was then transferred into a 500 mL separatory funnel and swirled gently before allowing it to stand for 10 min on a retort stand until the aqueous and ACN layer completely separated. 150 mL ACN layer was collected and used for DLLME. The procedure was repeated for the other extracts until completed.

Figure 2.3: Salting-out extraction.

2.5.2 DLLME

25 mL of the ACN extract from SOE was transferred into a 250 mL measuring cylinder and completed to the mark with deionized water. The solution was transferred into a 500 mL volumetric flask and 2.5 mL each of acetic acid and chloroform were added and ultrasonicated for 1 min. 10 mL fractions were then collected into separate screw cap conical tubes and centrifuged for 3 min at 6000 rpm. Approximately, 60-70 µL chloroform extract were recovered from each tube and collected into a clean 50-mL screw cap conical tube. The process was repeated until completion. The chloroform extracts were combined and evaporated to dryness in a rotary evaporator to get 1.66 g of a solid residue.

2.5.3 Preparation of the Sample for Isolation by Column Chromatography

1.66 g of the solid residue was dissolved in dichloromethane (DCM). The choice of DCM is due to its lesser toxicity than chloroform and that it is more easily evaporated. A small volume (2 mL) was collected as reference solution into a vial and then 5 g of silica gel were added to the remaining solution to serve as an adsorbent. Then, DCM was evaporated in a rotary evaporator at 40°C and atmospheric pressure until a solid residue was obtained after approximately 30 min. The solid residue was scratched off the surface of the round bottom flask and homogenized in a dry clean mortar making it ready for packing into the column.

2.5.4 Column Chromatography

Seventy reagent tubes were loaded into a rag. Three solvent systems were used, namely, 200 mL EtOAC: toluene 1:9 (v/v) (SS1), 200 mL EtOAC: toluene 2:8 (v/v) (SS2), 650 mL EtOAC: toluene 3:7 (v/v) (SS3). The column was conditioned with the first solvent system before adding 70 g of silica gel into the column. SS1 was added until the volume reached 2 cm from the top level of the column. The sample was then packed into the column by dry packing and covered with cotton wool to get a definite sample zone. A solvent reservoir was attached to the top of the column. The tap of the column was then released and

15 mL fractions from the column were collected into the reagent tubes manually while adding the solvent system into the solvent reservoir until 69 tubes were filled.

TLC was used to identify the content of the fractions by comparing the retardation factors (R_f) values of the fractions and the reference sample collected from DCM extract. Similar fractions were combined and a representative sample was injected into HPLC after evaporation to dryness and reconstituting into the mobile phase because the solvent system used for column chromatography was not compatible with the reversed-phase HPLC mobile phase. HPLC was used to confirm if the capsaicinoids have been isolated and to check for the degree of purity. Fractions 48-69 contained the capsaicinoids of interest in their combined form. Hence, the fractions were combined and evaporated to dryness in a rotary evaporator before MPLC analysis.

2.5.5 Medium-Pressure Liquid Chromatography (MPLC)

An MPLC column was filled with a reversed-phase packing material [i.e., LiChroprep RP-18 (24-40 μm)]. The column was then conditioned with 20% (v/v) MeOH in DI water.

0.3436 g of the evaporated residue from fractions 48-69 of column chromatography was dissolved in 1.0 mL of 90% MeOH and injected into the column. A gradient elution was applied at a flow rate of 10 $mL\ min^{-1}$ starting with a constant composition of 20% MeOH for 15 min. The composition of MeOH was then increased to 60% in 60 min and kept constant for 20 min. Then, it was increased further to 75% MeOH in 30 min. Finally it was increased to 85% in 10 min. 10 mL of the fractions were collected automatically into the reagent tubes. TLC and HPLC were then used to identify the capsaicinoids composition of the fractions.

The capsaicinoids were successfully isolated with variable degrees of purity; the fractions that matched each of the capsaicinoids were evaporated separately and crystallized.

A sample of each of the three capsaicinoids was then collected for characterization by LC-MS and NMR.

CHAPTER 3
RESULTS AND DISCUSSION

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

Before commencement of the study, it was necessary to select the wavelength of maximum absorption for each capsaicinoid. The initial source of information was the literature. In the literature, capsaicin has been monitored at 222 nm [40] or 280 nm [41]. Injecting pure capsaicinoid standards and monitoring their absorption in 3D plot for a spectral scan revealed two absorption maxima at 228 nm and 280 nm as shown in **Figure 3.1** and **Figure 3.2**. 280 nm was selected as optimum wavelength even though 228 nm gave better absorption, which was due to the fact that at the lower border of the UV spectrum many other compounds that might be present in the sample were thought to absorb at that wavelength. Hence, it was better to select a wavelength that would suffer less from interferences in the matrix. Isoabsorbance plot of the studied capsaicinoids are given in **Figure 3.3**.

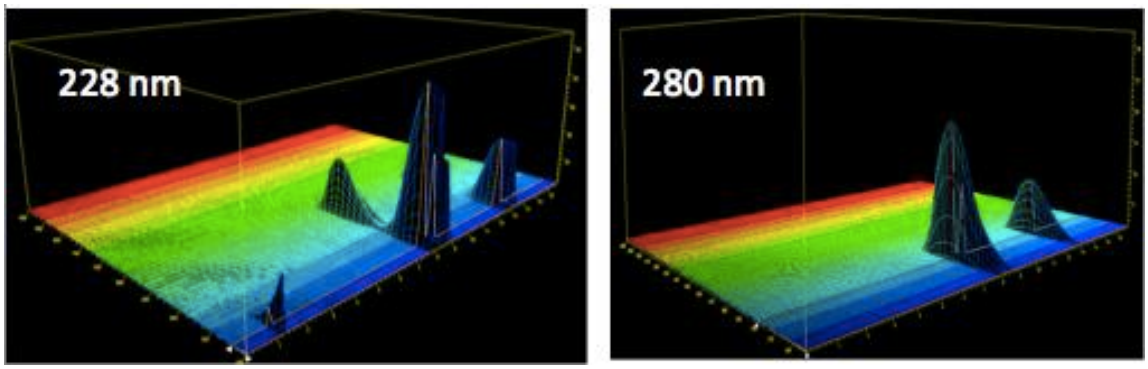


Figure 3.1: 3D Plot of capsaicinoids.

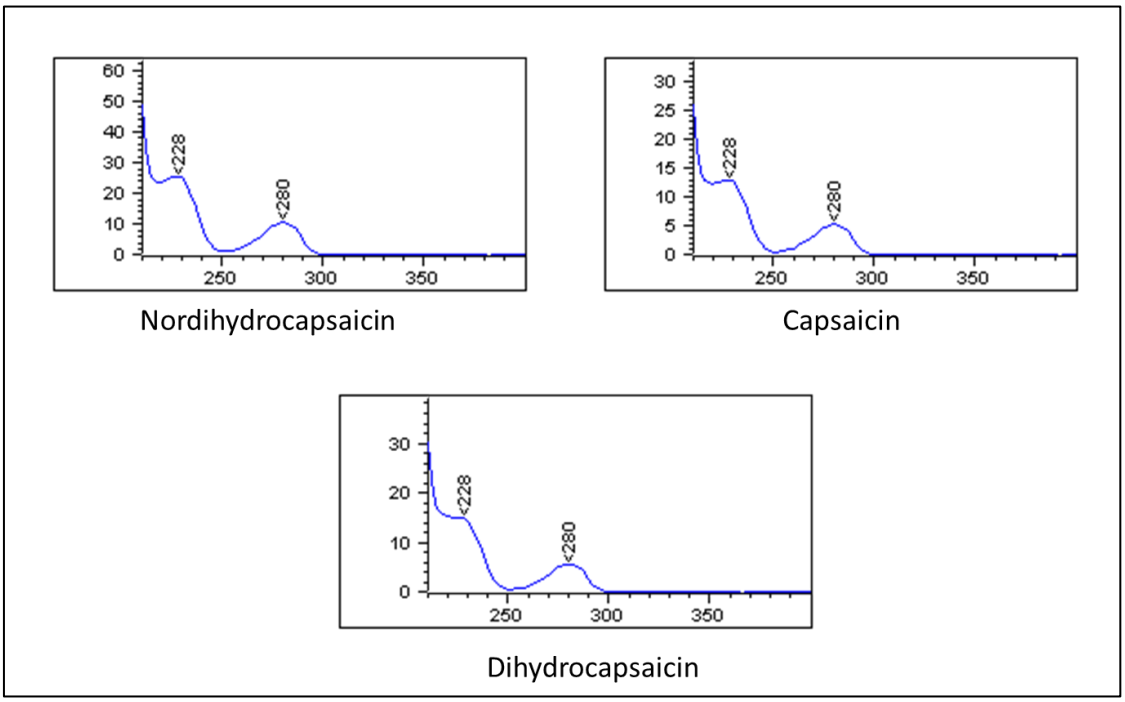


Figure 3.2: UV spectra of the studied capsaicinoids (at 50.0 mg L^{-1} each in the mobile phase).

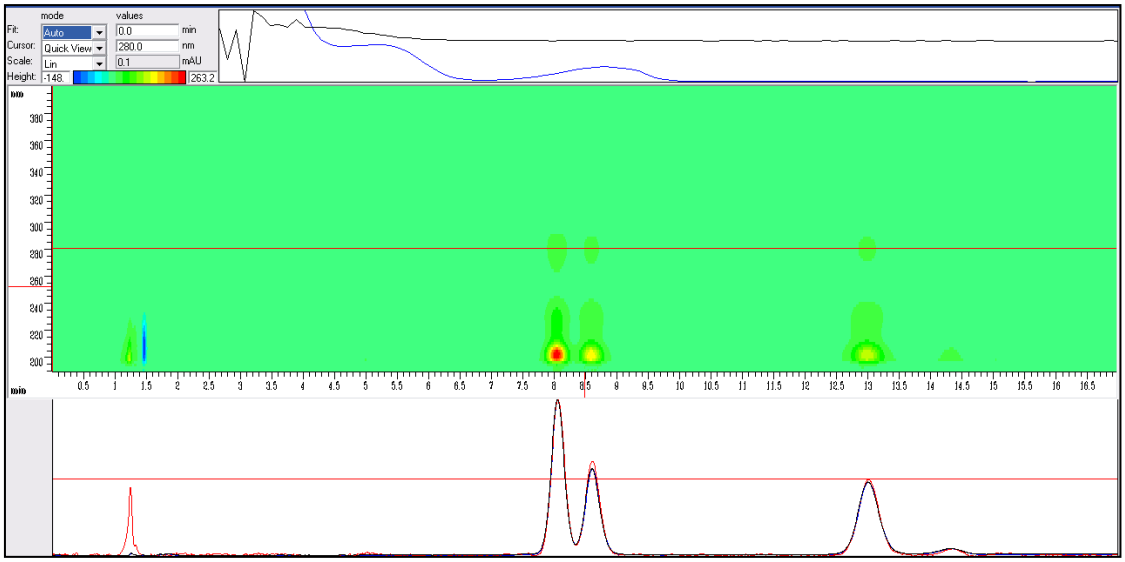


Figure 3.3: Isoabsorbance plot the studied capsaicinoids (at 50.0 mg L^{-1} each in the mobile phase).

3.2 Optimization of the Extraction Methods

Three extraction methods were considered which included salting-out extraction (SOE), DLLME with back-extraction (DLLME-BE) and DLLME with evaporation-to-dryness under the stream of nitrogen (DLLME-ETD).

3.2.1 Determination of the Extraction Parameters

The first step that was taken to be able to extract capsaicinoid from pepper was to consider the $logP$ values of all the capsaicinoids (**Figure 3.4**). Based on their $logP$ values, capsaicinoids are relatively non-polar with intermediate polarity so an organic solvent with intermediate polarity should be used for extraction, hence the choice for ACN.

ACN: H_20, 50:50 $(\%, v/v)$ was selected for leaching the analytes from the solid samples with the addition energy of ultrasonic bath. After extracting the analytes from the solid, SOE was performed to phase-separate the two solvents.

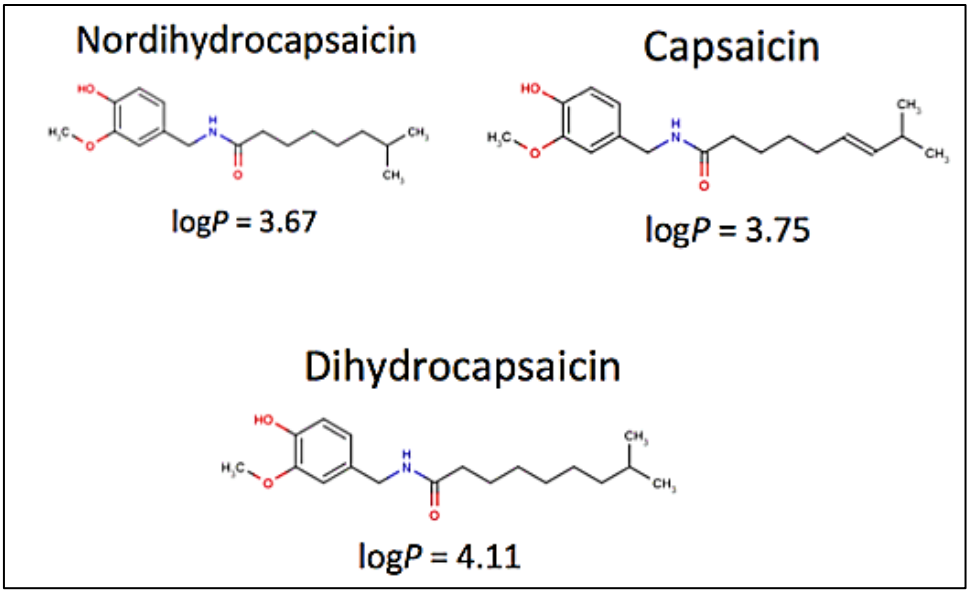
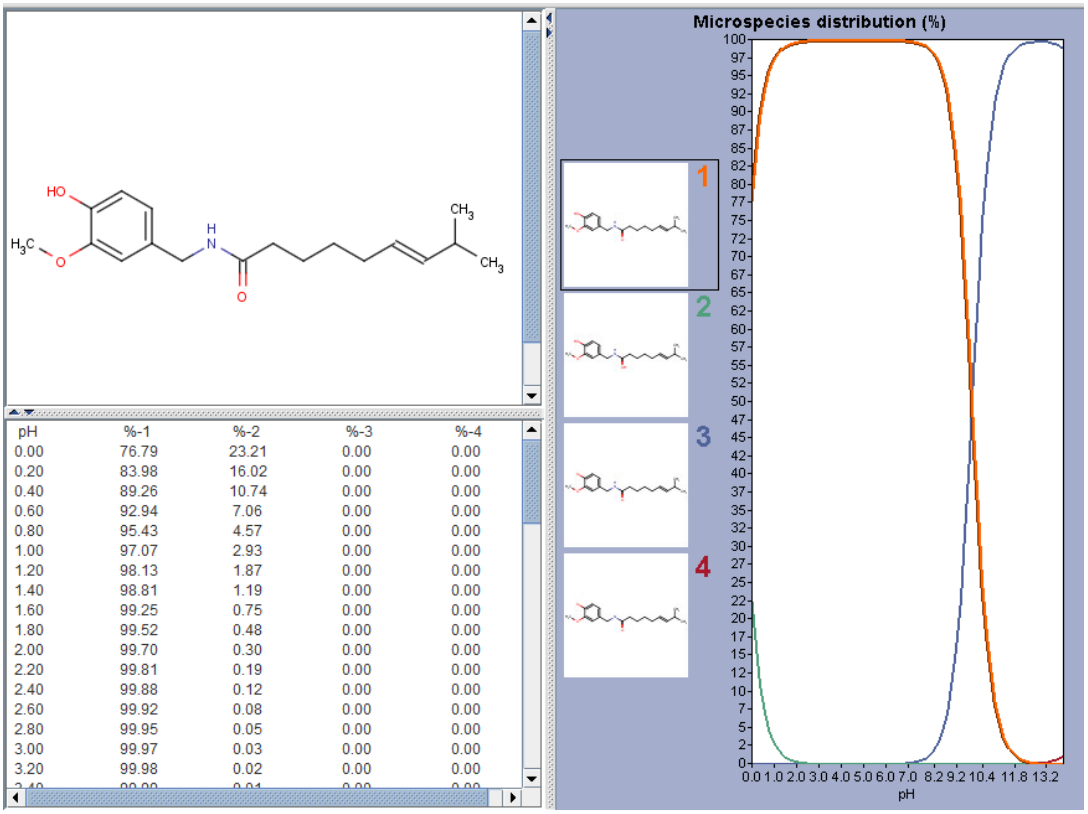


Figure 3.4: $logP$ values of capsaicinoids.

To be able to apply DLLME to extract the analytes, $logP$ value is not sufficient enough to determine the extraction parameters of the analyte. This is because RP-HPLC uses polar solvent as mobile phase, which is not compatible with the non-polar solvent that is required for the extraction of the analyte, so it was necessary to convert the analyte into a form that is going to be back extracted into aqueous solution. So, there was a need for analyzing the microspecie distribution of capsaicinoids at different pH.

Because capsaicinoids are structurally similar, they all possess similar forms at different pH so the microspecie distribution of capsaicin was taken as a reference for the others.

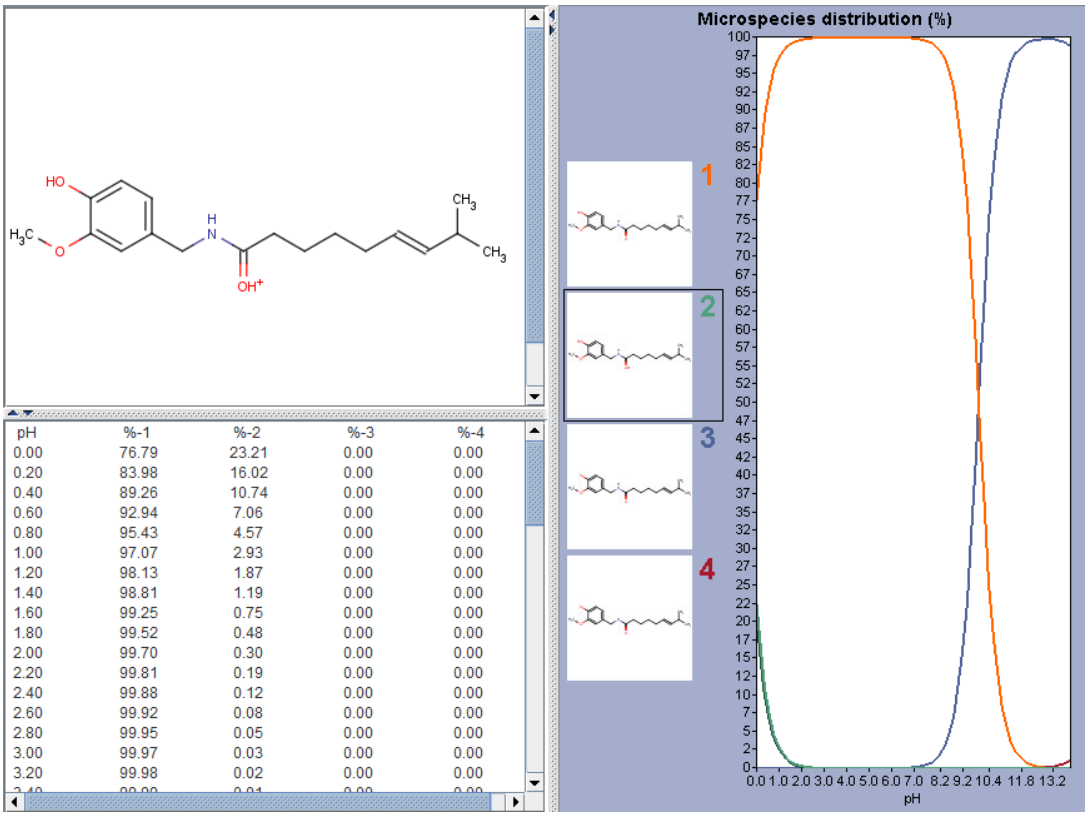
From its microspecie distribution, capsaicin can be present in four forms. The first form of capsaicin as shown in **Figure 3.5** is the neutral form, which is dominant from pH 2-7. This form is sufficient to extract capsaicin from the ACN extract to chloroform for DLLME since chloroform is more non-polar than ACN. But at pH 7, which is the present form of the solution from SOE, there is a risk that the form of capsaicin can be easily converted to ionizable form, so there was a need to adjust the pH to make it acidic. 100 µL of 14 M acetic acid would drop the pH to about 2.8 were the neutral form is dominant with less risk of ionization.



pH	%-1	%-2	%-3	%-4
0.00	76.79	23.21	0.00	0.00
0.20	83.98	16.02	0.00	0.00
0.40	89.26	10.74	0.00	0.00
0.60	92.94	7.06	0.00	0.00
0.80	95.43	4.57	0.00	0.00
1.00	97.07	2.93	0.00	0.00
1.20	98.13	1.87	0.00	0.00
1.40	98.81	1.19	0.00	0.00
1.60	99.25	0.75	0.00	0.00
1.80	99.52	0.48	0.00	0.00
2.00	99.70	0.30	0.00	0.00
2.20	99.81	0.19	0.00	0.00
2.40	99.88	0.12	0.00	0.00
2.60	99.92	0.08	0.00	0.00
2.80	99.95	0.05	0.00	0.00
3.00	99.97	0.03	0.00	0.00
3.20	99.98	0.02	0.00	0.00

Figure 3.5: First microspecies distribution form of capsaicin.

