METHOD DEVELOPMENT FOR TOTAL CAPSAICINOIDS DETERMINATION BY USING THIN-LAYER CHROMATOGRAPHY AND PAPER-BASED ANALYTICAL DEVICE

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ABSTRACT

A new analytical platform based on the use of thin-layer chromatography (TLC) coupled with paper-based analytical device (PAD) was developed for the determination of total capsaicinoids in chilli samples. This newly developed TLC-PAD is simple and low-cost without any requirement of special instrument or skillful person. The analysis consisted of two steps, i.e., extraction of capsaicinoids from chilli samples by using ethanol as solvent and separation of capsaicinoids by thin-layer chromatography (TLC) and elution of capsaicinoids from the TLC plate with in situ colorimetric detection of capsaicinoids on the PAD. For colorimetric detection, Folin-Ciocalteu reagent was used to detect phenolic functional group of capsaicinoids yielding the blue color. The blue color on the PAD was imaged by a scanner followed by evaluation of its grayscale intensity value by ImageJ program. This newly developed TLC-PAD method provided a linear range from 50 to 1000 mg L^{-1} capsaicinoids with the limit of detection as low as 50 mg L^{-1} capsaicinoids. The proposed method was applied to determine capsaicinoids in dried chilli and seasoning powder samples and the results were in good agreement with those obtained by high-performance liquid chromatography method.

KEY WORDS: PAPER-BASED ANALYTICAL DEVICE / THIN-LAYER CHROMATOGRAPHY / TOTAL CAPSAICINOIDS / CHILLI

73 pages

การพัฒนาวิธีการตรวจวัดปริมาณรวมของแคปไซซินอยด์ด้วยวิธีรงคเลขผิวบางและอุปกรณ์ตรวจวิเคราะห์ฐานกระดาษ

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บทคัดย่อ

งานวิจัยนี้ได้ทำการพัฒนาแพลตฟอร์มการวิเคราะห์ใหม่บนพื้นฐานของการใช้รงคเลขผิวบาง (thin-layer chromatography) ควบคู่กับอุปกรณ์ตรวจวิเคราะห์ฐานกระดาษ (paper-based analytical device) ได้รับการพัฒนาสำหรับการตรวจวัดปริมาณรวมของแคปไซซินอยด์ในตัวอย่างพริก อุปกรณ์ TLC-PAD ที่พัฒนาขึ้นใหม่นี้ ง่ายและราคาถูกโดยไม่ต้องการเครื่องมือพิเศษหรือบุคคลที่มีทักษะมาก การตรวจวิเคราะห์ประกอบด้วยสองขั้นตอนคือ การสกัดแคปไซซินอยด์จากตัวอย่างพริกโดยใช้เอทานอลเป็นตัวทำละลายและการแยกของแคปไซซินอยด์ด้วยรงคเลขผิวบางและการชะของแคปไซซินอยด์จากแผ่นรงคเลขผิวบาง ใช้การตรวจวัดความเข้มสีของแคปไซซินอยด์บนอุปกรณ์ตรวจวิเคราะห์ฐานกระดาษ สำหรับการตรวจวัดทางสี น้ำยาฟอลินซิโอแคลตูถูกใช้ในการตรวจสอบกลุ่มฟีนอลของแคปไซซินอยด์ให้ผลผลิตสีน้ำเงิน ถ่ายภาพสีน้ำเงินบนอุปกรณ์ตรวจวิเคราะห์ฐานกระดาษโดยใช้สแกนเนอร์ตามด้วยการประเมินผลของค่าความเข้มของสีเทาโดยโปรแกรมอิมเมจเจ วิธี TLC-PAD ที่พัฒนาขึ้นใหม่นี้ให้ช่วงความเป็นเส้นตรงที่ 50-1000 มิลลิกรัมต่อลิตรของแคปไซซินอยด์กับขีดจำกัดของการตรวจวัดที่ 50 มิลลิกรัมต่อลิตรของแคปไซซินอยด์ วิธีที่นำเสนอถูกนำไปใช้ในการตรวจวัดปริมาณรวมของแคปไซซินอยด์ในพริกแห้งและตัวอย่างผงปรุงรสและผลลัพธ์ที่ได้สอดคล้องกับค่าที่ตรวจวัดได้โดยเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง

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LIST OF ABBREVIATIONS

%	Percentage
µg g^{-1}	Microgram per gram
µm	Microgram
cm	Centimeter
CS	Capsaicinoids
DC	Dried chilli
FC	Folin-Ciocalteu
g	Gram
h	Hour (s)
HPLC	High performance liquid chromatography
L	Liter
M	Molar
mg L^{-1}	Milligram per liter
mg mL^{-1}	Milligram per milliliter
mg	Milligram, 10^{-3} g
Min	Minute
mL	Milliliter, 10^{-3} L
mm	Millimeter
MΩ cm^{-1}	Mega ohm per centimeter
nm	Nanometer, 10^{-9} m
^{o}C	Degree Celsius
PAD	Paper-based analytical device
PVC	Polyvinyl chloride
R	Resolution
R_f	Retention factor or retardation factor
SP	Seasoning powder

LIST OF ABBREVIATIONS (cont.)

TLC	Thin-layer chromatography
v/v	Volume by volume
W	Width
w/v	Weight by volume
μL	Microliter

THE RELEVANCY OF THE RESEARCH WORK TO THAILAND

The food industry is rapidly growing in Thailand. Chilli is widely used in Thai cuisine to provide hotness and aroma and its quality depends on amount of capsaicinoids. Therefore, a simple, easy and inexpensive method for determination of capsaicinoids is needed. This work presents the use of thin-layer chromatography (TLC) to separate capsaicinoids from other matrix compounds in chilli. After separation from the TLC, the plate was cut into a small piece and placed onto the paper-based analytical device (PAD) for further determination of capsaicinoids by using Folin-Ciocalteu colorimetric assay. The proposed method offers wide working linear detection range with low detection limit (50 mg L^{-1}) and the results obtained were in good agreement with those obtained from the high performance liquid chromatography (HPLC) method. Clearly, the TLC-PAD method is suitable to be used as a screening test for capsaicinoids in raw chilli and its products.

CHAPTER I
INTRODUCTION

Capsaicinoids (CS) are a group of vanillylamides of fatty acids with varying length of their aliphatic side chains [1]. They are mostly found in chilli, one of the most commercial agro-products used both as condiment or culinary supplement in Thailand [2]. The two most abundant capsaicinoids in chilli are capsaicin and dihydrocapsaicin [3]. Capsaicinoids are added in food and consumed daily because they are famously known for their sensory attributes of hotness, aroma, and color. Furthermore, they provide health benefits such as high antioxidant activity [4], antitumor [5], antibacterial [6], antimutagenic [7], and anticarcinogenic properties [8]. Thus, capsaicinoids in chilli is one of major parameters for quality control of commercial chilli and pharmaceutical products [9].