The second form of capsaicin is ionizable below pH 2. But the maximum percentage of that form is 17%, which is not a suitable form as shown in in **Figure 3.6.**



pH	%-1	%-2	%-3	%-4
0.00	76.79	23.21	0.00	0.00
0.20	83.98	16.02	0.00	0.00
0.40	89.26	10.74	0.00	0.00
0.60	92.94	7.06	0.00	0.00
0.80	95.43	4.57	0.00	0.00
1.00	97.07	2.93	0.00	0.00
1.20	98.13	1.87	0.00	0.00
1.40	98.81	1.19	0.00	0.00
1.60	99.25	0.75	0.00	0.00
1.80	99.52	0.48	0.00	0.00
2.00	99.70	0.30	0.00	0.00
2.20	99.81	0.19	0.00	0.00
2.40	99.88	0.12	0.00	0.00
2.60	99.92	0.08	0.00	0.00
2.80	99.95	0.05	0.00	0.00
3.00	99.97	0.03	0.00	0.00
3.20	99.98	0.02	0.00	0.00

Figure 3.6: Second microspecies distribution form of capsaicin.

The third microspecies of capsaicin is the ionized form that is dominant from pH 12-13 as shown in **Figure 3.7**. So, to be able to back-extract the analyte from chloroform to BES, 50 mM NaOH was used to obtain a pH of approximately 12.8 that is sufficient to back-extract the analyte from chloroform to BES.

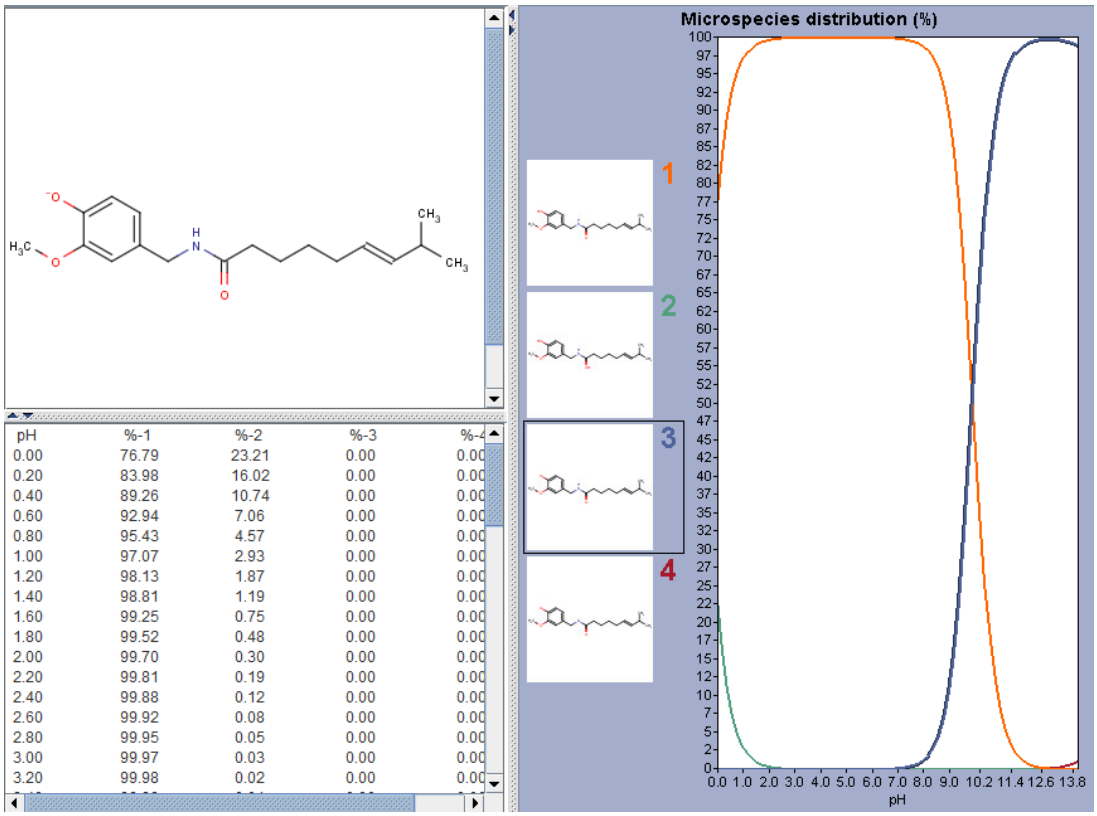
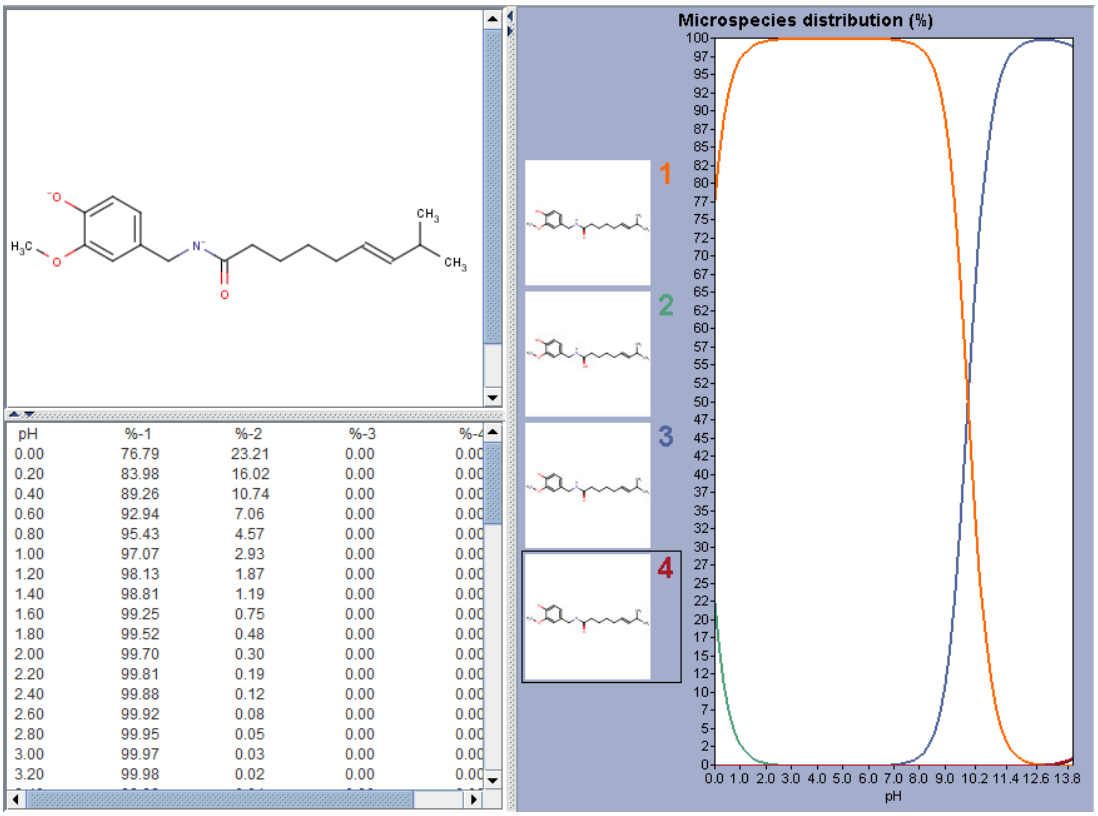


Figure 3.7: Third microspecies distribution form of capsaicin.

The fourth form of capsaicin is ionized but at negligible percentage below 1% at all pH values, so it is insignificant for the extraction (**Figure 3.8**).



pH	%-1	%-2	%-3	%-4
0.00	76.79	23.21	0.00	0.00
0.20	83.98	16.02	0.00	0.00
0.40	89.26	10.74	0.00	0.00
0.60	92.94	7.06	0.00	0.00
0.80	95.43	4.57	0.00	0.00
1.00	97.07	2.93	0.00	0.00
1.20	98.13	1.87	0.00	0.00
1.40	98.81	1.19	0.00	0.00
1.60	99.25	0.75	0.00	0.00
1.80	99.52	0.48	0.00	0.00
2.00	99.70	0.30	0.00	0.00
2.20	99.81	0.19	0.00	0.00
2.40	99.88	0.12	0.00	0.00
2.60	99.92	0.08	0.00	0.00
2.80	99.95	0.05	0.00	0.00
3.00	99.97	0.03	0.00	0.00
3.20	99.98	0.02	0.00	0.00

Figure 3.8: Fourth microspecies distribution form of capsaicin.

3.2.2 Preparation of Crude Extract with 50/50 (%, v/v) ACN/H_2O

Because SOE was decided to be used, it was necessary to prepare the crude extract in a solvent that contained ACN because it is the most suitable solvent for SOE.

3.2.3 Salting-Out Extraction (SOE)

ACN resulting from the salting-out was injected into HPLC. Although, separation was achieved, the baseline of peaks in the chromatogram (**Figure 3.9**) was “dirty” showing the need for further sample cleanup for better separation and improvement of the lifetime of the column.

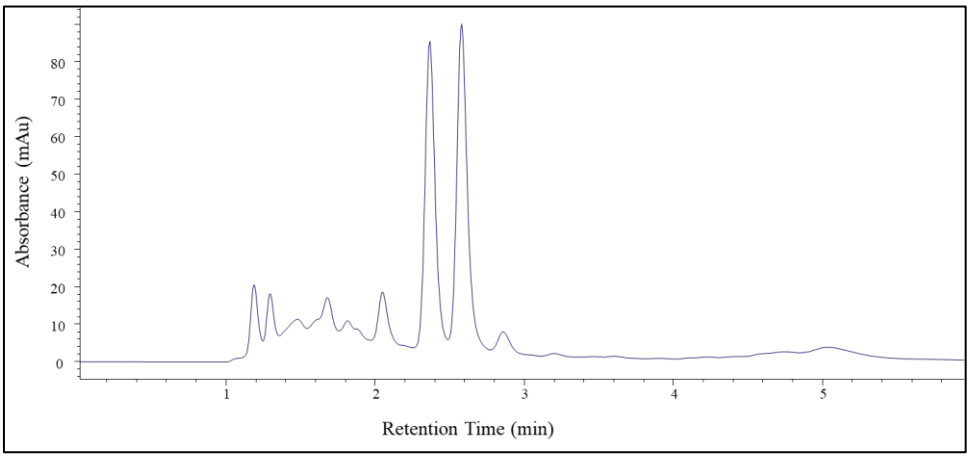


Figure 3.9: Representative chromatogram after SOE.

3.2.4 DLLME with Back-Extraction

Due to the combined cleanup of DLLME and back-extraction, some interfering peaks were eliminated but the peak area was more than six times less for the two major peaks compared to those obtained after SOE (**Figure 3.10**). Even though DLLME with back-extraction provided better sample cleanup, analyte loss is not desirable bearing in mind that the pepper sample containing the highest concentration of the analytes (i.e., GPP) was used for preliminary experiments. It implied that when the least concentrated ones were eventually analyzed, peaks might not be detected. Hence, this method was rendered unsuitable.

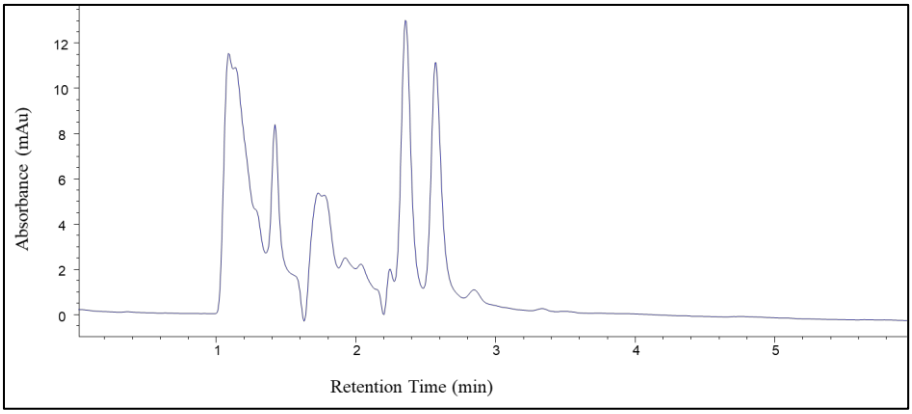


Figure 3.10: Representative chromatogram after DLLME-BE.

3.2.5 DLLME with Evaporation-to-Dryness and Reconstituting into the Mobile Phase (ETD)

The same DLLME procedure was used but 100 µL of the chloroform layer was transferred into a microvial and evaporated-to-dryness under a stream of nitrogen. The residue was dissolved in 200 µL of the mobile phase. 5 µL of the supernatant was injected into HPLC. This method gave better sample clean-up than with SOE alone as can be observed from the chromatogram in **Figure 3.11**. Also, a new capsaicinoid that was not observed with SOE was detectable. It was believed to be a capsaicinoid because the UV spectrum was similar to the other capsaicinoids. However, the average peak area after this method was still approximately 1.7 times less than that of SOE, which implies that SOE gave a better enrichment factor (EF).

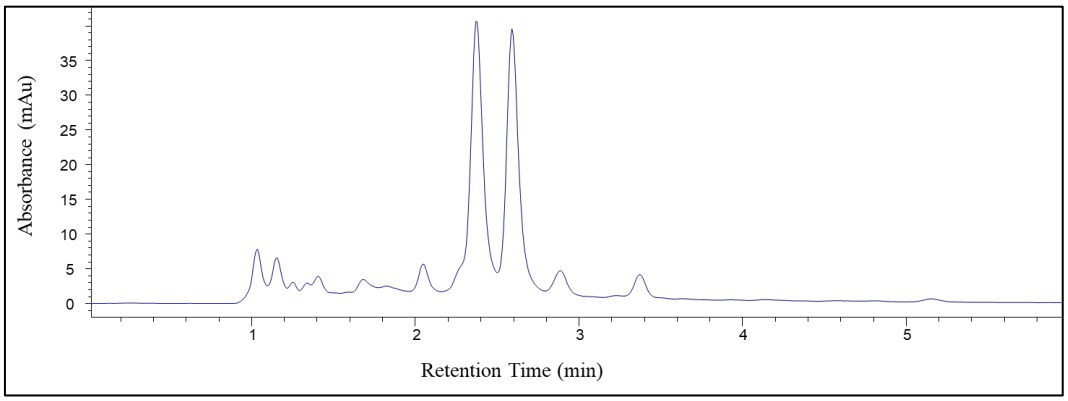


Figure 3.11: Representative chromatogram after DLLME-ETD.

3.3 Concluding Remarks on the Three Extraction Methods

After these experiments, it was assumed that at least five capsaicinoids were present in the sample since the peaks obtained all had similar UV spectra, which matched the ones present in the literature for capsaicinoids [40]. A representative chromatogram containing the five resolved capsaicinoids is given in **Figure 3.12.**

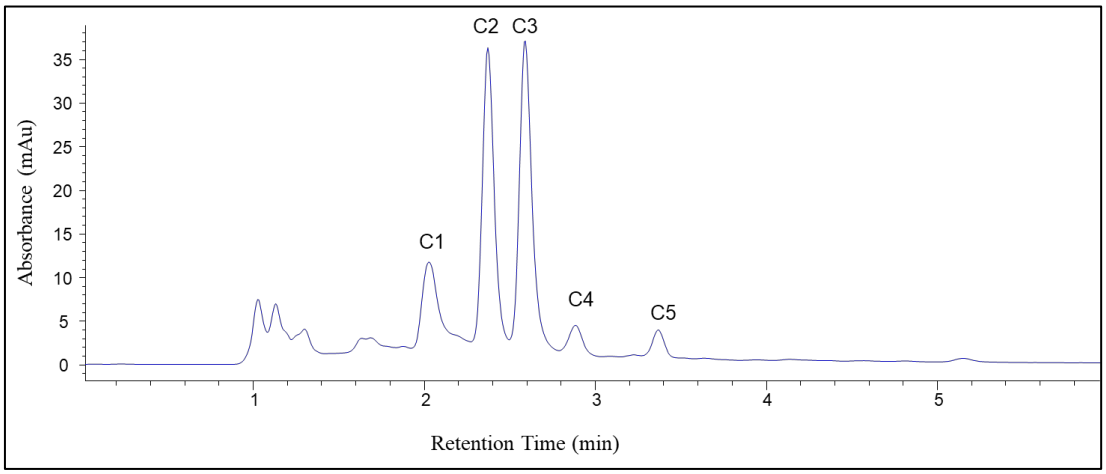


Figure 3.12: A representative chromatogram of the five suspected capsaicinoids.

The plot shown in **Figure 3.13** compares the peak areas obtained after the three extraction methods. Obviously, DLLME-BES is out of contention due to low EF. The choice was either SOE or DLLME-ETD, a compromise between higher EF or better selectivity and sample clean-up, respectively. It was decided to combine these two methods.

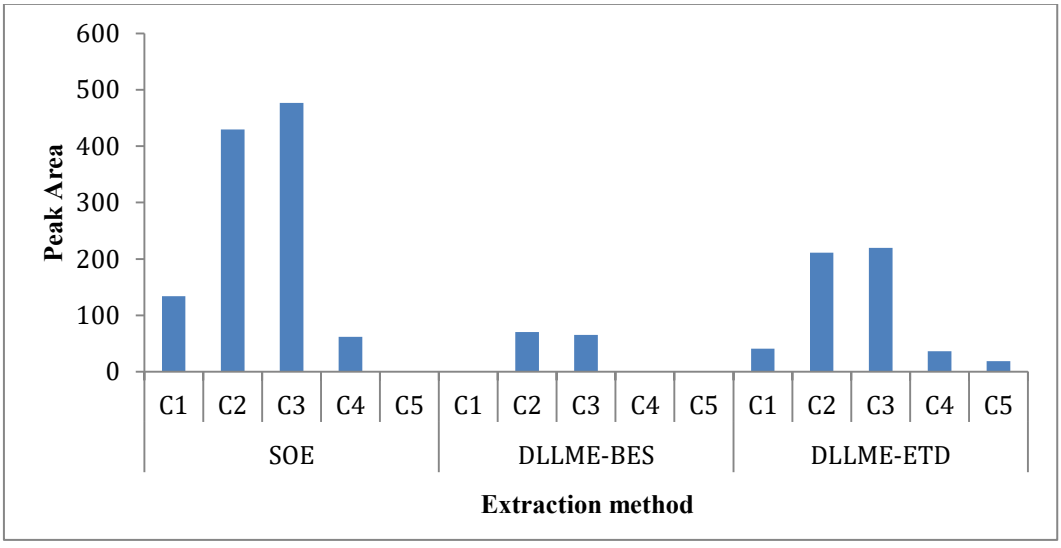


Figure 3.13: Comparing SOE, DLLME-BES and DLLME-ETD.

3.4 Combining SOE with DLLME-ETD

The extract from SOE was considered as the sample solution for DLLME-ETD. From the chromatogram shown in **Figure 3.14**, SOE combined with DLLME-ETD gave almost 14 times higher average peak area than with SOE alone. Also, all five capsaicinoids are present which shows the synergy of combining these two powerful methods. Even though combining the two methods means slightly longer analysis time, the higher EF, higher selectivity and better sample cleanup is worth the few additional minutes of extraction time that will be added especially with samples containing lower concentration of the analyte.

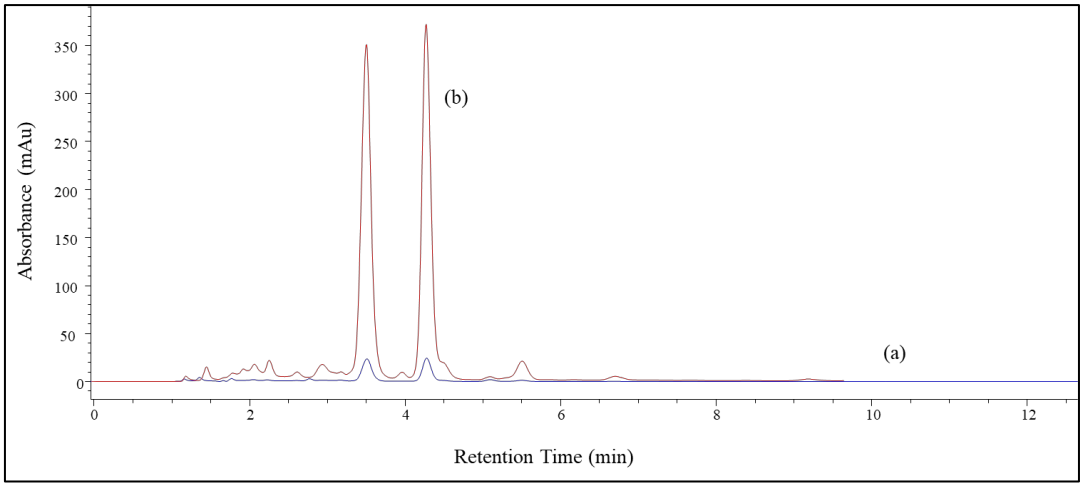


Figure 3.14: Comparing SOE (a) with SOE-DLLME-ETD (b).