Various methods can be used for determination of capsaicinoids in chilli including chromatographic analysis, such as high performance liquid chromatography (HPLC) with UV–visible detection [10], fluorescence detection [11], mass spectrometry detection [12], and gas chromatography (GC) [13]. Electrochemical analysis has also been used for capsaicin determination as capsaicin electrochemical sensors [14], and biosensors [15]. However, these techniques require expensive instrument and high skilled operators.

Paper-based analytical device (PAD) has gained recent interest as an analytical platform due to several advantages such as low cost, easy to fabricate, low reagent consumption, without requirement of high skilled operators. The PAD fabrication methods are mainly chemical modification and/or physical deposition processes for changing hydrophilic property of cellulose fiber on the paper (acting as channels and reservoirs) to hydrophobic area (acting as barriers) [16]. Many PAD fabrication methods can be used, such as photolithography [17], wax patterning [18], plotting [19], inkjet printing [20], inkjet etching [21], flexography printing [22], screen printing [23], and paper cutting [24]. These fabrication techniques offer advantages and

limitations which have been discussed in detail elsewhere [25-27]. In this work, paper cutting technique was used to prepare a simple PAD.

In this work, a simple and low cost technique for determination of capsaicinoids in chilli samples was developed. This proposed method was based on the separation of capsaicinoids from other components by using TLC. Then, the separated capsaicinoids on the TLC plate was eluted and further detected on the PAD by colorimetric method. Folin-Ciocalteu assay (FC) was used for capsaicinoids detection, which provided advantage over other colorimetric assays (such as vanadium oxytrichloride) due to its color stability [28]. The proposed method was applied to dried chilli and seasoning powder samples with reliable results.

CHAPTER II
OBJECTIVES

The objective of this work are as follows:

1) To develop a simple and low-cost method for determination of capsaicinoids by using thin layer chromatography coupled with paper based analytical device

2) To confirm the proposed method with high performance liquid chromatography

3) To apply the proposed method for the determination of total capsaicinoids in dried chilli and seasoning powder

CHAPTER III
LITERATURE REVIEWS

4.1 Capsaicinoids

In 1846, Thresh was the first crystallized the primary pungent principle and he named it capsaicin [29]. Subsequently, Nelson identified the compounds by hydrolysis and demonstrated that capsaicin was composed of 21 basic units (vanillylamine) and an acid component (an isomeric decenoic acid). Further, Nelson *et al.* established the structure of capsaicin as 8-methyI-6-nonenoyl vanillylamide [30, 31]. In 1962 Kosuge *et al.* suggested the term "capsaicinoids" for the mixture of these components [32].

Capsaicinoids (CS) are a group of vanillylamides of fatty acids with varying length of their aliphatic side chain (see Table 3.1). This is a family of related compounds from capsicum fruits. Depending on the variety of chilli, the concentration of capsaicinoids ranges from 0.003% to 1% of the total weight [33]. In 1968, Bennett *et al.* [34] used mass spectrometer and nuclear magnetic resonance spectrometer for measuring the crystalline material. Mass spectroscopic analysis showed the presence of 69% capsaicin (*m/z* 305), 22% dihydrocapsaicin (*m/z* 307), 7% nordihydrocapsaicin (*m/z* 307), 1% homocapsaicin (*m/z* 319), and 1% homodihydrocapsaicin (*m/z* 321). Capsaicinoids only differ in the group attached to the carbonyl group of the parent structure. Figure 3.1 shows the parent structure of the capsaicinoids, while Table 3.1 shows structures of various capsaicinoids compounds.

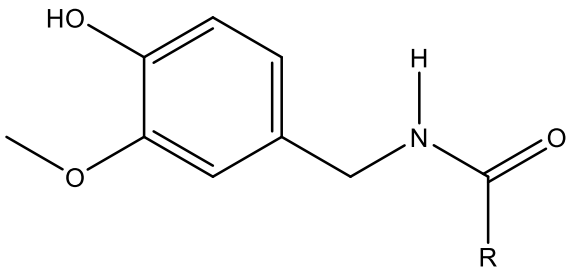


Figure 3.1 The parent structure of capsaicinoids

Table 3.1 The structure of different capsaicinoids compounds

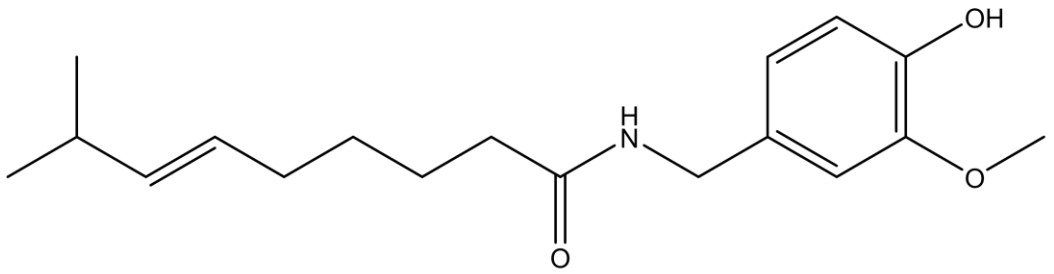
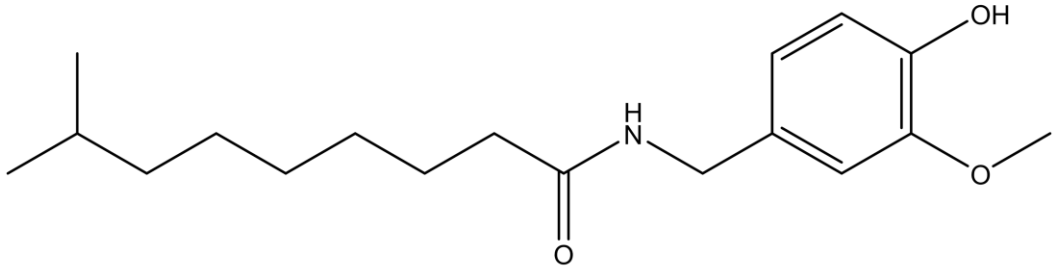
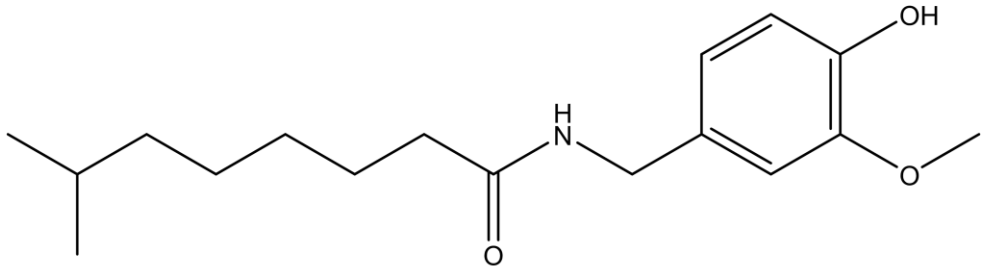
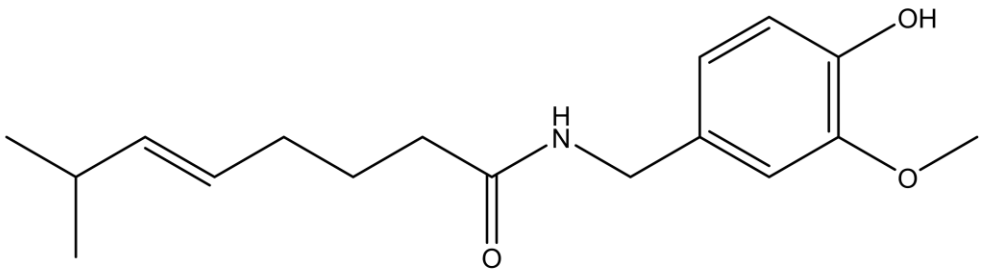
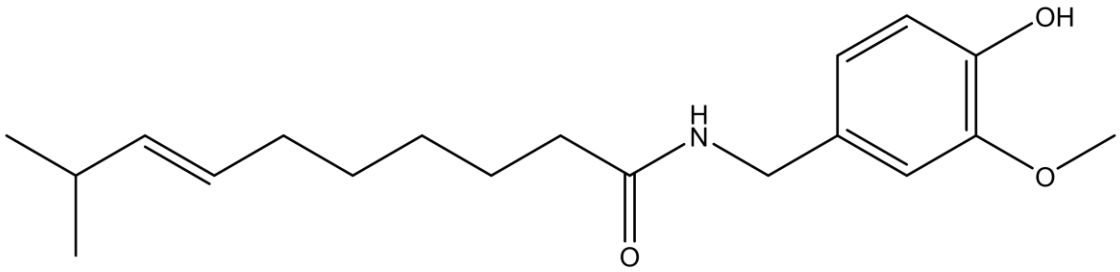
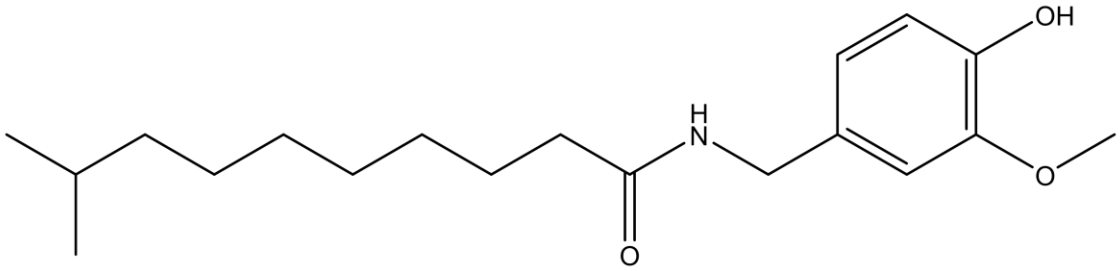
Name	Systematic name	Structure
Capsaicin	*trans*-8-methyl-*N*-vanillyl-6-nonenamide	
Dihydrocapsaicin	8-methyl-*N*-nonamide	
Nordihydrocapsaicin	7-methyl-*N*-vanillyl-octamide	

Table 3.1 The structure of different capsaicinoids compounds (cont.)

Name	Systematic name	Structure
Norcapsaicin	*trans*-7-methyl-*N*-vanillyl-5-octamide	
Homocapsaicin	*trans*-9-methyl-*N*-vanillyl-7-decamide	
Homodihydrocapsaicin	9-methyl-*N*-vanillyl-decamide	

Capsaicin has been widely used in the pharmaceutical industry because of its pharmacological properties [9]. They are mostly found in chilli, one of the most commercial agro products used both as condiment or culinary supplement in Thailand. Capsaicinoids are daily consumed in our diet and are mixed with many foods as a naturally present component of chilli. Due to its very pungent favor, chilli are famously known for their sensory attributes of "hotness", aroma and color, but they are less well known for their health benefits. For example, they have high antioxidant activity [4], antitumor [5], antibacterial [6], anti-mutagenic [7], and anticarcinogenic properties [8].

3.2 Analytical techniques for quantitative analysis of capsaicinoids

Many research has been reported with the aim of identifying the most efficient and reliable method for identification and quantitation of capsaicinoids. Some of the methods for identification and quantification of capsaicinoids are discussed below.

3.2.1 Scoville organoleptic method (Taste Method)

Scoville organoleptic method was firstly performed by Scoville in 1912 [35]. This was the first reliable method for measuring capsaicinoids content and it is commonly used in food industry. The method involves a test panel of five persons who each validate a chilli sample and then record the pungency level. Then, sample was diluted until there is no more detectable pungency that can be orally detected. The dilution is referred to as Scoville Heat Unit.

For ages, scoville organoleptic method has been the most important and the only sensory method for evaluation of hot in the chilli. Nevertheless, the main disadvantages of this method are due to insufficient accuracy and precision. There is also rapid taste fatigue and increased taste threshold as a result of the five samples required for tasting. Ethanol consumption interferes with the hotness. Above all, the organoleptic method cannot determine the amount of the individual capsaicinoids contain in the sample. Accordingly, chemical instrumental analysis methods were developed instead of using organoleptic methods. Because of variations among batches of chilli, the concentration range cannot be expressed in percentage but must be

calculated for each batch from quantitative analytical data [36]. Historically, the organoleptic method is preferred by the food industry because it is a direct measure of levels of hotness. Nonetheless, organoleptic technique requires extensive training of panelists and the monitoring of their sensitivity to environmental factors to get reliable results

3.2.2 Colorimetric methods

Colorimetric method involves reactions of capsaicinoids with either vanadium oxytrichloride or mixture of phosphomolybdate and phosphotungstate (call Folin-Denis reagent) to produce a colored species compound.

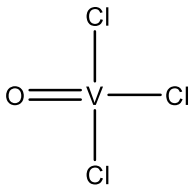


Figure 3.2 Chemical structure of vanadium oxytrichloride

Although not specific for capsaicinoids, the test can give a result that is proportional to the amount of hot. This method is thus rarely used [37].

Kosuge *et al*. [38] extracted capsaicinoids in ether-extracted concentrates taken in carbon tetrachloride, washed with acetic acid, and reacted with a Folin-Denis reagent. The blue color was measured at 760 nm and pure vanillyl was used as a standard with a conversion factor of 2:15 to calculate the amount of capsaicinoids. The repeatability, sensitivity, and reproducibility of this method were not reported. Vanadium oxytrichloride reagent suffers from of stability both with the reagent itself and the blue color product formed.