In conclusion, SOE-DLLME-ETD was considered as the optimum extraction method and was used for further analysis.

3.5 Optimization of HPLC Conditions

The systematic approach described in **Figure 1.5** was applied in the optimization of HPLC conditions starting with the type of mobile phase. So far in this study, 70% ACN/H_2O was used as the mobile phase composition.

3.5.1 Type of the Mobile Phase

ACN, MeOH and THF were used for investigating the effect of type of the mobile phase on the chromatographic behavior. The "solvent triangle" for selecting the three solvents is given in **Figure 3.15**.

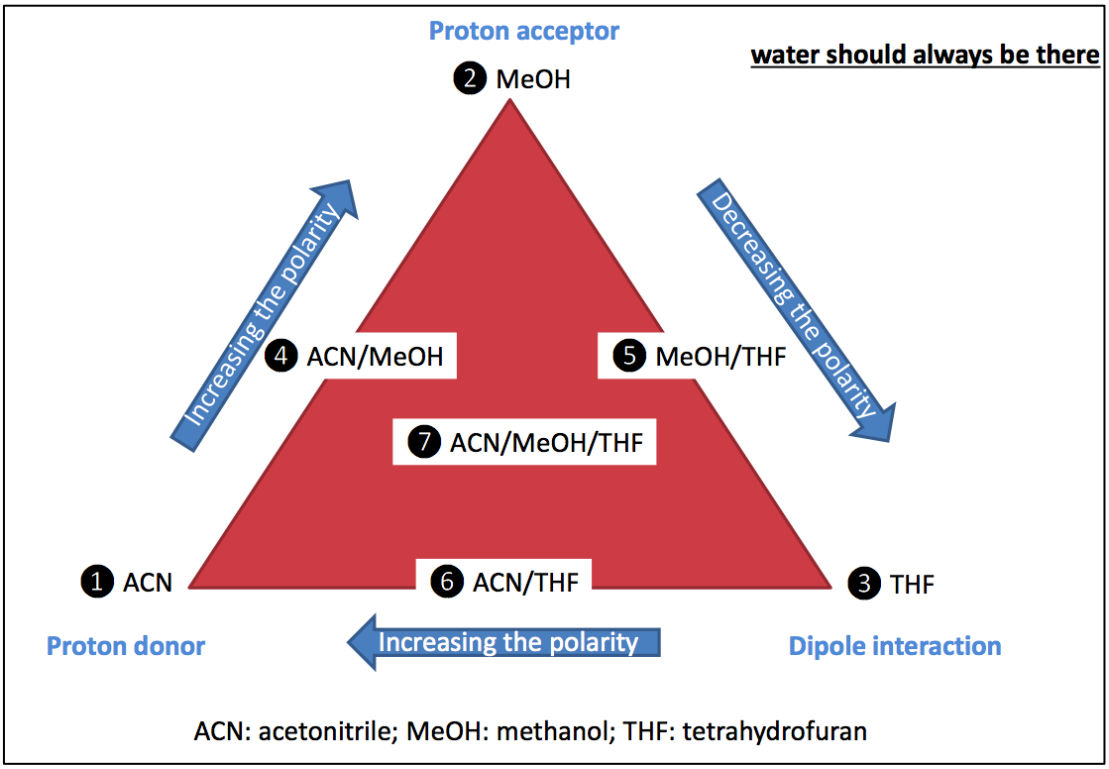


Figure 3.15: The solvent triangle.

From the solvent triangle given in **Figure 3.15**, the order of polarity is MeOH>ACN>THF. Also, from the $logP$ values for the studied capsaicinoids (i.e., NDHC 3.67, CAP 3.75 and DHC 4.11), they are relatively non-polar. Therefore, a non-polar solvent in the mobile phase would be a strong solvent that would not allow enough time for the analytes to interact with the reversed-phase column.

From **Figure 3.16**, it was observed that with THF, the analyte overlapped and all peaks co-eluted within 2 min. With ACN, the resolution was better but two peaks obviously co-eluted while with MeOH, their elution took about 6 min. There was more peak broadening in MeOH because the analyte were retained more strongly in the column due to the higher polarity of MeOH as can be inferred from the solvent triangle in **Figure 3.15**, but the peak area was similar to those obtained with ACN due to higher peak height in ACN.

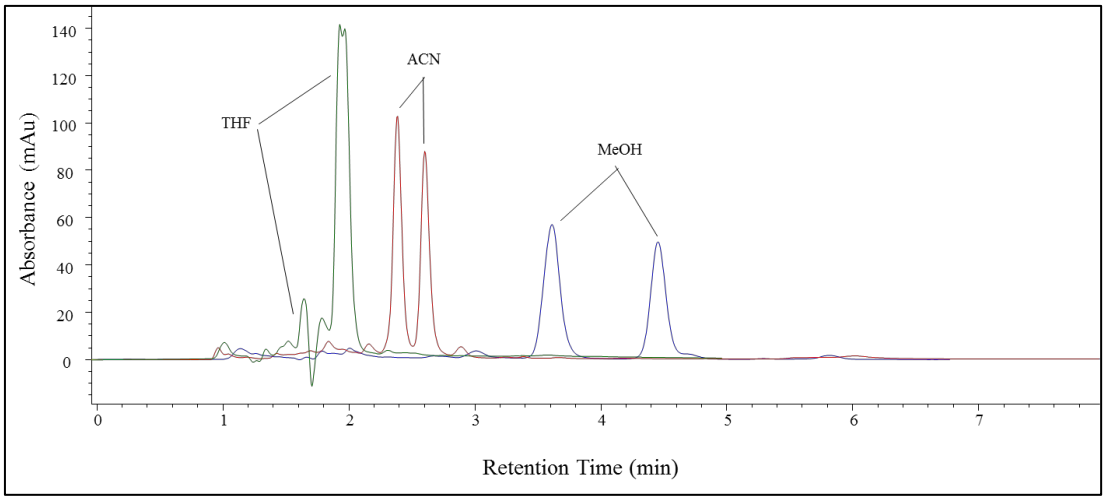


Figure 3.16: Representative chromatogram of the three types of mobile phase THF, ACN and MeOH.

MeOH was chosen for further analysis because even though there was more peak broadening in MeOH, the resolution was better. Since MeOH is relatively more polar than ACN, a longer interaction time with the column would enable better separation of the analytes. In addition, bearing in mind that capsaicinoids are very similar in structure, overlap might occur during optimization.

3.5.2 Optimization of the Mobile Phase Composition

Chromatographic behavior was investigated under the following compositions of MeOH: 90, 80, 70, 60, 55 and 50% (v/v) in water.

A short analysis time is always desired especially for routine analysis. It was observed that as the composition of MeOH was reduced, the retention time increased because of increase in the polarity of the mobile phase since water is more polar than MeOH. The resolution, on the other hand, was improving for the critical pairs since they interacted with the column for a longer time. At 60% MeOH and less, three peaks for major capsaicinoids were observed instead of the two in previous experiments with 70% implying that the two peaks which were overlapping were resolved with this new composition. A mobile phase composed 50% MeOH in water gave a good separation but the retention time was almost 50 min, which was too long and would defeat the aim of this study, i.e., developing a fast method for determination of capsaicinoids in peppers.

From the chromatograms given in **Figure 3.17**, 55% MeOH was considered optimum as a compromise between retention time and resolution.

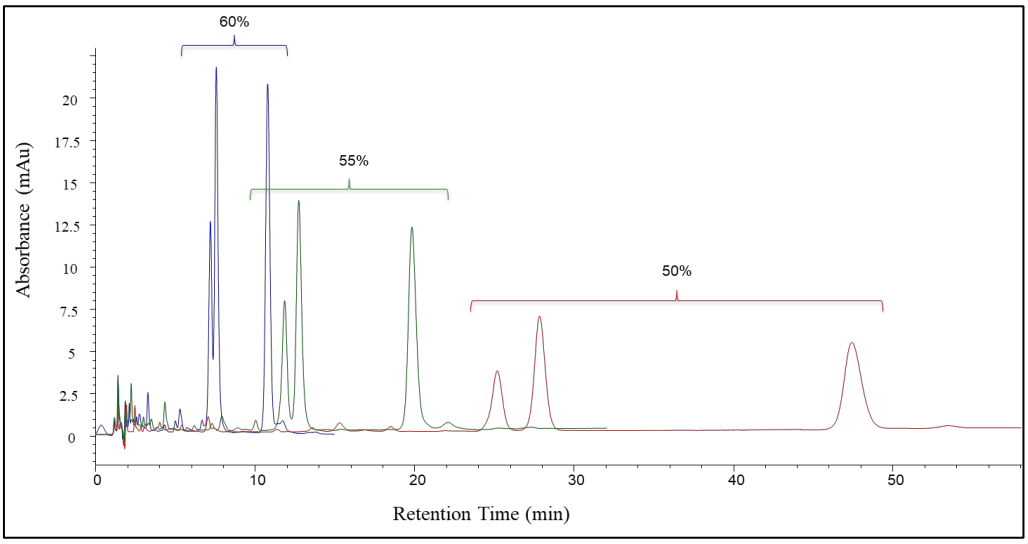


Figure 3.17: Comparing 60, 55 and 50% MeOH.

3.5.3 Effect of Acetic Acid as a Mobile Phase Modifier

Mobile phase modifiers are usually added to improve resolution and/or to reduce retention time. For this experiment, the effect of acetic acid was investigated up to 1.0% (v/v) acetic acid in water used in the mobile phase within intervals of 0.25% (v/v) of acetic acid.

Retention time improved with increasing in the concentration of acetic acid but 0.5% acetic acid was taken as optimum because there was no significant reduction in retention time after that point; lifetime of the column was also taken into consideration since consistent exposure to higher concentration of acid would reduce the life time.

3.5.4 Optimization of the Flow Rate

The purpose of this experiment was to reduce the retention time without affecting resolution by adjusting the flow rate until an optimum condition was reached. It was observed that the back-pressure of the column increased exponentially with increase in flow rate.

Peak area or retention time alone cannot be used as the basis for the selection of optimum condition for flow rate. This is due to the fact that increase in peak area and reduction in retention time can both reduce the resolution of the peaks because of peak overloading in the case of higher peak area and co-elution of analyte in the case of shorter retention time. It is, therefore, necessary to find a factor that would account for the effect on resolution. The corrected peak area is the factor required and it is calculated by dividing the peak area by the retention time and plotting the ratio against flow rate. The optimum flow rate is then selected as the point where a constant trend is achieved. 1.2 $mL\ min^{-1}$ was taken as optimum flow rate due to a constant trend as observed in **Figure 3.18**.

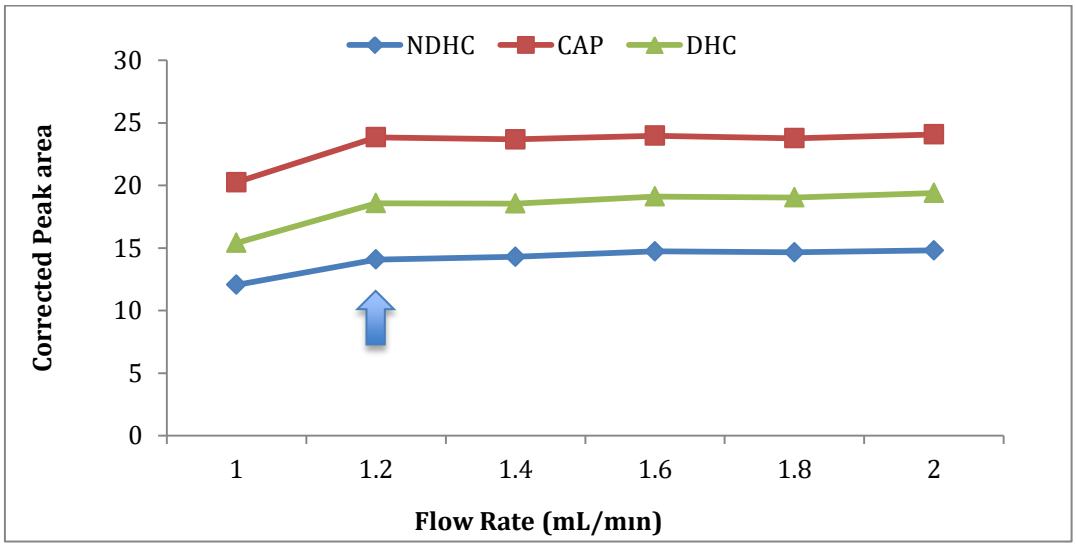


Figure 3.18: Effect of flow rate on corrected peak area.

3.6 Optimum HPLC Conditions

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

Table 3.1: Optimum HPLC conditions.

Physical parameters	Column	ZORBAX SB-Aq, 4.6 mm ID × 150 mm (5 µm)
	Flow Rate	1.2 $mL\ min^{-1}$
	Temperature	Room temperature
	Detector/wavelength	DAD 280 nm (BW 8). Reference: none
	Injection volume	5.0 µL
Chemical parameters	Mobile phase	MeOH:H_2O containing 0.5% acetic acid (v/v), 55:45 (v/v)

3.7 Dispersive Liquid-Liquid Microextraction (DLLME)

In DLLME, the factors that affect extraction efficiency include the following: identity and volume of extraction and disperser solvent as well as the extraction time [42]. Salt

addition has been shown to enhance the ability of some solvents to extract some analytes and improve recovery [29]. It was, therefore, necessary to find the optimum conditions for these parameters.

3.7.1 Optimization of the Type of Extracting Solvent for DLLME

Because the choice of extraction solvent in conventional DLLME is based on higher density than water [26], dichloromethane and chloroform were selected for this experiment. For solvents which are less dense than water, 1-undecanol, 2-dodecanol and diphenylether were initially used for DLLME based on solidification of floating organic drop (DLLME-SFOD) since they can solidify easily by cooling in a freezer for about 5 min, but because the method used so far relied on evaporation-to-dryness under a stream of nitrogen, evaporating them was not possible because they solidified under nitrogen and hot air was needed to be blown at regular interval to melt them; microwave also proved futile in evaporating them probably because of their high boiling point so they were dropped for this experiment and replaced with toluene and n-hexane both of which are less dense than water but do not solidify easily.

Equal volumes of 200 μL were collected from each of the extraction solvent (chloroform, CF; dichloromethane, DCM; toluene, TL; and n-hexane, n-Hex) and used for DLLME-ETD. The recovery of the solvent is an important factor to be considered in such experiments. So, a preliminary test of the relative recovery of each of the solvents was carried out and DCM gave the least recovery of about 40 μL, hence, for fair comparison, 40 μL of each of the recovered solvents after DLLME were evaporated to dryness under a stream of nitrogen and reconstituted in the same volume of the mobile phase (i.e., 200 μL).

Looking at the graph in **Figure 3.19** does not give the complete picture of the extraction efficiency of each solvent without considering the relative recovery of each solvent, which is given in **Figure 3.20.** This is because not all recovered volume of the solvents was evaporated. So, even though the peak area of DCM was the highest, it would

demand a larger volume of DCM to get similar recovery to the other solvents. CF was chosen as the extraction solvent on the basis of having the highest recovery implying that lower volume of the chloroform could be used to achieve considerable recovery and quantitation of the analyte, thereby, reducing the volume of the organic solvent required for the analysis.

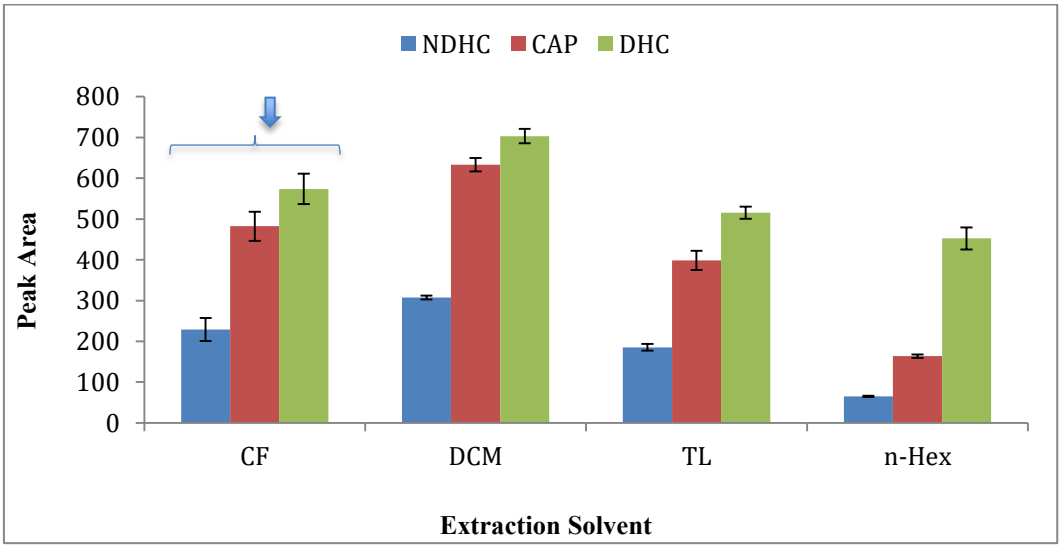


Figure 3.19: Effect of the type of extracting solvent in DLLME.

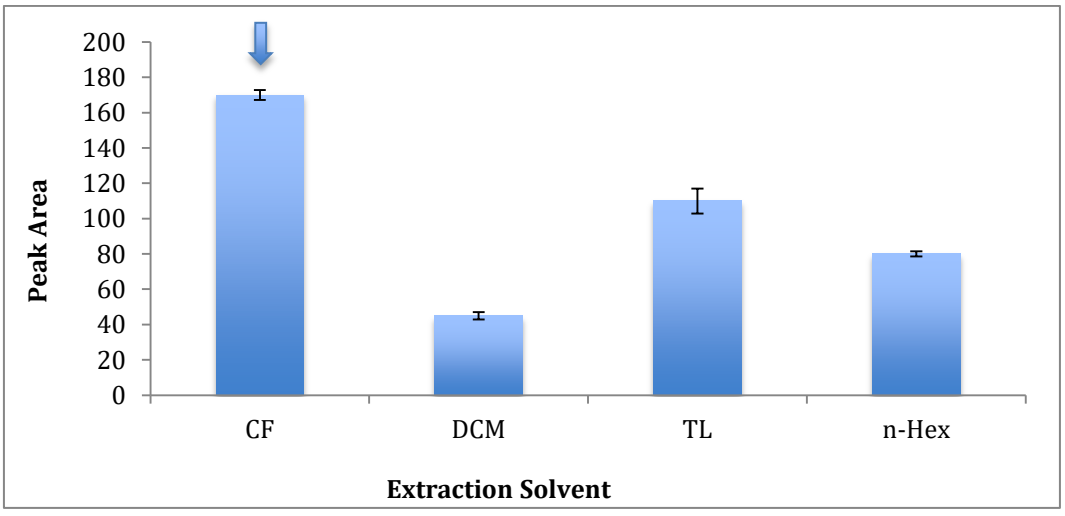


Figure 3.20: Relative recovery of the extracting solvent.

3.7.2 Optimizing the Volume of the Extraction Solvent

The volume of the extraction solvent significantly affects the extraction efficiency [43]. This is because EF decreases with increase in the extraction solvent volume that also results in the increase of the sedimented phase obtained after centrifugation. The optimum extraction solvent volume is expected to ensure both high EF and quantifiable volume of the sedimented phase after centrifugation [42].

3.7.2.1 Volume of the Extraction Solvent Using SOE-DLLME-ETD

For this experiment, the effect of the volume of chloroform was monitored starting from 100 µL to 250 µL within intervals of 50 µL. The whole volume of the recovered chloroform after centrifugation was evaporated-to-dryness and reconstituted into a 300 µL constant volume of the mobile phase. It was observed that lower volume of chloroform gave better enrichment factor EF as shown in **Figure 3.21**. To reduce the volume further, 50 µL of CF was used but it was observed that no CF sedimented after centrifugation when this volume was used. Hence, 100 µL of CF was taken as optimum. But, due to the large error observed, back-extraction after DLLME was tested again.

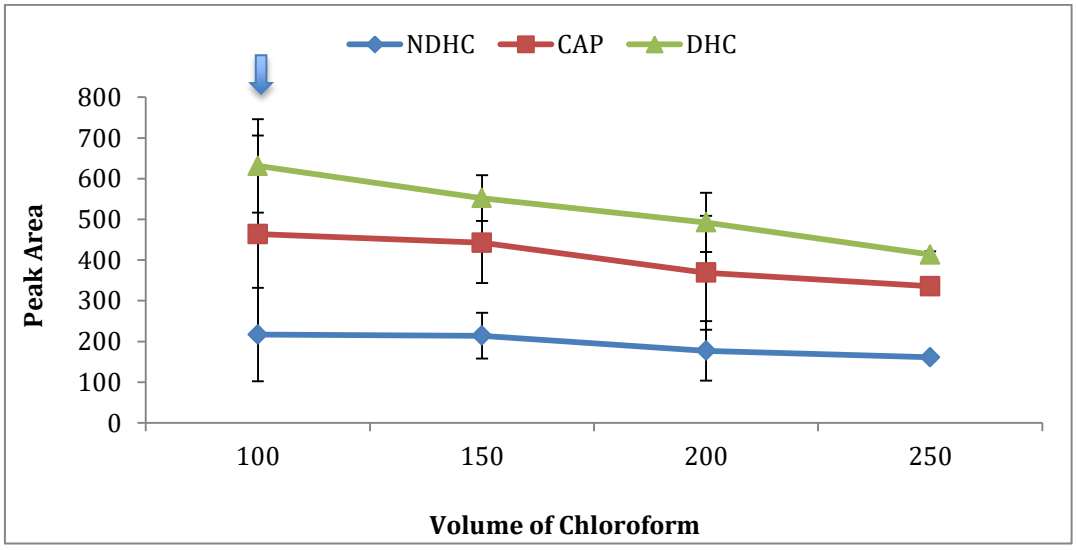


Figure 3.21: Effect of volume of extraction solvent (chloroform) using SOE-DLLME-ETD.

3.7.2.2 Optimization of the Volume of Extraction Solvent Using DLLME-BE

BES was prepared by mixing MeOH and 50 mM NaOH (55:45 %, v/v), which is similar to the composition of the mobile phase (i.e., 55 % MeOH in water). All recovered volume of CF was back-extracted with a constant volume of BES (i.e., 300 µL).

A similar trend was observed with less error as can be seen from the error bar at 100 µL in **Figure 3.22**. Comparing evaporation-to-dryness with back-extraction at this point as reflected by **Figure 3.23** gave a direct correlation. 100 µL of CF using back-extraction was taken as optimum and was used in further experiments. It is noteworthy that back-extraction is relatively easier, faster and cheaper than evaporation-to-dryness which requires a stream of nitrogen for about 4-5 min per sample.

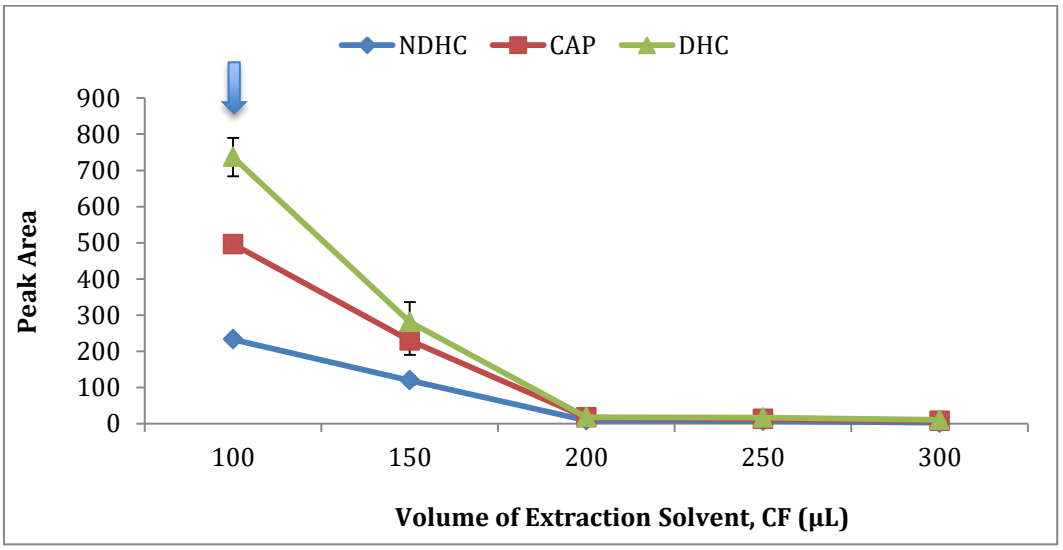


Figure 3.22: Effect of volume of extraction solvent (Chloroform) using SOE-DLLME-BE.

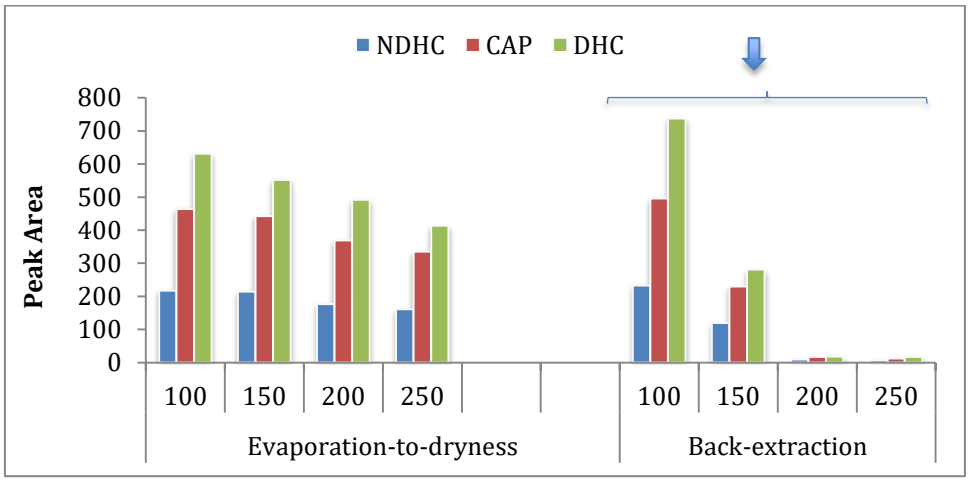


Figure 3.23: Effect of volume of extraction solvent with both methods.

3.7.3 Optimization of the Extraction Time

The extraction time in DLLME is defined as the time interval from injecting the disperser and extraction solvent to the moment just before centrifugation [26]. The vortex time was varied from 0 to 120 s within 30 s intervals until maximum extraction was achieved by getting a constant peak area after 30 s (**Figure 3.24**).

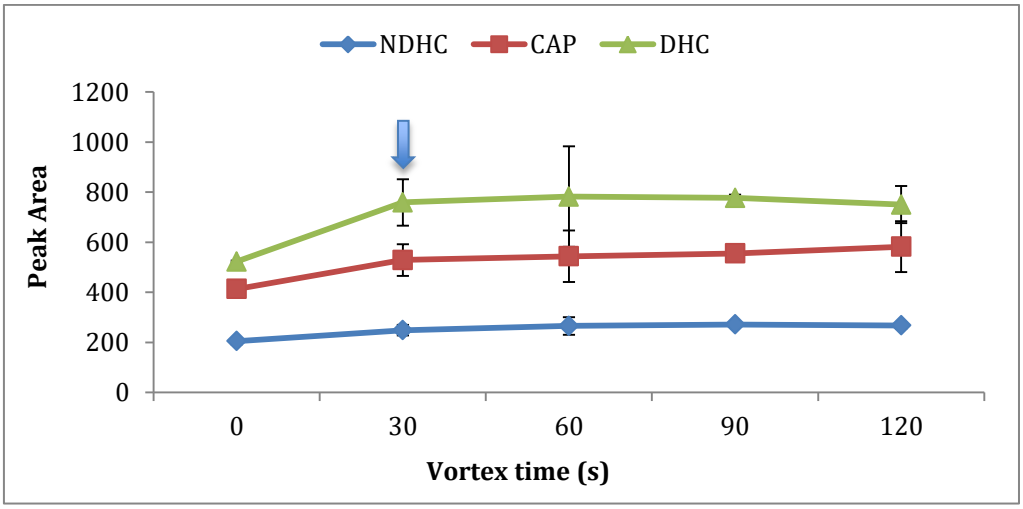


Figure 3.24: Effect of extraction time.

There was a sharp increase in peak area from 0 to 30 s vortex time where it remained constant. Therefore, 30 s was considered as the optimum vortex time.

3.7.4 Effect of the Volume of the Disperser Solvent (ACN)

Since SOE was used prior to DLLME, there was no point of trying different types of disperser solvent. ACN is well known as the most suitable solvent for SOE. The disperser solvent volume has a direct effect on the formation of cloudy solution. Consequently, the degree to which the extraction solvent is dispersed in the aqueous phase affects the extraction efficiency [29].

The effect of this parameter was investigated by taking 1000 µL of the salted-out ACN after SOE and being subjected to DLLME without and with pure ACN added at intervals of 250 µL. The effect of the ACN was experimented within the range of 0-1125 µL. The optimum volume, as can be seen from **Figure 3.25** was 1250 µL. Starting from this point, peak area remained constant up to 2000 µL where further addition of ACN reduced peak area, which was thought to be due to increased solubility of the analytes in the aqueous solution.

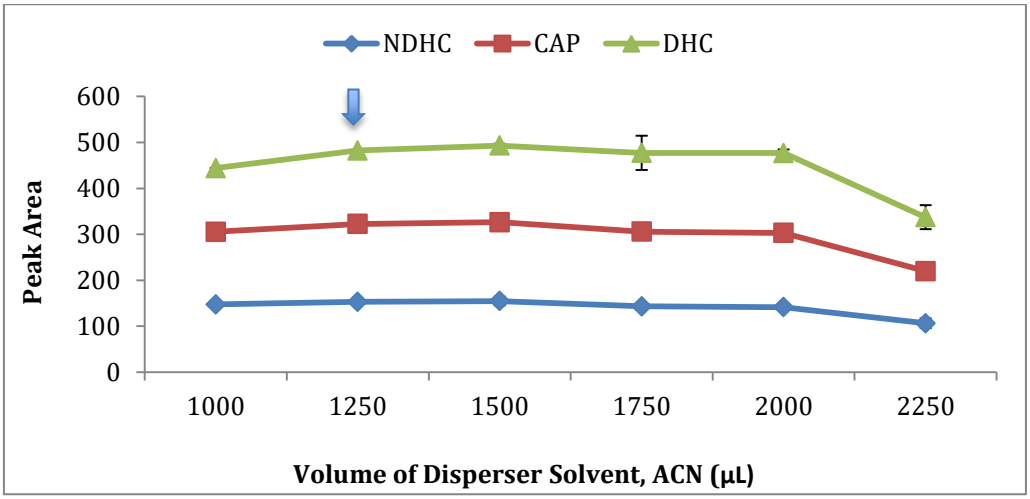


Figure 3.25: Effect of disperser solvent volume.

3.7.5 Effect of Salt Addition

Generally, the addition of salt can assist in decreasing the solubility of the analyte in the aqueous phase and enhancing its solubility in the organic extraction phase [44]. Salts can also act as alternative demulsifier by reducing the zeta potential, thereby decreasing the stability of the hydrophobic colloid and increasing the ionic strength of the solution, which lead to flocculation and coalescence of fine organic solvent droplets. This could eventually lead to separation of the two phases [45].

The effect of the concentration of NaCl on extraction efficiency was evaluated from 0 to 8% (w/v). The addition of NaCl gave a negative effect on the extraction efficiency as seen from the consistent decrease in peak area with increase in the concentration of NaCl (**Figure 3.26**). Therefore, no NaCl was added in further experiments.

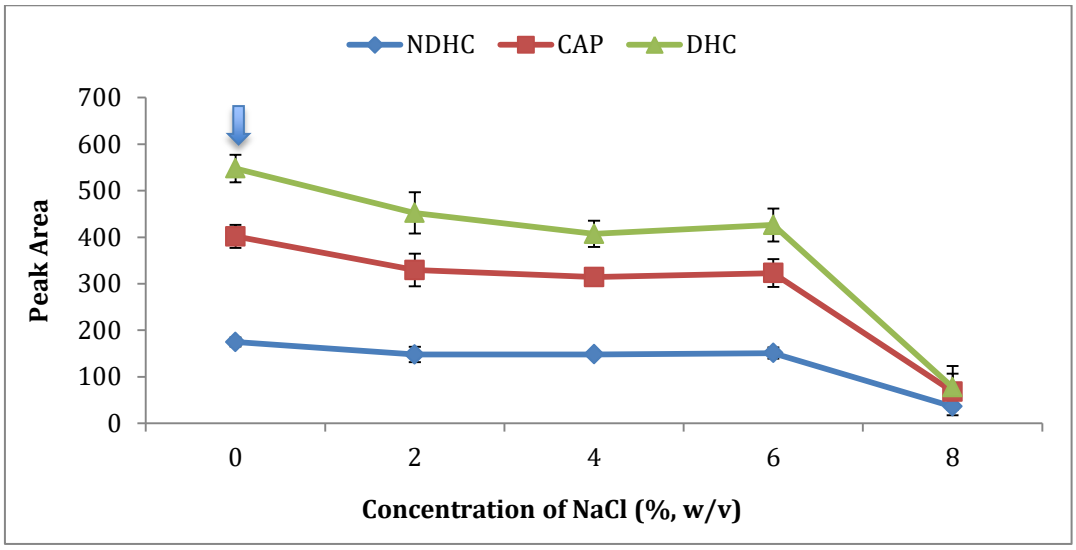


Figure 3.26: Effect of salt addition on extraction efficiency.

3.7.6 Effect of Back-Extraction Volume

The effect of BES volume on extraction efficiency was evaluated starting from 100 up to 400 µL. The extraction efficiency increased with increase in BES volume until 300 µL (**Figure 3.27**), which signaled the maximum extraction capability. Further increase in BES volume resulted to decrease in extraction efficiency, which could be due to dilution of the analyte due to excess volume of BES. Thus, 300 µL BES was taken as optimum.

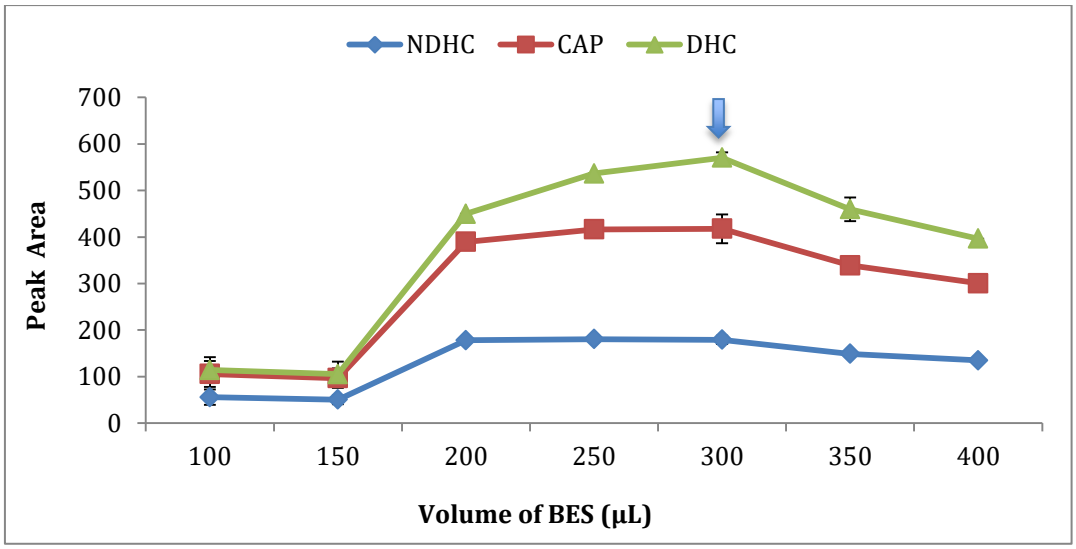


Figure 3.27: Effect of BES volume on extraction efficiency.

3.7.7 Effect of Back-Extraction Time

The effect of back-extraction time was evaluated starting from 0-60 s. There was a significant increase from 0 to 15 s after which relatively constant peak areas were obtained as shown in **Figure 3.28**. This value (i.e., 15 s) was taken as the optimum back-extraction time.

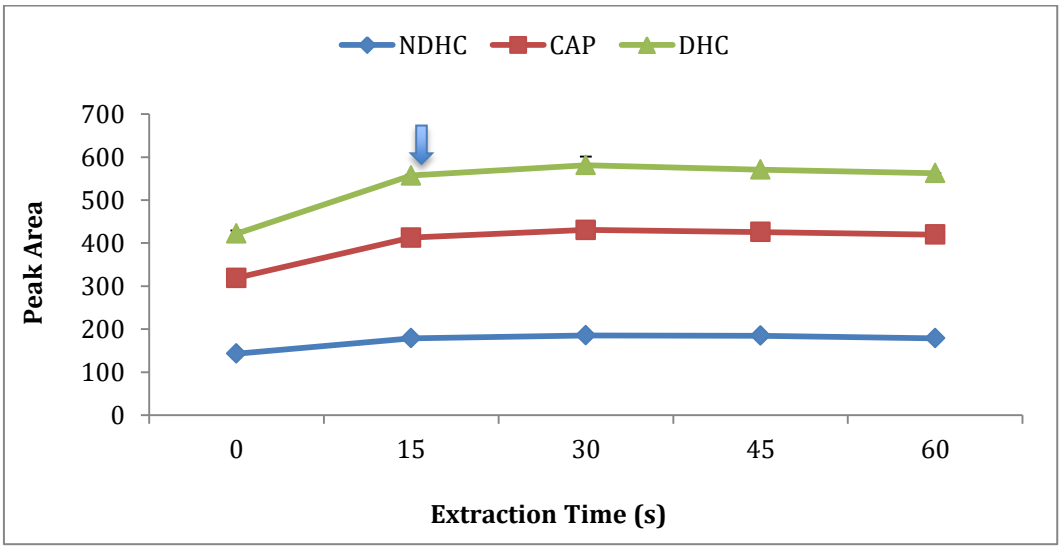


Figure 3.28: Effect of BE time on extraction efficiency.

3.8 Optimum DLLME-BE Conditions

The optimum DLLME-BE conditions for this study are summarized in **Table 3.2**.

Table 3.2: Optimum DLLME-BE conditions.

DLLME	Extraction solvent	Chloroform
	Volume of extraction solvent	100 µL
	Disperser solvent	Acetonitrile
	Volume of disperser solvent	1250 µL
	Acidification with	Acetic acid, 100 µL
	Extraction time	30 s
BE	Back-extraction solution	55/45% (v/v) MeOH/50 mM NaOH
	Volume of back-extraction solution	300 µL
	Back-extraction time	15 s

3.9 Peak Characterization with HPLC

Peak characterization was done by injecting individual standards into HPLC and comparing their retention times with the mixed standard solution, each standard at a concentration of 50.0 $mg\ L^{-1}$. Since the identity of the standards is now known, each peak was assigned a name based on the retention time of the standards. The chromatograms obtained with the standards and the mixtures are given in **Figure 3.29**.

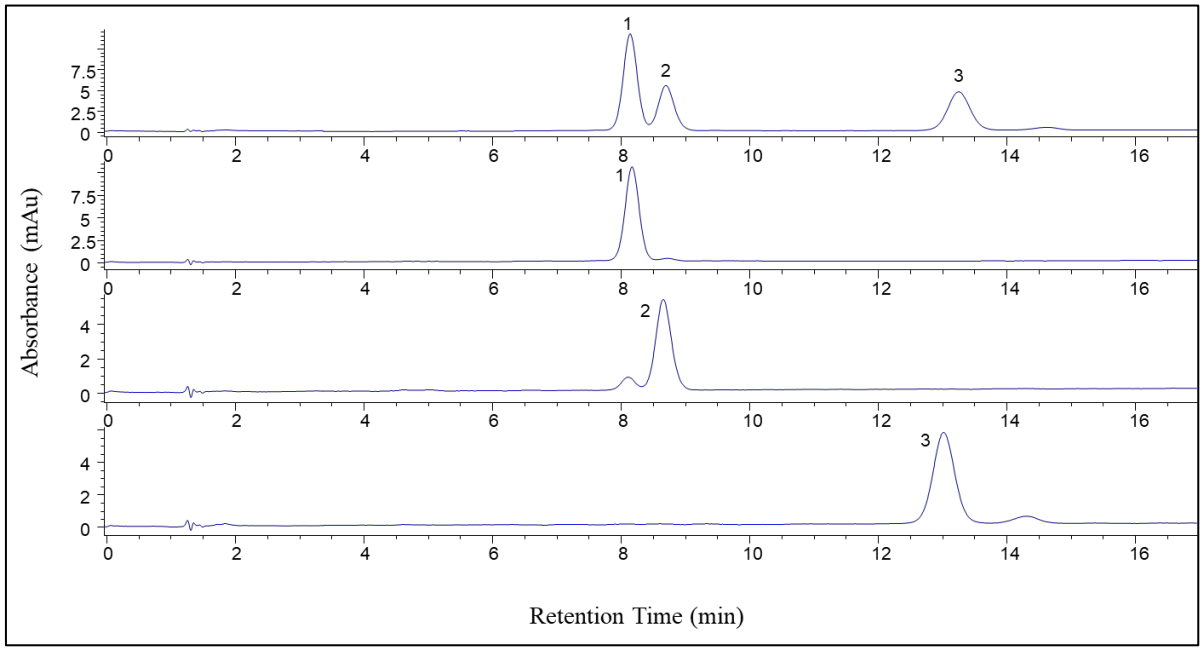


Figure 3.29: Peak characterization of capsaicinoids. Peaks: 1, NDHC; 2, CAP; 3, DHC.