North *et al.* [39] reported the separation of capsaicinoids from pigments in the extract by repeated partition between alkaline polar and non-polar solvents. Capsaicinoids were estimated by reacting with phenolic reagents such as phosphomolybdic and phosphotungstic acid. Pure vanillyl was used as a standard and the value obtained was multiplied by 2 due to the molecular ratio between vanillin and capsaicin. The recovery and reproducibility were often reported to be poor.

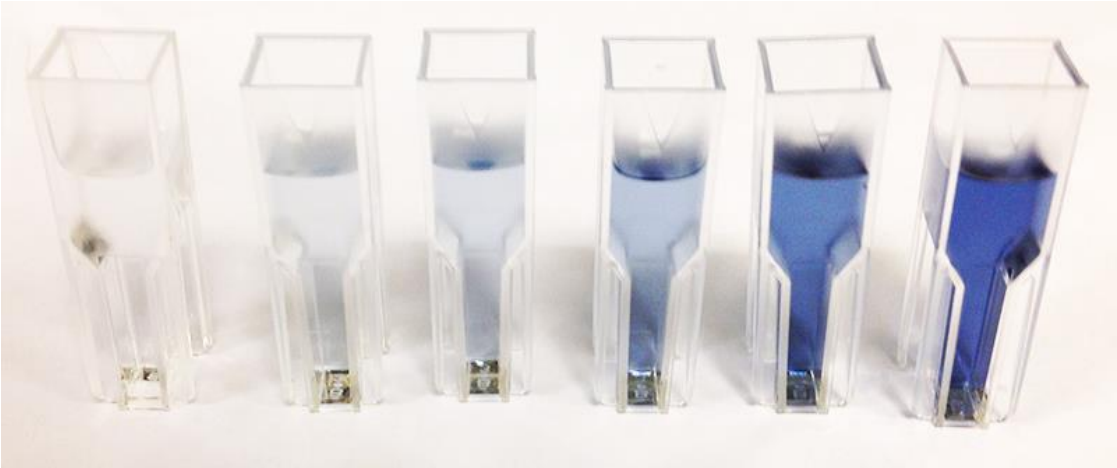


Figure 3.3 The blue colors from the positive results of the Folin -ciocalteu method.

The possible containing capsaicinoids are reacted with 1% solution of vanadium oxytrichloride in carbon tetrachloride. If a green color product is produced in the reaction, then capaicinoids are present [38]. Still, colorimetric method is used exploiting vanadium oxytrichloride for direct and rapid method for detecting the presence or absence of capsaicinoids. However, the method is unpopular because vanadium oxytrichloride has provided poor stability, sensitivity, and reproducibility.

However, the method based on Folin-ciocalteu reagent (mixture of phosphomolybdic and phosphotungstic acid) has been continuously developed to provide high stability, sensitivity, and reproducibility.

3.2.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in the mobile phase at high pressure through a column with stationary phase. Schematic of HPLC system is shown in Figure 3.4. Sample retention time varies depending on the interaction between the stationary phase, the molecules being analyzed, and the solvent used. As the sample passes through the column it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least amount of interaction with the stationary phase will exit the column faster. HPLC has the ability to separate, and identify the compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility,

HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals.

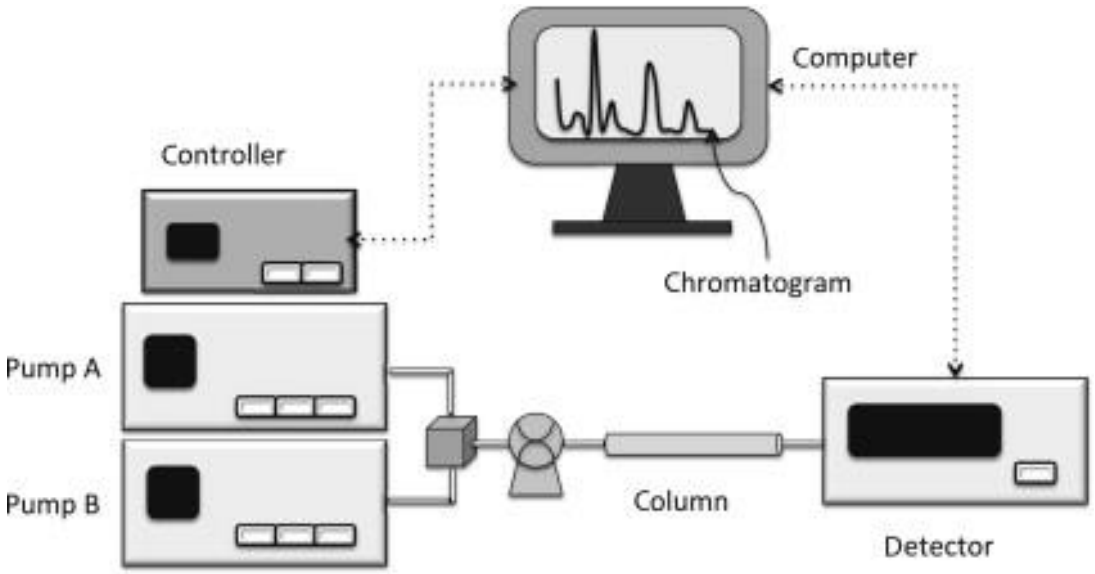


Figure 3.4 Schematic of HPLC system showing component modules.[40]

HPLC methods have been developed that are based on underivatized direct injections of the samples. HPLC technique shows separation capabilities and it provides accurate and efficient analysis of content and type of capsaicinoids present in a chilli sample [41].

HPLC can also accurately determine the homologs and analogs of capsaicin. Combined with mass spectral analysis, HPLC can identify the structural isomers of the minor components. Nano-gram levels of the individual capsaicinoids as required in biosynthetic and metabolic studies can be determined using HPLC [41]. The development of HPLC method that allowed analysis as many as 50 samples a day that varied from less than 300 to 13000 mg L^{-1} in capsaicinoids. This was possible due to spectroflurometric measurement that was more sensitive and selective even at low levels of capsaicinoids. HPLC was used for the estimation of capsaicinoids and correlate them with Scoville Heat Units (SHU) determined by organoleptic test. HPLC method developed by Woodbury *et al*. [41] is the foundation for all subsequent modifications to the HPLC method. It involved extraction of the chilli powder at 60 ºC for 5 hours using 95% methanol.