3.10 Calibration, Quantitation and Figures of Merit

Calibration graphs were plotted under optimized RP-HPLC conditions using the standards of capsaicinoids to evaluate the performance of the method. Standards were prepared in BES within a concentration range of 10-150 $mg\ L^{-1}$ and a calibration graph was plotted for each standard as peak area versus concentration as shown in **Figure 3.30.**

Under optimized RP-HPLC conditions, LODs (calculated based on $3S_b/m$, where; S_b is the standard deviation of the intercept and m is the slope of the regression equation), ranged from 1.4 to 3.1 mg L^{-1} (**Table 3.3**) and limits of quantitation (LOQ, based on $10S_b/m$) ranged from 4.5 to 10.3 mg L^{-1}. The average enrichment factor for capsaicinoids was calculated as 4.4 for NDHC, 10.2 for CAP and 9.5 for DHC.

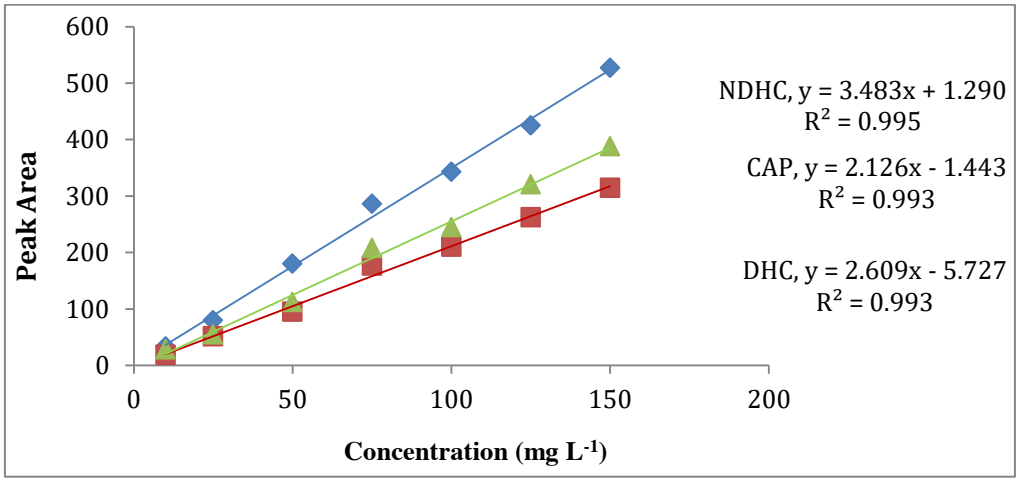


Figure 3.30: Calibration curves for capsaicinoid standards.

Table 3.3: Analytical performance parameters of DLLME-HPLC.

Analyte	Regression equation[a]	R^2	RSD[b]		LOD[c]	LOQ[d]	LDR[e]	EF[f]
			Intraday	Interday				
NDHC	y=3.48(±0.05)x+1.25(±4.73)	0.9957	5.1	5.9	2.7	9.0	9.0-150	4.4
CAP	y=2.13(±0.02)x-4.65(±1.47)	0.9991	3.7	4.7	1.4	4.5	4.5-150	10.2
DHC	y=2.58(±0.04)x-1.45(±4.18)	0.9954	6.9	7.8	3.1	10.3	10.3-150	9.5

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

[b] Percentage relative standard deviation, n = 3

[c] Limit of detection

[d] Limit of quantitation

[e] Linear dynamic range

[f] Enrichment factor

For evaluating the applicability, recovery and possible matrix effect of the proposed DLLME–HPLC method, six pepper samples were examined. Typical chromatograms of unspiked and spiked samples are shown in **Figure 3.31**. Absence of interfering peaks at the migration times of the three capsaicinoids indicated good selectivity of the method. Furthermore, the three analytes were baseline-separated in less than 15 min. The recoveries obtained for unspiked and spiked samples at two concentration levels (i.e., 2.5 and 5 mg L^{-1} of each capsaicinoid) are listed in **Table 3.4**.

Table 3.4: Percentage recoveries of capsaicinoids from pepper.

Pepper	Added (mg L^{-1})	Found (mg L^{-1})			%R[a]		
		NDHC	CAP	DHC	NDHC	CAP	DHC
SGC	-	<LOQ	26.6 (105.2 mg kg^{-1})	17.7 (99.6 mg kg^{-1})	-	-	-
	2.5	11.54	31.6	21.2	27.7	75.8	50.9
	5.0	27.50	63.2	46.5	33.0	75.8	55.8
GPP	-	9.48 (333.2 mg kg^{-1})	38.2 (449.2 mg kg^{-1})	38.78 (467.9 mg kg^{-1})	-	-	-
	2.5	17.20	52.0	50.14	41.3	124.9	120.3
	5.0	22.62	66.3	65.51	27.1	79.5	78.6
LGP	-	<LOD	<LOD	<LOD	-	-	-
	2.5	8.90	20.5	25.33	21.4	49.2	60.8
	5.0	19.80	39.7	42.63	23.8	47.7	51.2
YP	-	<LOD	<LOD	<LOD	-	-	-
	2.5	10.47	16.56	16.54	25.1	39.7	39.7
	5.0	18.13	36.05	41.66	21.8	43.3	50.0
LRP	-	<LOD	<LOD	<LOD	-	-	-
	2.5	9.0	22.69	20.51	21.6	54.5	49.2
	5.0	25.75	50.5	47.06	30.9	60.6	56.5
RBP	-	<LOD	<LOD	<LOD	-	-	-
	2.5	9.92	17.4	13.78	23.8	41.8	33.1
	5.0	16.60	32.22	34.02	19.9	38.7	40.8

[a] % Recovery, obtained by considering extraction yield compared with theoretical yield.

The percentage recovery was used to estimate the actual concentration of the capsaicinoid in SGC, which was found to contain 105.2 mg kg^{-1} of CAP and 99.6 mg kg^{-1} of DHC. Although a small peak was obtained for NDHC, the concentration was still below LOQ. For GPP, all three capsaicinoids were identified and quantified with a concentration of 333.2 mg kg^{-1} of NDHC, 449.2 mg kg^{-1} of CAP and 467.9 mg kg^{-1} of DHC. The other peppers may have contained the analytes at concentrations below the LOD of this method, hence could not be detected.

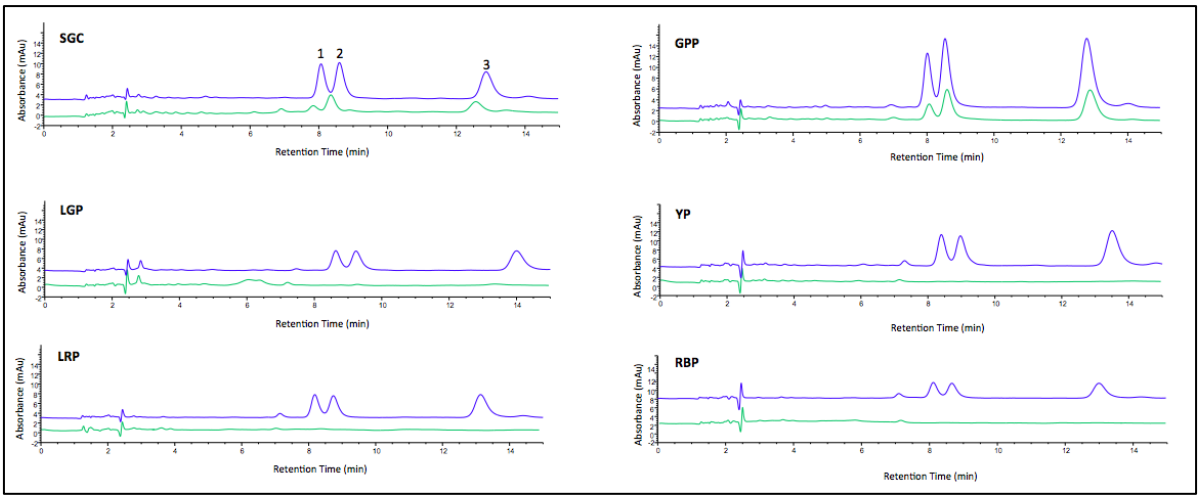


Figure 3.31: Representative chromatograms of samples extracted and analyzed under optimum DLLME-HPLC conditions. Top chromatogram; spiked. Bottom chromatogram unspiked. Spiked concentration level: 5.0 mg L^{-1} of each analyte. Peaks: 1, NDHC; 2, CAP; and 3, DHC.

3.11 Isolation of Capsaicinoids

For the isolation of the three most abundant capsaicinoids (i.e., CAP, DHC and NDHC), an initial attempt was made using column chromatography. However, with this simple chromatographic technique, isolation of the capsaicinoid was not possible due to their very closely related structures. Therefore, medium-pressure liquid chromatography (MPLC) was used to isolate the capsaicinoids.

3.11.1 Column Chromatography

Determination of the solvent system was the first step to be considered. Given that the optimum HPLC mobile phase for the separation of the analytes was 55% MeOH in water, a reversed-phase TLC experiment was carried out with 60% MeOH solvent system similar to the composition of the mobile phase but no separation was achieved as shown in **Figure 3.32**.

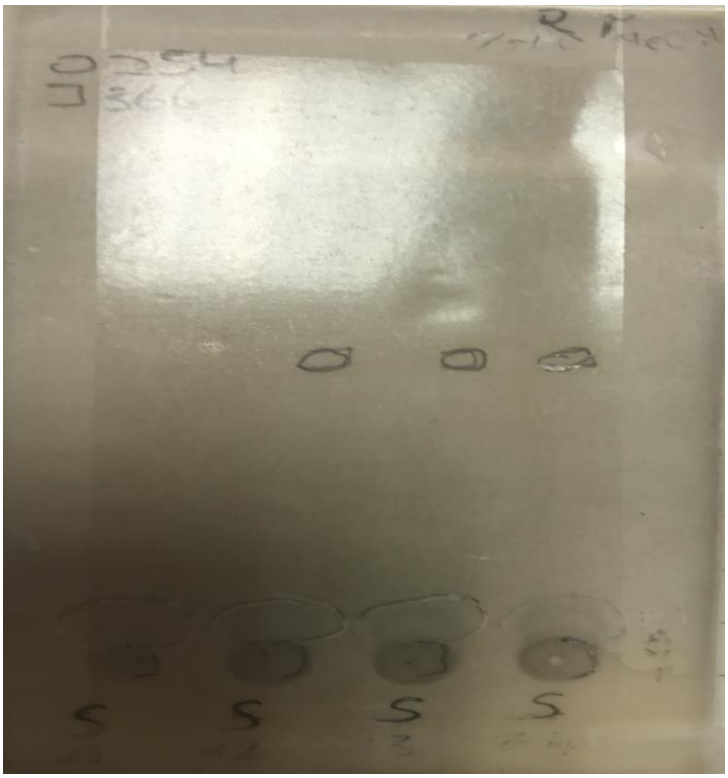


Figure 3.32: RP-TLC with 60% MeOH solvent system.

A silica gel plate was then used using a mobile phase composition of EtOAC-Toluene (3:7, v/v) and separation was observed as shown in **Figure 3.33**.

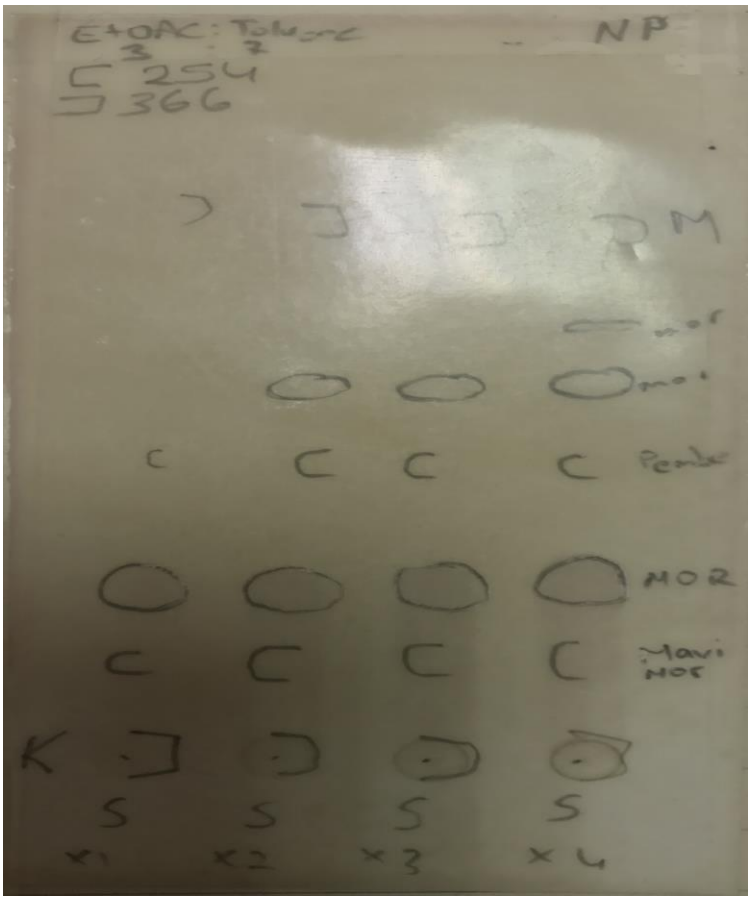


Figure 3.33: NP-TLC with AtOAC:Toluene (3:7, v/v) solvent system.

A normal-phase column (silica gel 60 (0.063-0.200 mm) was then selected for column chromatography with a three-solvent system similar to the one applied with TLC. Starting with 200 mL each of EtOAC-toluene 1:9 (v/v) and EtOAC-toluene 2:8 (v/v), respectively, and then EtOAC-toluene 3:7 (v/v) until no spots were observed from the fractions by TLC signaling the end of analysis.

Fractions were collected at regular intervals for TLC analysis to check if they contained the analytes and, subsequently, HPLC analysis were carried out to confirm the identity of the fractions based on their UV spectra. A deep green color was observed in the column as can be seen in **Figure 3.34** showing that chlorophyll from the pepper was probably strongly retained by the silica gel.

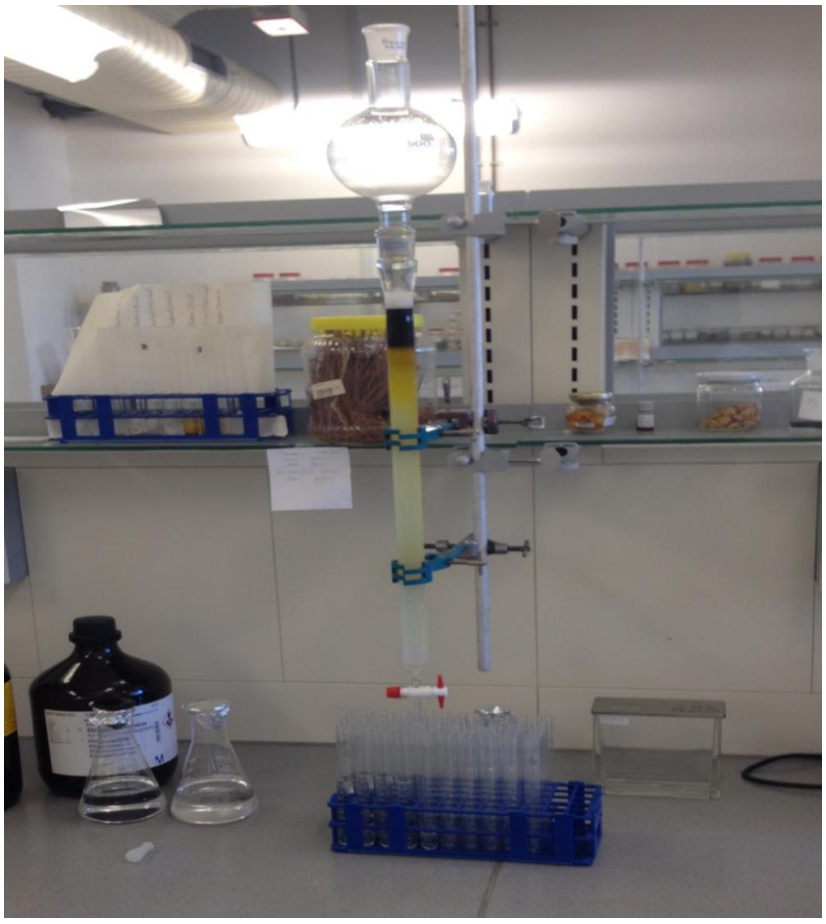


Figure 3.34: Setup of column chromatography containing the crude extract.

TLC analysis was carried out by numbering and spotting fractions 1 cm apart and comparing their R_f values with the reference sample from the initial crude extract spotted at the middle of the plate. A solvent system of EtOAC-toluene (3:7, v/v) and diethylether were compared as shown in **Figure 3.35** for the TLC.

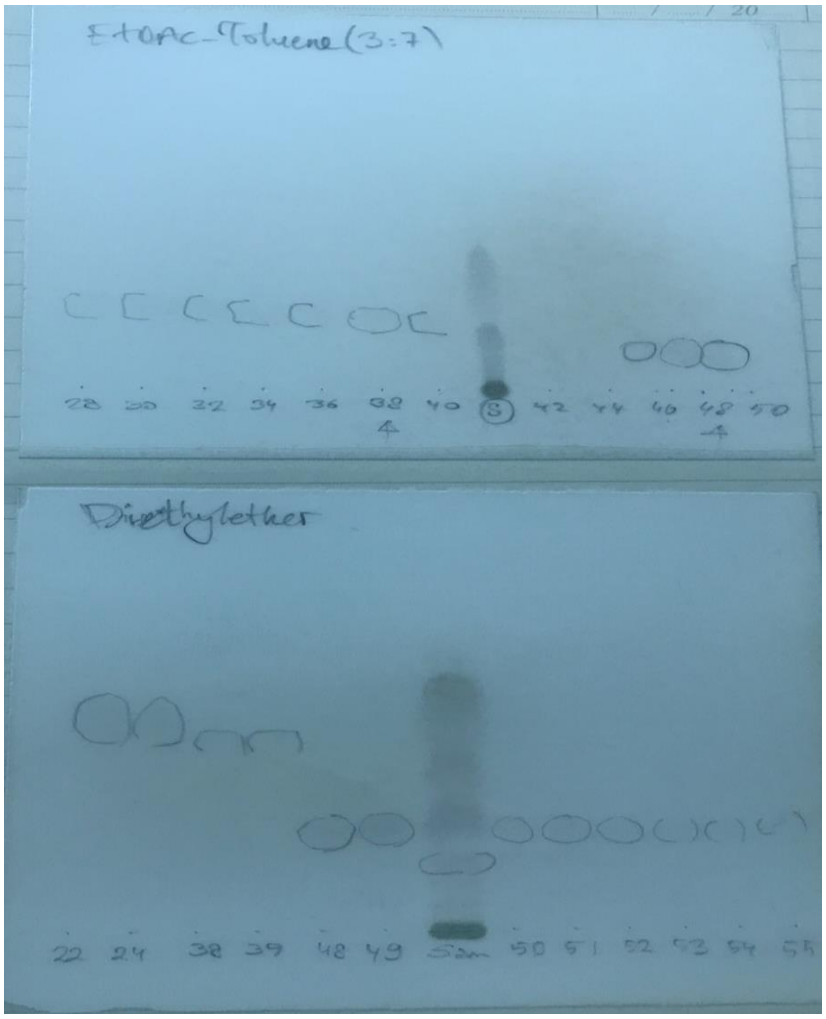


Figure 3.35: TLC Chromatogram of fractions obtained column chromatography.

It was observed from HPLC results that fractions 48-69 contained capsaicinoids but in their mixture form with varying composition from individual fractions; an unknown compound was also isolated below fraction 48.

The fractions containing the capsaicinoids were then evaporated-to-dryness to be used for MPLC.

3.11.2 Medium-Pressure Liquid Chromatography (MPLC)

Since the attempt made to isolate capsaicinoids using a normal-phase column chromatography was not completely successful, a reversed-phase MPLC was considered using a similar composition of MeOH/H_2O as with the optimum HPLC mobile phase composition, but a gradient elution was applied in this case because the result from column chromatography revealed how closely related the capsaicinoids were.

MPLC separation was then carried out using LiChroprep RP-18 (25-40 µm) column at a flow rate of 10 $mL\ min^{-1}$ with the gradient elution program as shown in **Figure 3.36.**

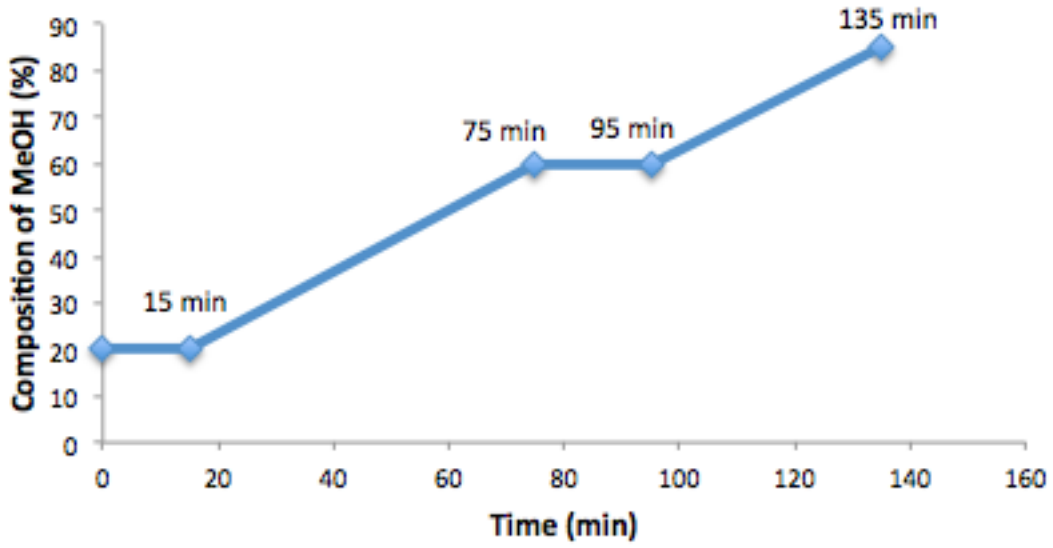


Figure 3.36: MPLC gradient elution program.

Fractions collected were spotted onto both a RP-C18 TLC plate with 60% MeOH (v/v) mobile phase as shown in **Figure 3.37** and a NP silica gel plate with diethylether mobile phase as shown in **Figure 3.38**.

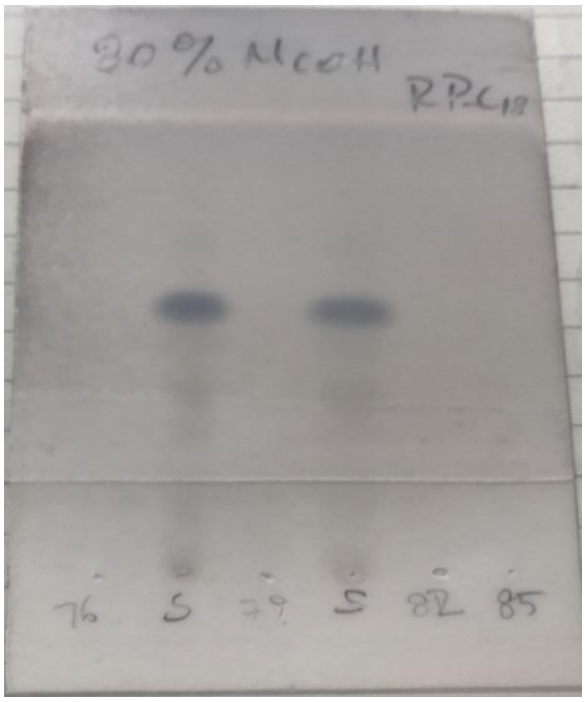


Figure 3.37: RP-TLC from MPLC fractions.

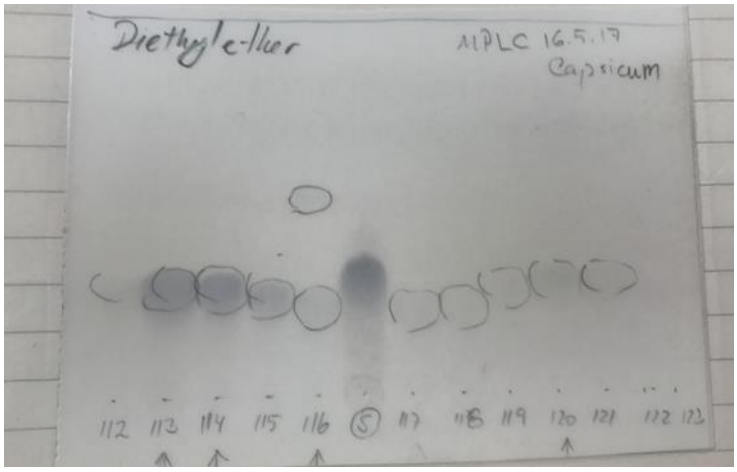


Figure 3.38: NP-TLC from MPLC fractions.

Fractions were injected into HPLC and the results obtain revealed that the three capsaicinoids were isolated successfully. The fractions associated with each of the analytes were evaporated using a rotary evaporator and lyophilized to obtain the pure crystals. The

purity of the crystals was determined by NMR and LC-MS. Pure standards obtained were used for quantitation studies with HPLC.

3.12 Characterization of Purified Standards by LC-MS and NMR

LC-MS and NMR were used for structural determination of the isolated capsaicinoid standards.

3.12.1 MS and NMR Spectra Analysis of Capsaicin

The positive ion ES-MS spectrum of capsaicin (CA-114) exhibited a quasi-molecular ion peak at m/z = 306.5 $[M+H]^+$ and 328.5 $[M+Na]^+$ indicating a molecular formula of $C_{18}H_{27}NO_3$ (calculated molecular weight = 305.4 g/mol) as shown in **Figure 3.39**.

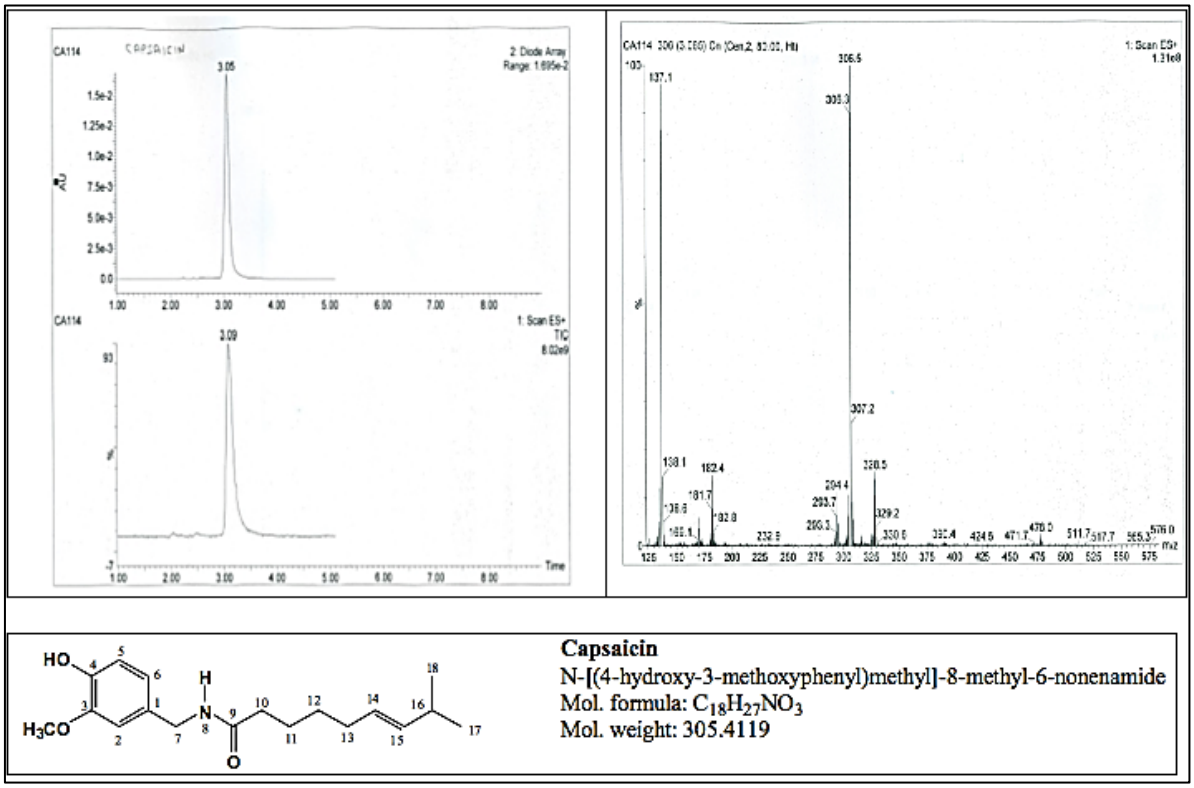


Figure 3.39: MS Spectra of Capsaicin.

The ^{1}H-NMR spectrum of capsaicin showed the signals between 0.80 and 7.0 ppm (**Figure 3.40**). In the low field of the spectrum (aromatic region), three protons were observed as an ABX system arising from the 1,3,4-trisubstituted phenyl unit of vanillylamine. These signals observed at δ 6.75 (dd, J 8.1 and 1.6 Hz), 6.80 (d, J 1.6 Hz) and δ 6.85 (d, J 8.1 Hz) were assigned as H-6, H-2 and H-5, respectively, of vanillylamine moiety of the structure together with an aromatic methoxy signal at δ 3.85. Additional signals in this region were two olefinic protons at δ 5.30 (ddt, J = 15.4, 5.7 and 5.7 Hz) and δ 5.36 (dd, J = 15.4, 5.7 Hz) assigned to trans located H-14 and H-15 of the acyl unit. The signal observed as a broad singlet at δ 5.73 was assigned to the NH of amide functionality. The signal at 4.35 ppm (d, J = 5.6 Hz) with two proton intensity were assigned to the methylene protons of the vanilliylamine. The rest of the signals were observed at the high field of the spectrum arising from the aliphatic protons of the acyl unit.

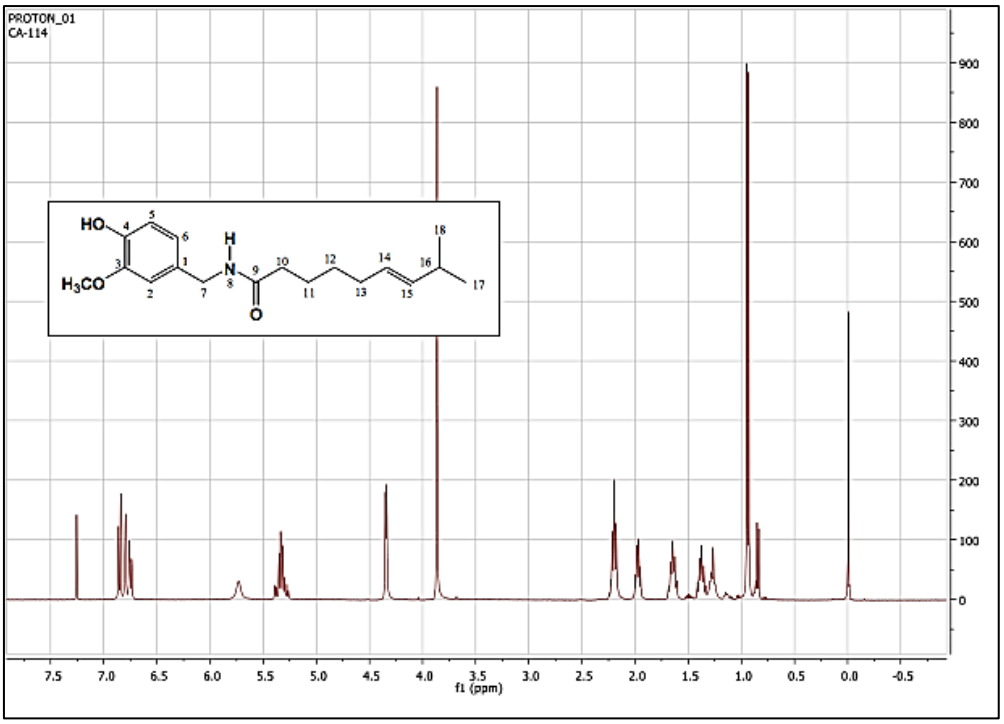


Figure 3.40: ^{1}H-NMR Spectrum of Capsaicin (CA-114)(400 MHz, $CDCl_3$).

A magnified version of the ^{1}H-NMR Spectra of capsaicin is given in **Figure 3.41** and **Figure 3.42** at different δ range for clarity.

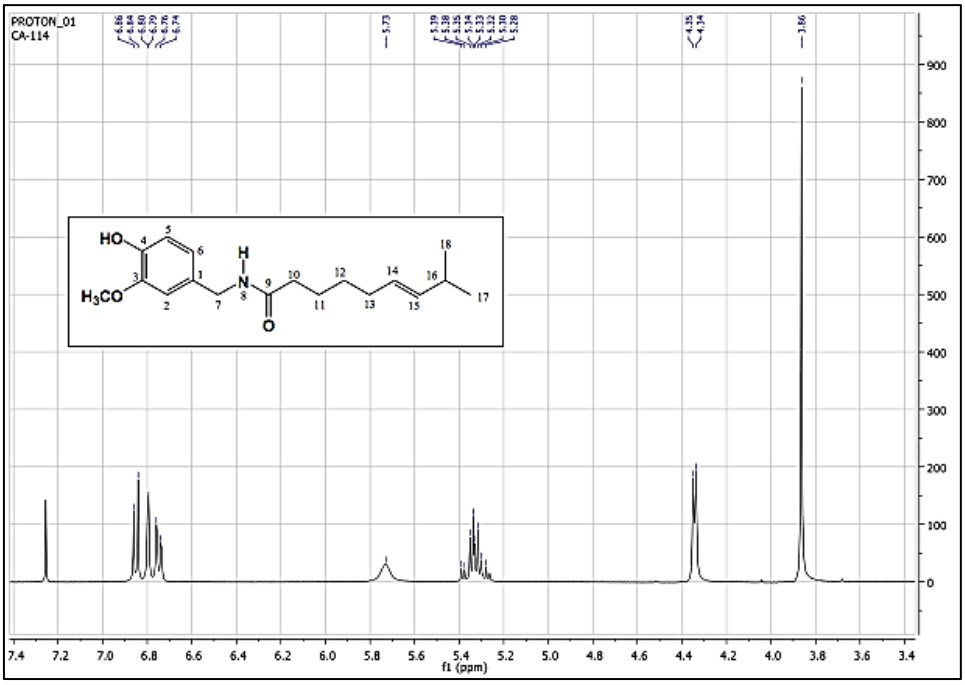


Figure 3.41: Magnified ^{1}H-NMR Spectrum of capsaicin from 7.4-3.4 ppm.

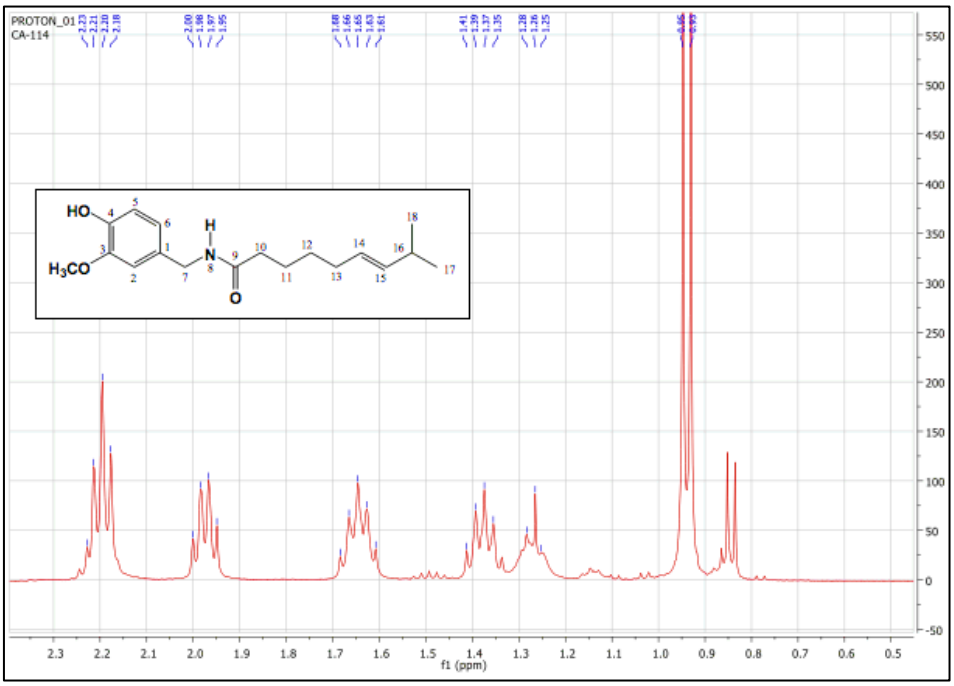


Figure 3.42: Magnified [1]H-NMR Spectrum of capsaicin from 2.3-0.5 ppm.

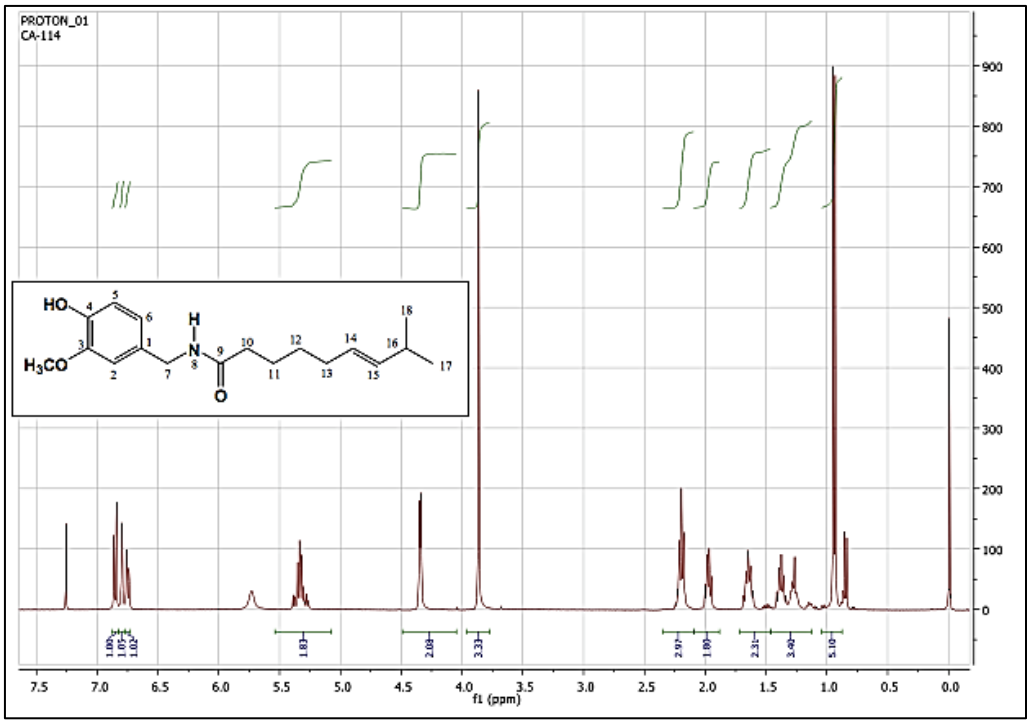


Figure 3.43: [1]H-NMR Spectrum of capsaicin with integration.

In the COSY spectrum (**Figure 3.44**), all protons were observed in three spin systems of which the first was the aromatic protons of the vanilliylamine unit while the second spin system was consisting of the two protons, NH and H_2-7 of the same unit. The remaining protons were observed in the third spin system attributed to acyl unit. For the interpretation of the protons of acyl unit, the signals with six proton intensity assigned as the terminally located two secondary methyl resonances at 0.94 ppm (d, J = 6.7 Hz) were selected. With the help of COSY correlations, a methine signal was assigned to H-16 (δ 2.20). Further COSY correlation was between the H-16 and one of the two olefinic protons, H-15 (δ 5.36 dd, J = 15.4 and 5.7 Hz). H-15 showed further correlation with the second olefinic proton H-14 (δ 5.30 ddt, J = 15.4, 5.7 and 5.7 Hz). Apart from H-14, the rest of the third spin system was found to be consisting of four methylene protons of H_2-13, H_2-12, H_2-11 and H_2-10, respectively.

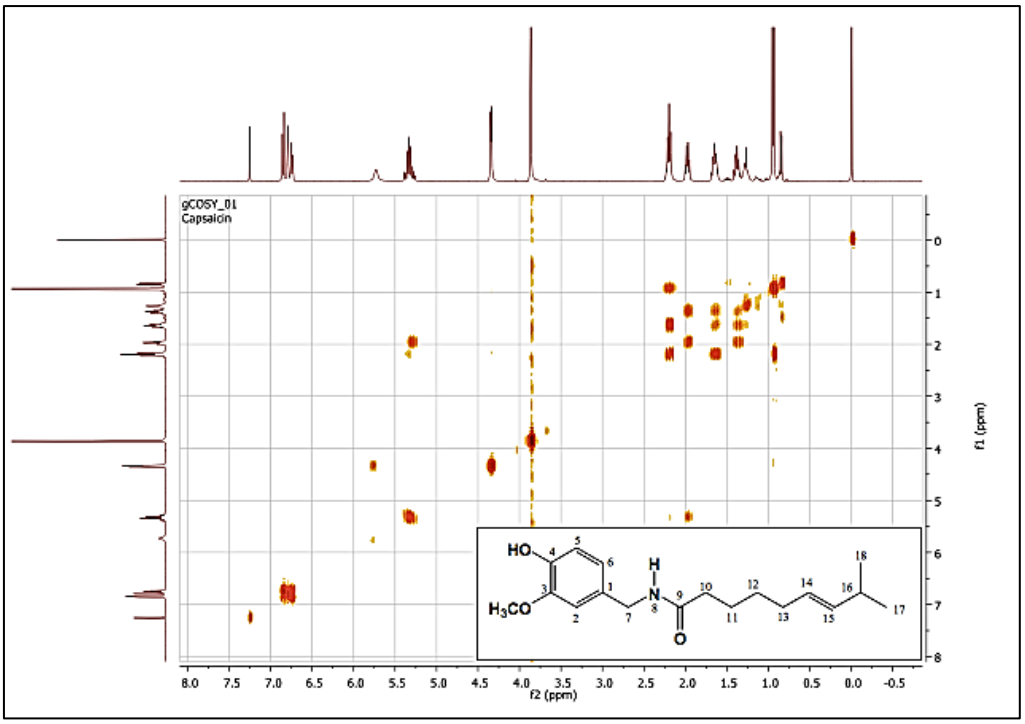


Figure 3.44: COSY (=^{1}H, ^{1}H-correlated spectrum) of capsaicin (CA-114).