Margarita *et al*. [42] used HPLC equipped with Novapak C18 reversed phase column, with 73% methanol and water used as a mobile phase at a flow rate of 1.0 mL min^{-1}. The detector used for this analysis was a photodiode array. The standards used were 98% capsaicin and 90% dihydrocapsaicin. The method was used to quantify the level of capsaicin during development, maturation, and senescence of chilli pepper. It was found that capsaicinoids accumulation increased during the development of the fruits and reached a maximum of 200 µg of capsaicinoids g^{-1} in green house grown fruit, and 120 µg of capsaicinoids g^{-1} in field grown fruit after 45 days from fruit set. Capsaicinoids reached minimum after 55 days from fruit set in field and 80 days from fruit set in green house fruit with 60 µg g^{-1}, and 120 µg g^{-1} of capsaicinoids, respectively.

Krishnamurthy *et al*. [43] evaluated the amount of capsaicin in red and green pepper using Hewlett-packard's HP-1050. HPLC equipped with HP-3396 series II integrator, Lichrosphere 100, RP-18 column. The mobile phase was acetonitrile: 1% acetic acid water (55:45) with the flow rate of 2 mL min^{-1}. The detection was carried out at 281 nm. From this study the percentage of capsaicin obtained in green chilli was very low owing to the high water content in green chilli, although water level did not interfere with the analysis of capsaicin.

Karnka *et al*. [44] developed a method for the analysis of capsaicinoids compounds, using simplex method to optimize the chromatography response function to assess the quality of separation by varying the chromatographic parameters, the separation was achieved in 11 min using a C8 column with a UV detector. A flow rate of 1.15 mL min^{-1} at a column temperature of 43.5 ºC using 63.7% methanol in water gave the most efficient separation. The standard used was capsaicin and dihydrocapsaicin and prepared using acetonitrile as solvent. The calibration solutions were made after appropriate dilutions. The recovery for this method was 93% and 89.6% for capasaicin and dihydrocapsaicin, respectively.

Robert *et al*. [45] determined eight naturally occurring capsaicinoids with norcapsaicin used as an internal standard. The solid standards were rigorously checked for purity. The sensitivity of electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and coordination ion-spray (CIS; with silver) toward the capsaicinoids were measured and compared. The highest sensitivity was found for positive ion ESI. Method validation of the liquid chromatography-ESI-mass

spectrometry (LC-ESI-MS) determination is reported, including tests for repeatability (4%), detection limit (5 pg injected), linear range (20 ng injected), quantitation (excellent linearity; < 2% relative standard deviation), and recovery (99-103%).

Nazari *et al*. [46] developed a method for the direct and simultaneous determination of capsaicin and dihydrocapsaicin in Capsicum fruit extracts by using liquid chromatography-electrospray ionization/time-of-flight mass spectrometry. Chromatographic separation was achieved with a reversed-phase chromatography column, using a gradient of methanol and water. Quantification was done using (4,5-dimethoxybenzyl)-4-methyloctamide, synthetic capsaicin analogue not found in nature, as an internal standard. Analytes were base-peak resolved in less than 16 min, and limits of detection were 20 pmol for capsaicin and 4 pmol for dihydrocapsaicin. The analyte recoveries were found to be 83% and 93% for capsaicin and dihydrocapsaicin, respectively. The method developed has been applied to the identification and quantification of capsaicin and dihydrocapsaicin in fruit extracts from different Capsicum genotypes, and concentrations found ranged from 2 to 6639 mg kg^{-1}.

Veronika *et al*. [47] used electrochemical detection after high performance liquid chromatography separation. The aim of that work was to determine capsaicin content in different fruit parts (ovary, lower flesh, upper flesh and seeds). The most suitable conditions for capsaicin determination were as follows: working electrode potential of 750 mV, mobile phase of acetate buffer (pH 4) and methanol in ratio 40: 60 (v/v, %).

Barbero *et al*. [48] developed a rapid and reproducible method of HPLC with fluorescence detection for the determination and quantification of the main capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin) present in hot peppers by employing a monolithic column. The type of column employed is a RP-18 monolithic column. A gradient method was utilized for the chromatographic separation as follows: solvent A: water (0.1% acetic acid) and solvent B: methanol (0.1% acetic acid). A study of the robustness of the method was also made in respect of the conditions of temperature in the separation column (15-40 ^{o}C), the solvent flow rate (4-7 mL min^{-1}), the injection volume (10-50 µL), and the percentage of methanol in the sample (25-100%). The repeatability and reproducibility of the method showed relative standard deviations of less than 2%. The robustness of

the method was determined by utilizing different injection volumes and different percentages of methanol in the extracts. The method developed has then been utilized for the quantification of the major capsaicinoids present in different varieties of hot peppers grown in Spain. The capsaicinoids have been separated in less than 8 min.

Othman *et al*. [49] investigated samples consisted of hot chilli, red chilli, green chilli, green peppers, red peppers and yellow peppers. Extraction of capsaicinoids was done using ethanol as solvent, while high performance liquid chromatography (HPLC) was used for separation, identification and quantitation of the components. The limit of detection of the method was 0.09 and 0.10 μg g^{-1} for capsaicin and dihydrocapsaicin, respectively. Hot chilli showed the highest concentration of capsaicin (4249.0 ± 190.3 μg g^{-1}) and the highest pungency level (67984.60 SHU), whereas green peppers had the lowest detected concentration (1.0 ± 0.9 μg g^{-1}); green peppers, red peppers and yellow peppers were non pungent.

Uboh *et al*. [50] developed method involving ultra high performance liquid chromatography-tandem mass spectrometry and validated for the analysis of capsaicin and dihydrocapsaicin in equine plasma. The analytes were recovered from plasma by liquid-liquid extraction using methyl *tert*-butyl ether and separated on a sub-micron column. The mobile phase was composed of 2 mmol L^{-1} ammonium formate and methanol. A triple quadrupole mass spectrometer was used to detect the analytes in positive electrospray ionization mode with selected reaction monitoring. The limits of detection, quantification and confirmation for both analytes were 0.5, 1.0 and 2.5 pg mL^{-1}, respectively. The linear dynamic range of quantification was 1.0-1,000 pg mL^{-1}. During storage, both analytes in equine plasma were unstable at room temperature but stable at -20 and -70 ºC. The retention time and product ion ratios were employed as the criteria for confirmation of the presence of the analytes in plasma. The total analysis time was 2 min. The method is fast, selective, sensitive, reproducible, reliable and fully validated.

Perjési *et al.* [11] developed reverse-phase high performance liquid chromatographic method with fluorescence detection for determination of capsaicin and dihydrocapsaicin in samples generated in rat small intestine luminal perfusion experiments. The experiments were designed to study the biotransformation of capsaicinoids in the small intestine in the rat. The chromatographic separation was

performed at room temperature on a ZORBAX Eclipse®XDB-C8 column using isocratic elution with a mobile phase consisting 0.05 M orthophosphoric acid solution and acetonitrile (60:40, v/v; pH 3.0) with a flow rate of 1.5 mL min^{-1}. Fluorescence detection was performed at excitation and emission wavelengths of 230 and 323 nm, respectively. The limit of detection was 50 ng mL^{-1} for both capsaicinand dihydrocapsaicin.