The ^{13}C-NMR spectrum of capsaicin exhibited 18 carbon resonances as shown in **Figure 3.45**.

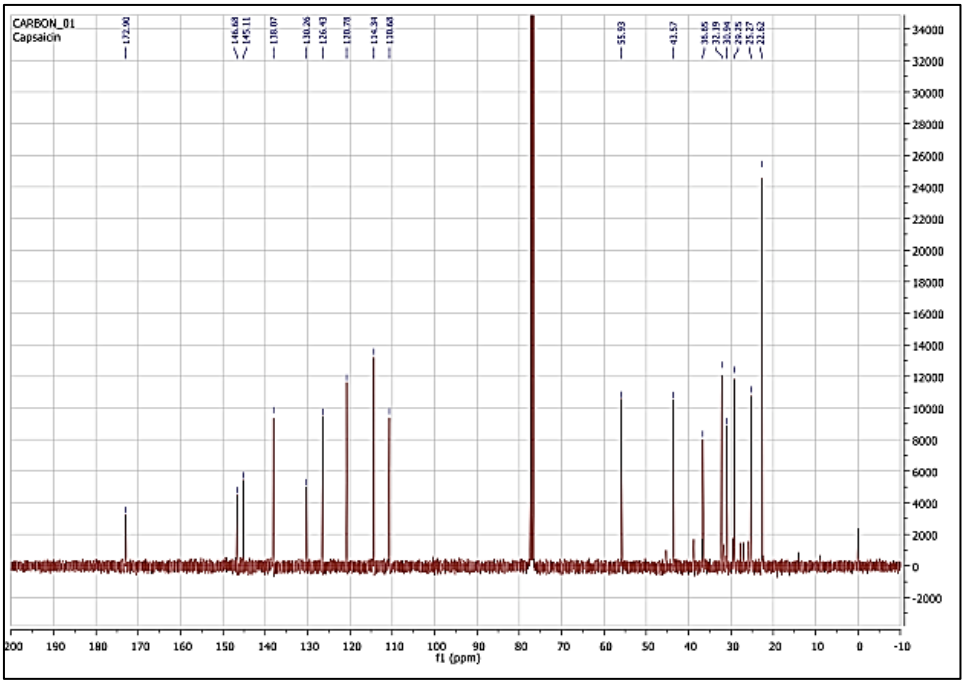


Figure 3.45: ^{13}C-NMR Spectrum of Capsaicin (CA-114) (100 MHz, $CDCl_3$).

An HSQC experiment made clear the assignments of each carbon resonance involved in the three spin systems. By the help of this experiment, the carbon resonances of vanillylamine and the acyl unit were clearly assigned. The HSQC spectrum of capsaicin is given in **Figure 3.46**.

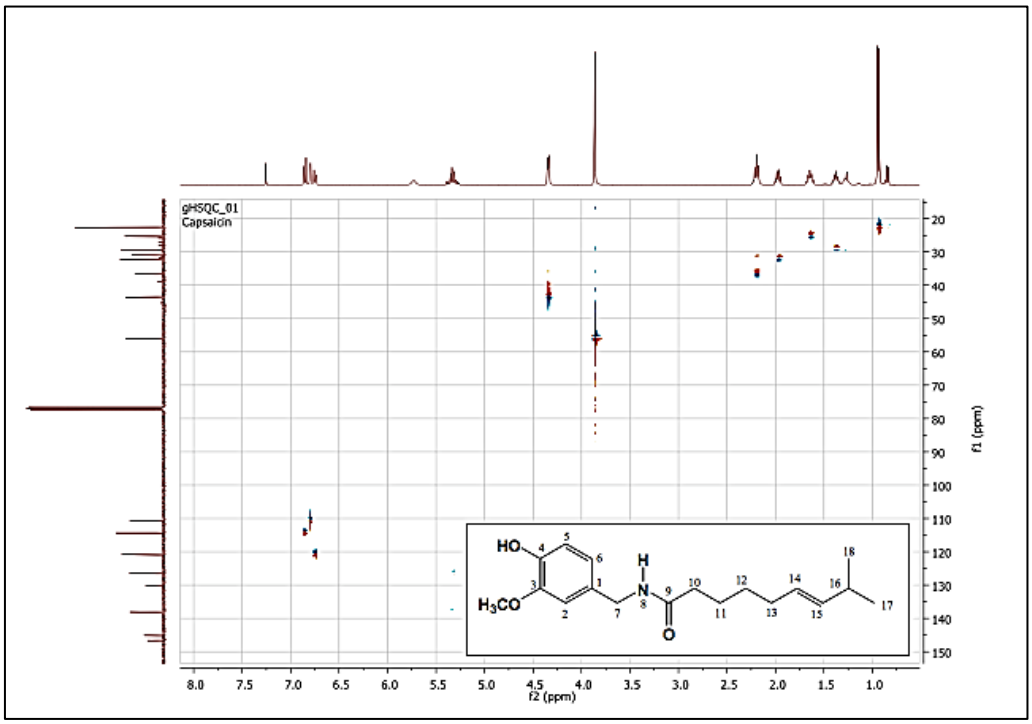


Figure 3.46: HSQC (=^{1}H, ^{13}C- short-range correlation spectrum of capsaicin) (CA-114).

The HMBC experiment made clear the intermolecular connectivity showing the ^{1}H and ^{13}C heteronuclear long-range correlations (**Table 3.5**). The significant HMBC correlations were observed between the C-3/ Ar-OCH_3, C-1/H_2-7, H-2 and H-6, C-7/ H-2 and H-6, C-9/H_2-7, C-16/H_3-17, H_3-18, H-14 and H-15.

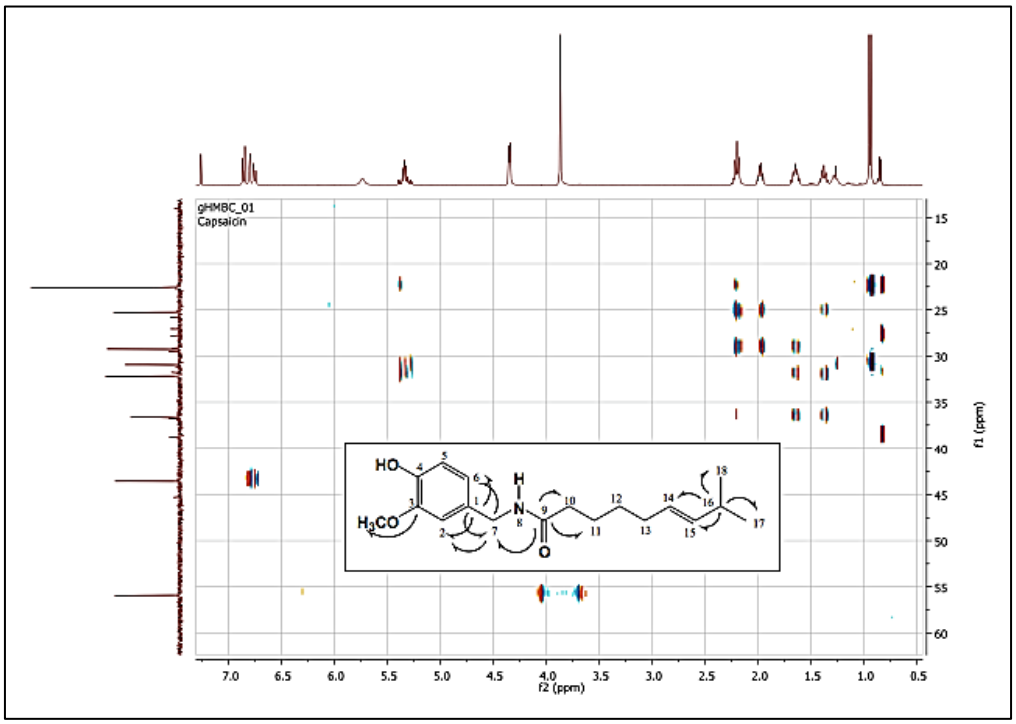
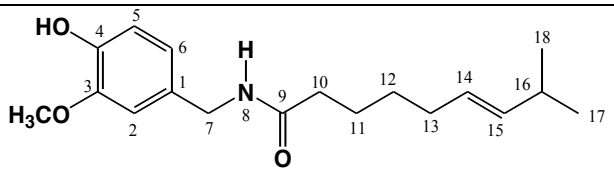


Figure 3.47: HMBC (=^{1}H, ^{13}C- long-range correlated spectrum) of capsaicin (CA-114).

The NMR spectral results for capsaicin are summarized in **Table 3.5**.

Table 3.5: ^{1}H and ^{13}C-NMR data of capsaicin and HMBC correlations (^{1}H: 400 MHz; ^{13}C: 100 MHz, $CDCl_3$).



CA-114: Capsaicin				
C/H		δ_c ppm	δ_H ppm, *J* (Hz)	HMBC (from C to H)
1	C	130.26	-	H-2, H-5, H_2-7
2	CH	110.68	6.75 dd (8.1 / 1.6)	H-6, H_2-7
3	C	146.68	-	OMe, H-2, H-5
4	C	145.11	-	H-2, H-6
5	CH	114.34	6.85 d (8.1)	-
6	CH	120.78	6.80 d (1.6)	H-2, H-5, H_2-7
7	CH_2	43.57	4.35 d (5.6)	H-2, H-6
8				
9	C	172.90	-	H_2-7, H_2-10, H_2-11
10	CH_2	36.65	2.20 dd "t" (7.8)	H_2-11, H_2-12
11	CH_2	25.27	1.65 q (7.8)	H_2-10, H_2-12
12	CH_2	29.25	1.37 q (7.8)	H_2-10, H_2-11, H_2-13
13	CH_2	32.19	1.98 q (7.8)	H-14, H-15, H_2-12, H_2-11
14	CH	126.43	5.30 ddt (15.4 / 5.7 / 5.7)	H-16, H_2-12
15	CH	138.07	5.36 dd (15.4 / 5.7)	H_3-17, H_3-18, H-16, H_2-13
16	CH	30.94	2.20 dq†	H_3-17, H_3-18, H-14, H-15
17	CH_3	22.62	0.94 d (6.7)	H-15, H-16
18	CH_3	22.62	0.94 d (6.7)	H-15, H-16
OCH_3	CH_3	55.93	3.85 s	-
NH	-	-	5.73 br s	-

Based on these experiments, the full assignment of proton and carbon resonances of capsaicin was determined.

3.12.2 MS and NMR Spectra Analysis of Nordihydrocapsaicin

The positive-ion ES-MS spectrum of NDHC (CA-112) exhibited a molecular ion peak at m/z 294 $[M+H]^+$, and 316.5 $[M+Na]^+$ indicating a molecular formula of $C_{17}H_{27}NO_3$ (calculated molecular weight = 293 g/mol). The MS spectrum of NDHC is given in **Figure 3.48**.

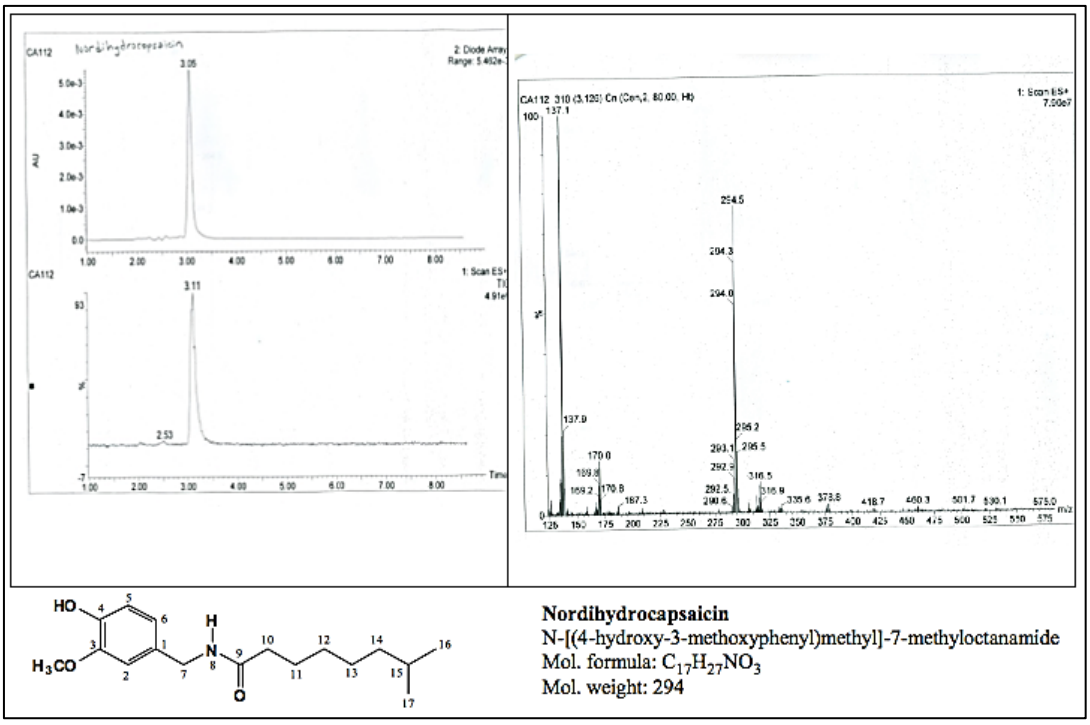


Figure 3.48: MS spectrum of nordihydrocapsaicin.

The ^{1}H-NMR spectrum of NDHC was very similar to that of capsaicin. All chemical shifts associated with the proton in the vanillylamine portion of the molecule and the first two carbons along with the acyl chain are identical. The major difference is the length of the acyl chain, which is shorter by a single unit, and also the absence of an olefinic proton that was observed in capsaicin. The ^{1}H-NMR spectrum of NDHC is given in **Figure 3.49**, while the integrated version is given in **Figure 3.50** for better details. The ^{1}H-NMR data for NDHC is summarized in **Table 3.6**.

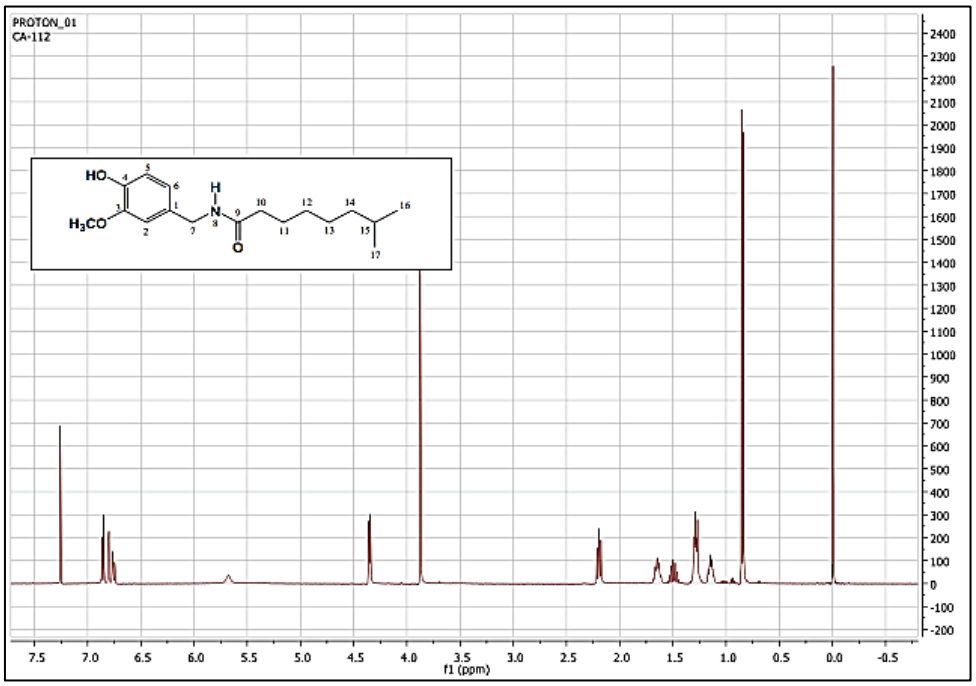


Figure 3.49: ^{1}H-NMR spectrum of NDHC (CA-112) (400 MHz, $CDCl_3$).

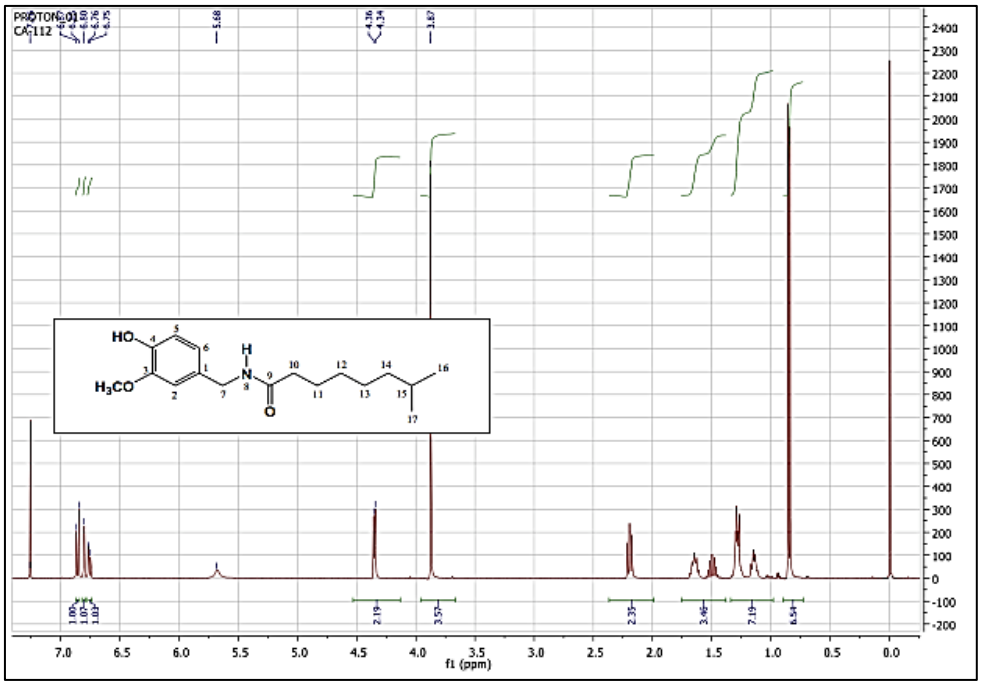


Figure 3.50: ^{1}H-NMR spectrum of NDHC with integration.

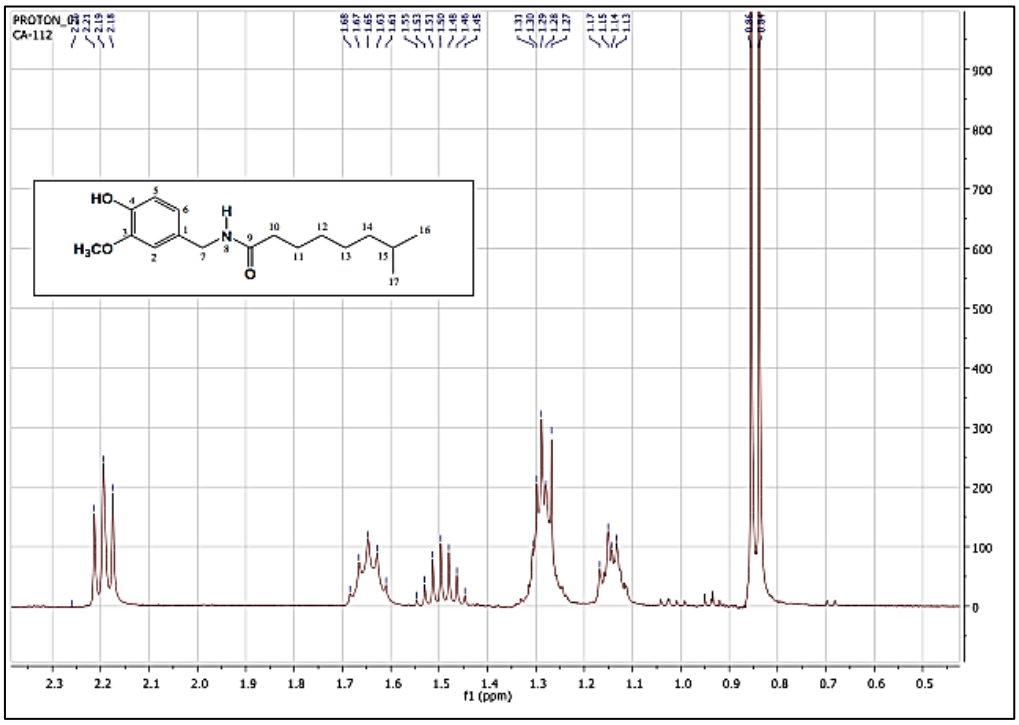


Figure 3.51: Magnified ^{1}H-NMR spectrum of NDHC.