Recently, Shim *et al.* [51] developed simultaneous analytical method for determination of piperine, capsaicin, and dihydrocapsaicin in Korean instant-noodle soup base using HPLC. The HPLC separation was performed on a reversed-phase C18 column using a UV detector fixed at 280 nm. The LOD and LOQ of the HPLC analyses ranged from 0.25 to 1.03 mg kg^{-1}. The intraday and interday precisions of the individual piperine, capsaicin, and dihydrocapsaicin were <10.55%, and the recovery values ranged from 85.43 to 94.68%. The calibration curves exhibited good linearity (r^2 = 0.999) within the tested ranges. These results suggest that the analytical method in this study can be used to classify Korean instant noodles based on their levels of spiciness.

3.2.4 Gas Chromatography (GC)

In gas chromatography (GC), an inert carrier gas serves as a mobile phase that elutes the components of a mixture from a column containing the stationary phase.

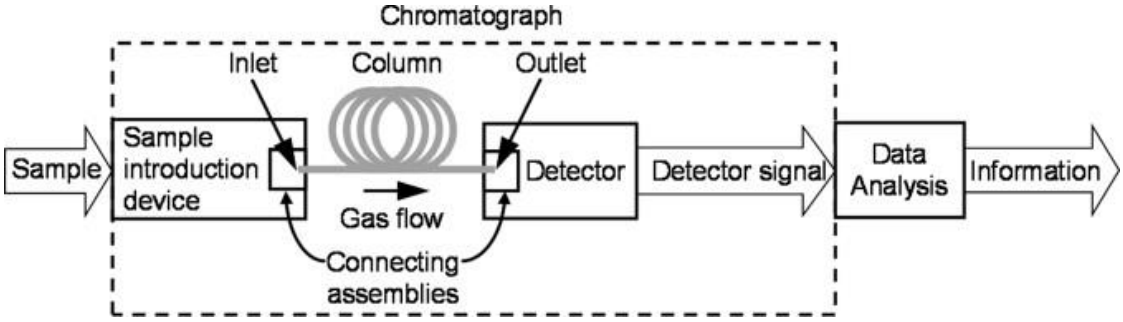


Figure 3.5 Block diagram of a chromatographic system. [52]

GC method has been used for the separation and analysis of capsaicinoids similarly to HPLC.

Gas chromatography was used for quantitative determination of individual homologs and analogs of capsaicinoids in mixtures from a natural source and of vanillyl pelargonic amide as adulteration in 1971 [53].

Dicecco *et al*. [54] developed a simple and accurate method for quantitative determination of capsaicin in capsicum spices and their oleosresins. The capsaicin was quantitated by gas-liquid chromatography, using a Carbowax 20M-Teflon column and piperine as an internal standard.

Suzuki *et al*. [55] used a combination of gas chromatography-mass spectrometer with a column packed with 3% SE-52 on chromosorb W. The column temperature was kept at 260 °C. The carrier gas flow rate was 30 mL min^{-1}. Quantitation of capsaicinoids was carried out with 0.1-1.0 μg of authentic capsaicinoids, together with 0.5 μg of 5α-cholestane as the internal standard.

Bretty *et al*. [56] developed a method to quantify capsaicinoids using capillary gas chromatography of pepper tissue extracted with acetone. The extract was analyzed via capillary gas chromatography/ thermionic selective detection without derivatization. The column temperature program was as follows: 160 °C for 1 min, 20 °C min^{-1} to 250 °C for 0 min, 1 °C min^{-1} to 260 °C for 7 min, 5 °C min^{-1} to 270 °C for 5 min (30 min total). The carrier gas was helium with a flow rate of 1.8 mL min^{-1} at 160°C and a septum purge of 2.2 mL min^{-1}. The detector was a thermionic selective detector (TSD) operated at 290 °C. The instrumental detection limit for each capsaicinoid is 0.2 ng μL^{-1} in the final extract.

Jaeho *et al.* [57] developed gas chromatographic method for the analysis of capsaicin in Gochujang and validated by comparing with a column high-performance liquid chromatographic method (AOAC 995.03). The method validation parameters yielded good results, including linearity, precision, accuracy, and recovery. The GC separation was performed on a (5 phenyl)-methylpolysiloxane column followed by flame ionization detection. The conditions of temperature programming were initially 220 ºC for 1 min, ramp at 5 ºC min^{-1} to 270 ºC, and hold for 10 min. The recovery of capsaicin in Gochujang was more than 92%, and the detection limit and lower determination limit of the GC analysis were 1.0 and 5.0 μg g^{-1}, respectively. The calibration graph for capsaicin was linear from 1 to 250 g mL^{-1} for GC and 0.5 to 50 g

mL^{-1} for HPLC. The interday and intraday precisions (relative standard deviations) were < 4.02.

Araceli *et al*. [58] developed a method for the analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase micro extraction-gas chromatography-mass spectrometry. The analysis was performed without derivatization. The method was reported to be linear in the range 0.109- 1.323 mg mL^{-1} for capsaicin and 0.107-1.713 mg mL^{-1} for dihydrocapsaicin with LOD of 0.014 and 0.022 μg mL^{-1} for capsaicin and dihydrocapsaicin, respectively.

Alvarez *et al*. [13] developed a method for the analysis of capsaicin and dihydrocapsaicin in peppers by ultrasound assisted extraction (USAE) followed by gas chromatography–mass spectrometry (GC-MS). The GC-MS analytical method was linear in the range 10–100 μg mL^{-1} for capsaicin and dihydrocpsaicin with correlation coefficient r = 0.998 and peak area variability of ∼1% for both capsaicinoids. The method was applied to the analysis of 11 varieties of hot peppers cultivated in México. A large concentration range for capsaicin (101–6800 μg g^{-1}) and dihydrocapsaicin (110–2736 μg g^{-1}) was found in these pepper samples.

3.2.5 Electrochemical analysis

Electrochemical method for capsaicinoids determination was developed. Electrochemical techniques are powerful and versatile analytical techniques that offer high accuracy, sensitivity, and precision as well as large linear dynamic range, with relatively low-cost instrumentation. Literature survey revealed only three voltammetric studies made on capsaicin so far. All of them involve the use of modified electrodes. The first investigation performed by Kachoosangi *et al.* [59] was concerned with studying the electroactivity and determination of capsaicinoids using adsorptive stripping voltammetry at carbon nanotube-based electrochemical sensors. The proposed mechanism for the electrochemical oxidation/reduction of capsaicin is depicted in Figure 3.6. This research exhibits two linear ranges, from 0.5 to 15 μmol L^{-1} and from 15 to 60 μmol L^{-1} and a detection limit of 0.31 μmol L^{-1}. Its applicability was evaluated in a variety of hot pepper sauces. The proposed mechanism for the electrochemical oxidation/reduction of capsaicin is depicted in Figure 3.6. The electrochemical oxidation

of capsaicin in the first scan is coupled to an irreversible homogeneous chemical step, which results in the hydrolysis of the 2-methoxy group to form an o-benzoquinone unit in the structure of capsaicin. Then the *o*-benzoquinone part of the capsaicin falls in a redox electrochemical loop with catechol (C and D) which are observed as peaks II and III.

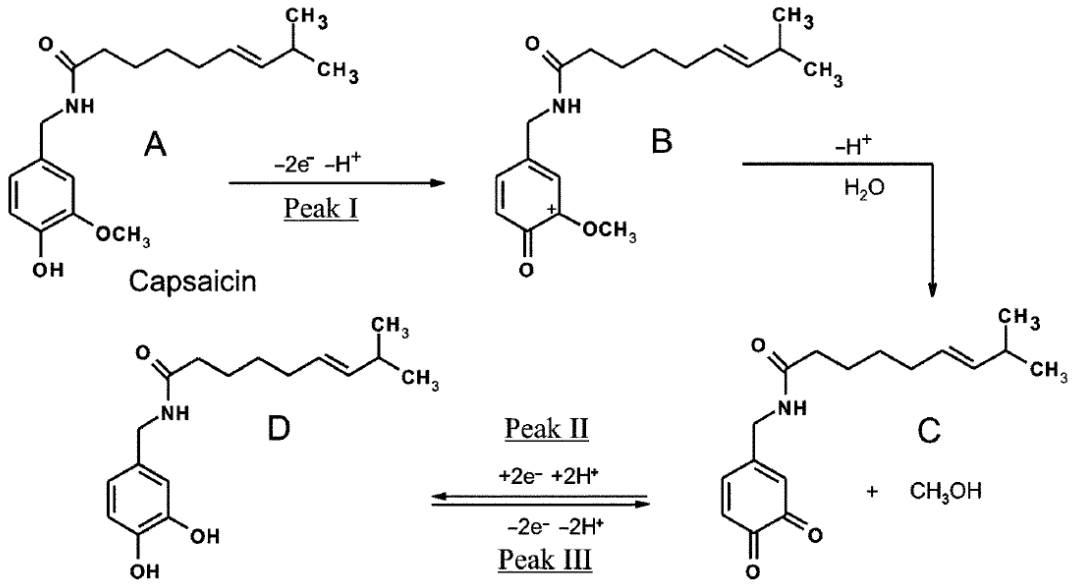


Figure 3.6 Mechanism for the electrochemical oxidation/reduction of capsaicin. [59]

Afterward, carbon paste electrode modified with amino-functionalized mesoporous silica for capsaicin detection was proposed by Liang *et al.* [60]. Figure 3.7 elucidates the mechanism of electrochemical reactions. Among these peaks, Pa1 was found to be more sensitive. Due to its enhanced sensitivity, it was selected for the determination of capsaicin. The characteristic anodic peak is 0.60 V with the cathodic peaks at 0.34 V and 0.38 V with saturated calomel reference electrode. Under optimal conditions, the oxidation peak current was proportional to capsaicin concentration in the range of 0.040-0.40 μmol L^{-1} and 0.40-4.0 μmol L^{-1}, and the detection limit was 0.020 μmol L^{-1}. The above method was successfully applied to determine capsaicin in hot pepper samples, yielding satisfactory results. The spiked recoveries were in the range of 93.1-100.7%.

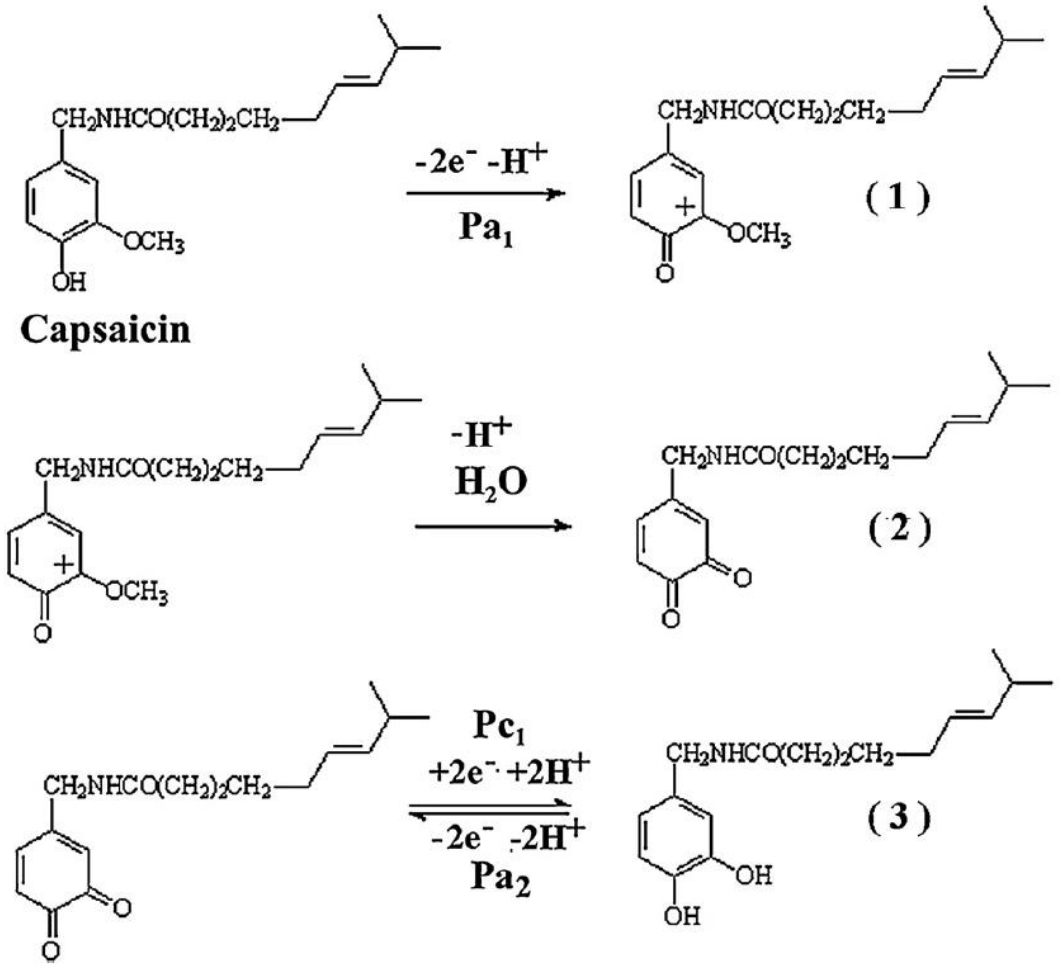


Figure 3.7 Electrochemical reaction mechanism of capsaicin. [60]

Furthermore, electrochemical impedance spectroscopy was reported by Banks *et al*. [61]. The authors used screen printed carbon nanotube electrodes as electroanalytical sensing platforms for the detection of capsaicin in both synthetic capsaicin solutions and capsaicin extracted from chilli and chilli sauces utilising both cyclic voltammetry and electrochemical impedance spectroscopy. The limit of detection was 5.76 mmol L^{-1}.