The ^{13}C-NMR spectrum of NDHC exhibited 17 carbon resonances, also one unit short of that of capsaicin. The spectrum is given in **Figure 3.52**, while the data is summarized in **Table 3.6**.

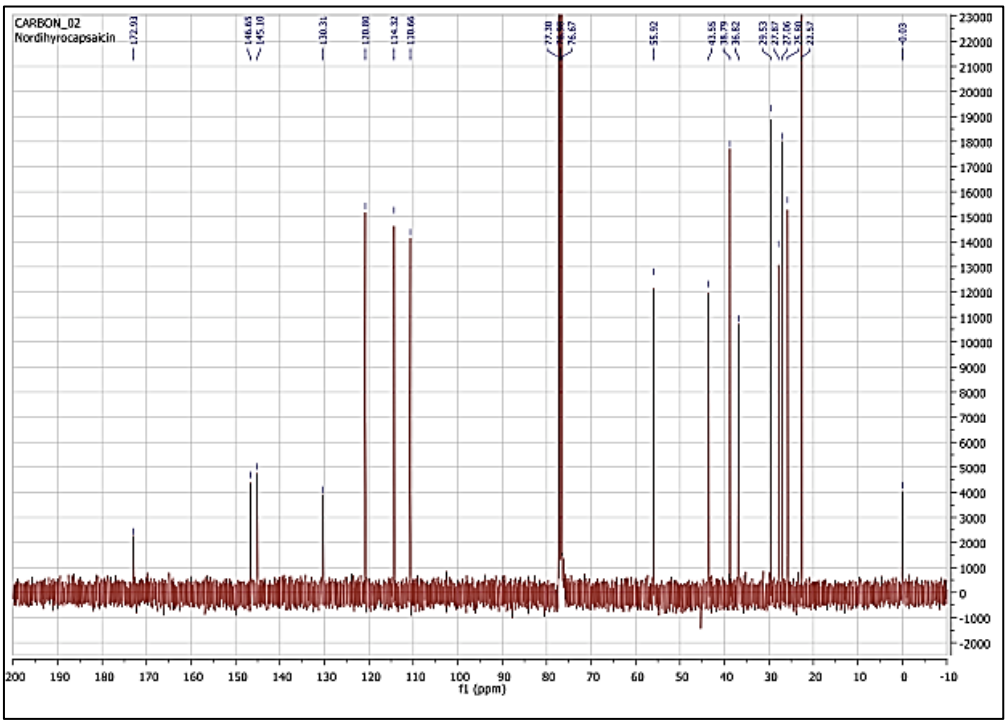
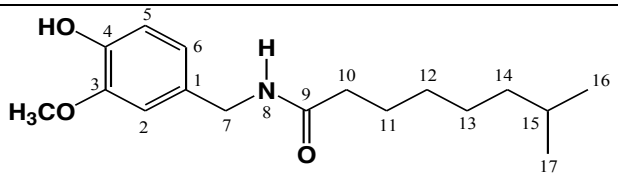


Figure 3.52: ^{13}C-NMR spectrum of NDHC (CA-112) (100 MHz, $CDCl_3$).

Table 3.6: ^{1}H and ^{13}C-NMR data of NDHC (^{1}H: 400 MHz; ^{13}C: 100 MHz, $CDCl_3$).



	CA-112: Nordihydrocapsaicin		
C/H		δ_c ppm	δ_H ppm, *J* (Hz)
1	C	130.31	-
2	CH	110.66	6.76 dd (8.1 / 1.8)
3	C	145.65	-
4	C	145.10	-
5	CH	114.32	6.86 d (8.0)
6	CH	120.80	6.81 d (1.8)
7	CH_2	43.55	4.35 d (5.1)
8			
9	C	173.08	-
10	CH_2	36.82	2.19 t (7.4)
11	CH_2	25.80	1.65 q (7.4)
12	CH_2	29.53	1.29 †
13	CH_2	27.06	1.29 †
14	CH_2	38.79	1.14 m
15	CH_2	27.87	1.50 septet (6.7)
16	CH_3	22.57	0.85 d (6.7)
17	CH_3	22.57	0.85 d (6.7)
OCH_3	CH_3	55.92	3.87 s
NH	-	-	5.74 br s

3.12.3 MS and NMR Spectra Analysis of Dihydrocapsaicin

The positive ion ES-MS spectrum of DHC (CA-120) exhibited a quasi molecular ion peak at m/z = 308.4 $[M+H]^+$ and 330 $[M+ Na]^+$ indicating a molecular formula of $C_{18}H_{19}NO_3$ (calculated molecular weight = 307.4 g/mol) as shown in **Figure 3.53**.

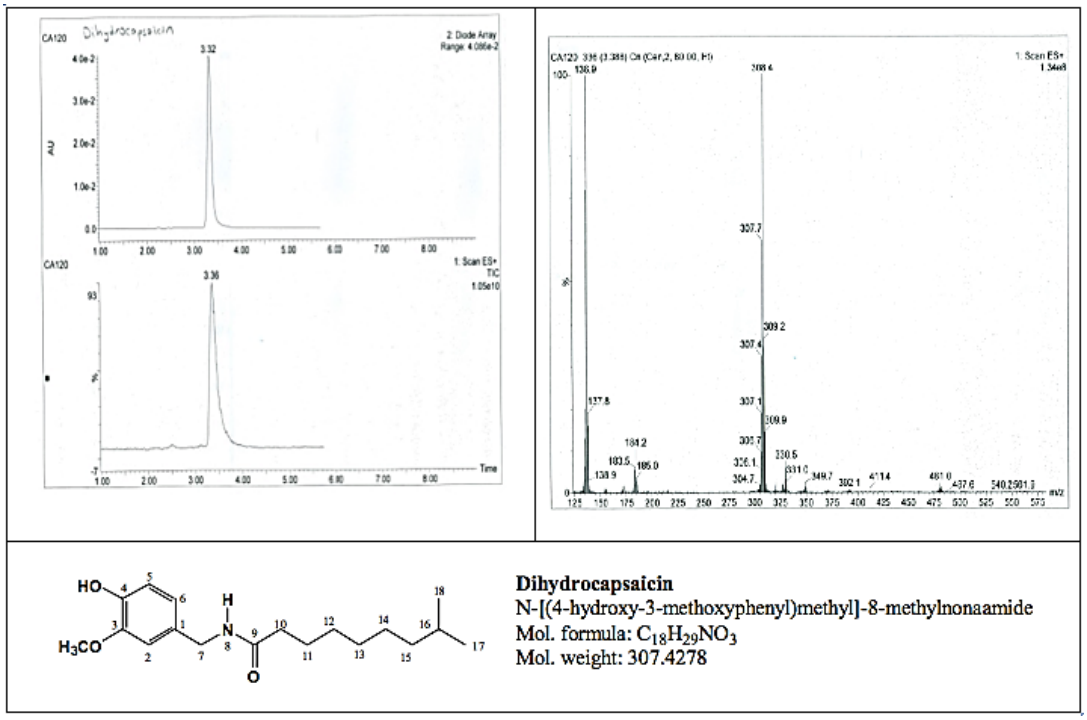


Figure 3.53: MS spectrum of DHC.

The ^{1}H-NMR spectrum of DHC is also very similar to that of capsaicin, also exhibiting similar chemical shifts associated with the protons in the vanillylamin portion of the molecule and the first two carbons along the acyl chain. The length of the acyl chain is also the same with that of capsaicin with the only difference being the absence of olefinic proton in the case of DHC. The ^{1}H-NMR spectrum of DHC is given in **Figure 3.54**, while the integrated version is given in **Figure 3.55**. NMR data of DHC is summarized in **Table 3.7**.

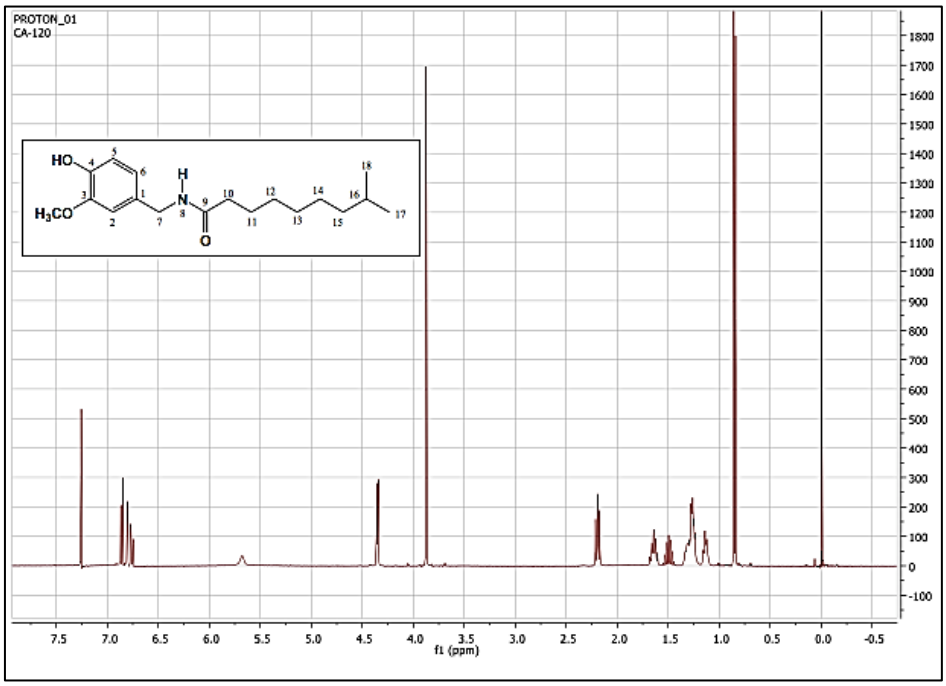


Figure 3.54: ^{1}H-NMR spectrum of DHC (CA-120) (400 MHz, $CDCl_3$).

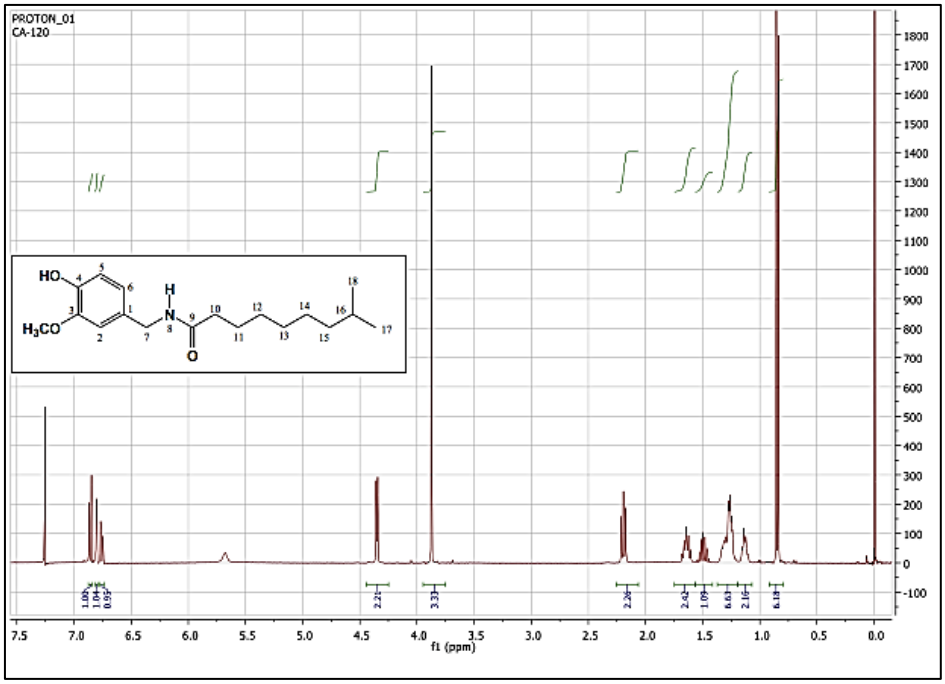


Figure 3.55: ^{1}H-NMR spectrum of DHC with integration.

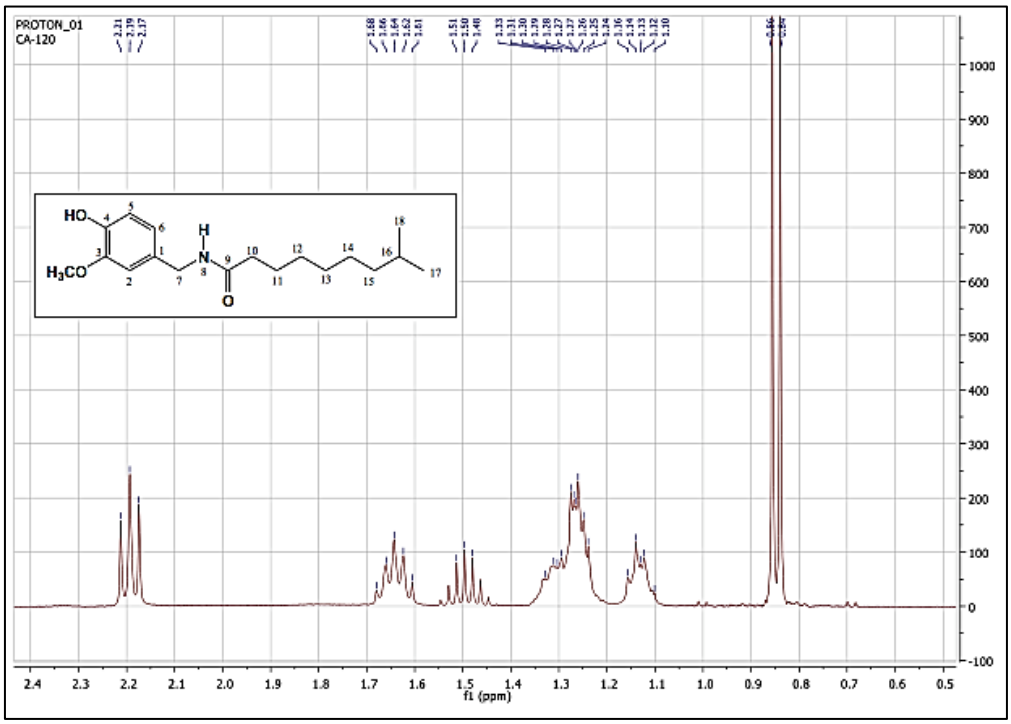


Figure 3.56: Magnified ^{1}H-NMR spectrum of DHC from 2.4-0.5 ppm.

The ^{13}C-NMR spectrum of DHC exhibited 18 carbon resonances as shown in **Figure 3.57**. The data is summarized in **Table 3.7**.

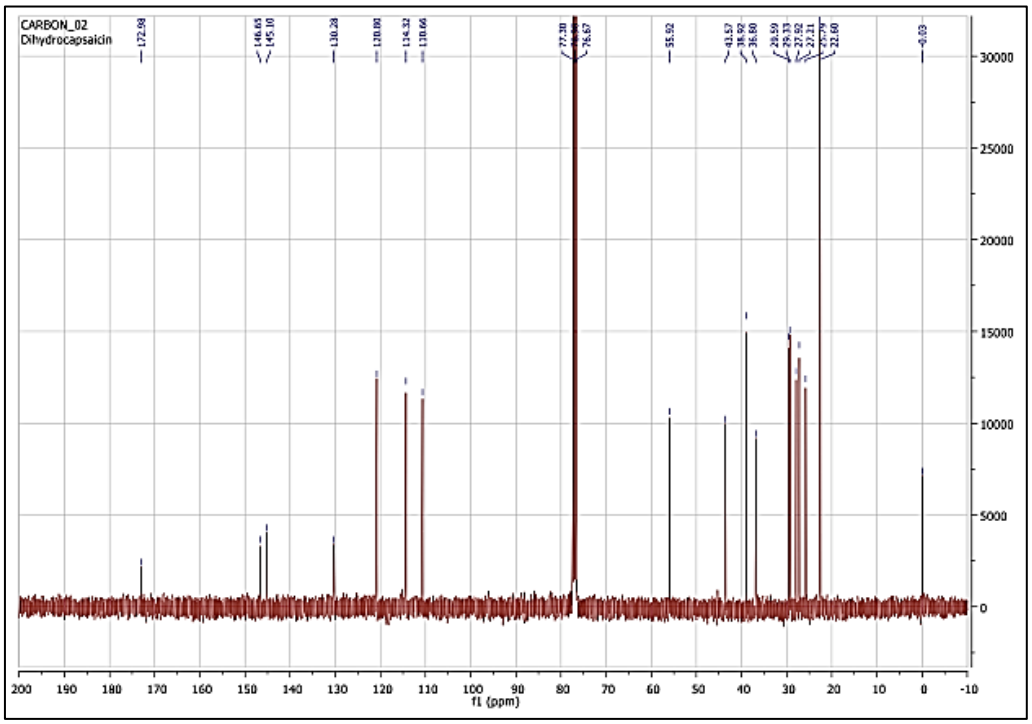
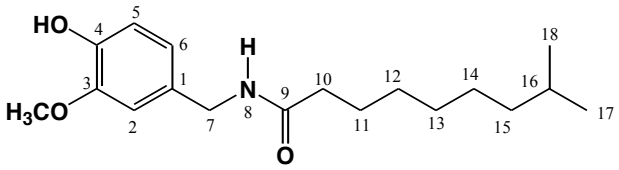


Figure 3.57: ^{13}C-NMR spectrum of DHC (CA-120) (100 MHz, $CDCl_3$).

Table 3.7: ^{1}H and ^{13}C-NMR data of dihydrocapsaicin (^{1}H: 400 MHz; ^{13}C: 100 MHz, $CDCl_3$).



CA-120: Dihydrocapsaicin

C/H		δ_c ppm	δ_H ppm, *J* (Hz)
1	C	130.28	-
2	CH	110.66	6.76 dd (8.1 / 1.8)
3	C	145.65	-
4	C	145.10	-
5	CH	114.32	6.86 d (8.0)
6	CH	120.80	6.81 d (1.8)
7	CH_2	43.57	4.36 d (5.1)
8			
9	C	172.82	-
10	CH_2	36.80	2.19 t (7.8)
11	CH_2	25.79	1.64 q (7.8)
12	CH_2	29.33	1.31 †
13	CH_2	29.59	1.28 †
14	CH_2	27.21	1.26 †
15	CH_2	38.92	1.13 m
16	CH	27.92	1.50 septet (6.7)
17	CH_3	22.60	0.85 d (6.7)
18	CH_3	22.60	0.85 d (6.7)
OCH_3	CH_3	55.92	3.87 s
NH	-	-	5.74 br s

CHAPTER 4
CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to provide a simple, efficient and robust reversed-phase high-performance liquid chromatography method that can be applied for the determination of major capsaicinoids in peppers.

Some targets set for the study were that the extraction method considered by many as the “bottle neck” of method development has to be selective for capsaicinoids by cleaning up the matrix from other interferences meanwhile preconcentrating the analytes of interest so that trace amounts in a sample could be detected.

The HPLC conditions had to be fast enough to be able to be applied for routine analysis and simple enough requiring the use of basic HPLC instrument that can be found in most laboratories, hence the preference for isocratic elution.

The method has to be also directly scaled up for the isolation of pure standards of capsaicinoids without further modification or additional steps and achieving high enrichment factors.

Dispersive liquid-liquid microextraction was demonstrated to perform efficiently for both determination of the amount of capsaicinoid as evident by the “clean” baseline obtained in the HPLC chromatograms of the sample and subsequent isolation of the pure standards.

The total extraction time for DLLME was 30 s, while the back-extraction time was 15 s making the total extraction time to be 45 s while all capsaicinoids eluted from HPLC within 15 min.

The study also demonstrated how various forms of chromatographic methods interact with each other. For example, the optimized HPLC conditions were used to estimate the solvent system and column packing material that will be suitable for MPLC. Thin-layer chromatography (TLC), which is relatively faster, was used for determining the identity of the fractions from MPLC before taking only the fractions containing some analytes and confirming their identity by HPLC reducing the amount of experiments needed. This shows how different chromatographic methods are complimentary to but not replacing each other.

This study also demonstrated the power of collaboration between the identification and quantitation strength of Analytical Chemistry and the isolation and purification strength of Pharmacognosy, mirroring the recent trend of interdisciplinary approach to solving problems facing the society because no single field is sufficient by itself.

It is the wish of the Authors that more collaboration is done in the future that would not only be limited to these two Departments, but that would also involve other Departments at the Faculty of Pharmacy and/or other faculties.

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