Meanwhile, Yardim *et al*. [62] study electrochemical properties of these compounds at pencil graphite electrode. Using the square-wave adsorptive stripping voltammetry with fixed accumulation potential at −0.1 V for 120 s and voltammetric response at +0.31 V. The limit of detection was achieved as 3.7 nmol L^{-1} for capsaicin and 0.91 nmol L^{-1} for dihydrocapsaicin. The electrochemical reactions mechanism

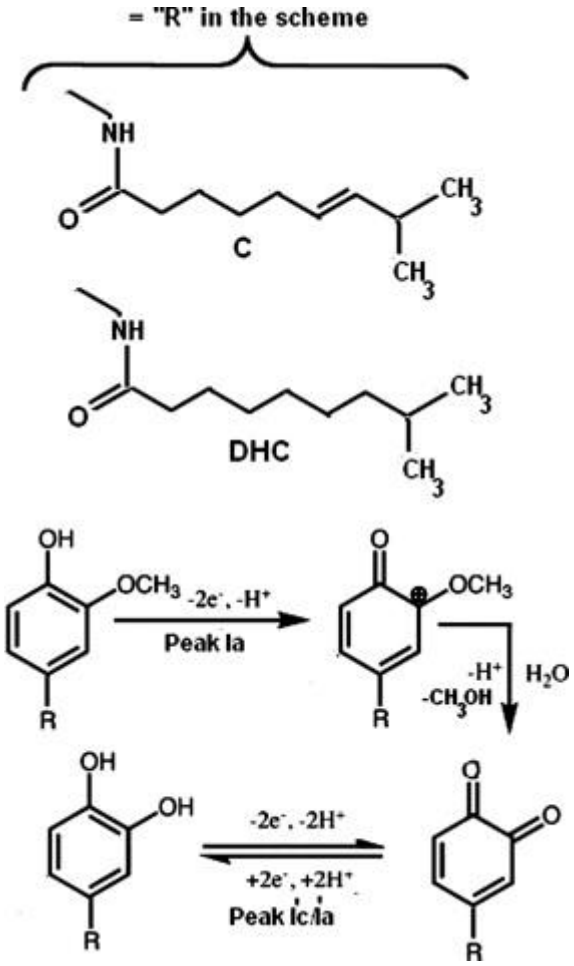


Figure 3.8 The structure of capsaicin and dihydrocapsaicin and their reaction pathway in acidic and less alkaline solutions. C, capsaicin; DHC, dihydrocapsaicin. [62]

Furthermore, biosensor with electrochemical detection was developed for capsaicinoids detection. Especially, enzymatic biosensor was modified on electrode such as horseradish peroxidase [63] and *L*-phenylalanine ammonia-lyase [14].

Amperometric biosensor based on a horseradish peroxidase enzyme-capsaicin reaction mediated by ferrocene has been successfully developed for capsaicinoids determination [63]. The biosensor was fabricated by immobilization of both ferrocene and horseradish peroxidase in a photocurable poly(2-hydroxyethyl methacrylate) hydrogel membrane. The reaction mechanism of peroxidase with capsaicin and hydrogen peroxide was proposed as shown in Figure 3.9. Biosensor measured capsaicin concentrations at a potential of 0.22 V with Ag/AgCl reference electrode. The limit of detection was achieved as 1.94 μmol L^{-1}.

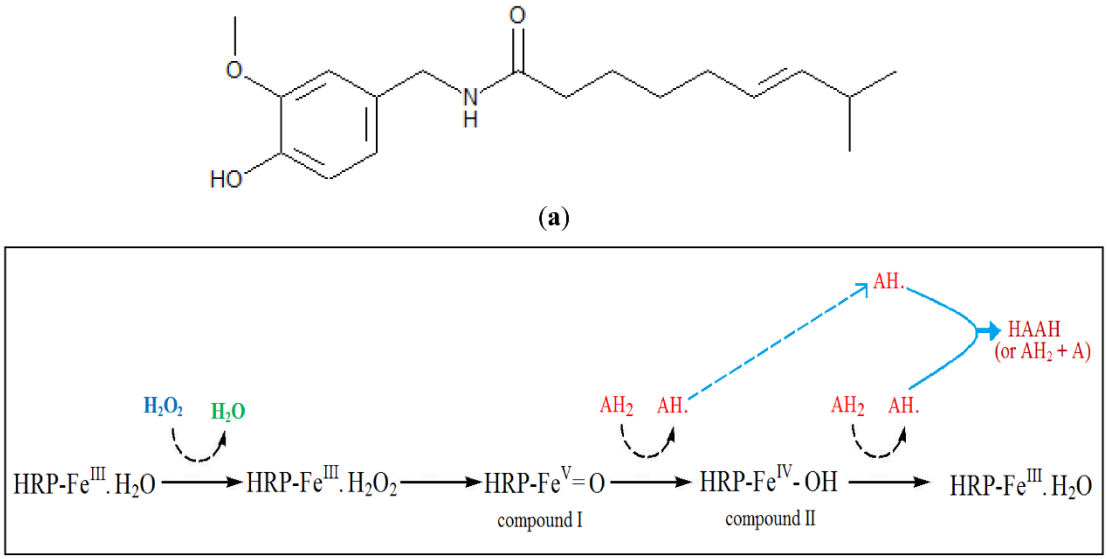


Figure 3.9 The structure of capsaicin (a); and the proposed reaction mechanism of peroxidase with capsaicin and hydrogen peroxide (b). AH2 & AH• = molecule & free radical from the hydrogen donor (capsaisin or phenolic compounds); HRP = Horseradish peroxidase; HAAH = polymerized product. [63]

Recently, Sabela *et al*. [14] developed novel nanocomposite of L-phenylalanine ammonia-lyase enzyme for the detection of capsaicin. The platinum electrode was modified with multiwall carbon nanotubes where phenylalanine ammonia-lyase enzyme was immobilized using nafion. The redox mechanism (see Figure 3.10) for capsaicinis aquasireversible reduction reaction between the -OH and R-O-R groups of the guaiacol ring and the residues of the enzyme resulting in the reversible catechol ring. Therefore, the electron density is in agreement with redox mechanism as shown in Figure 3.10. Docking studies were used to assess the chemical interaction between the capsaicin and the PAL enzyme. The limit of detection was achieved as 0.1863 µg mL^{-1}.

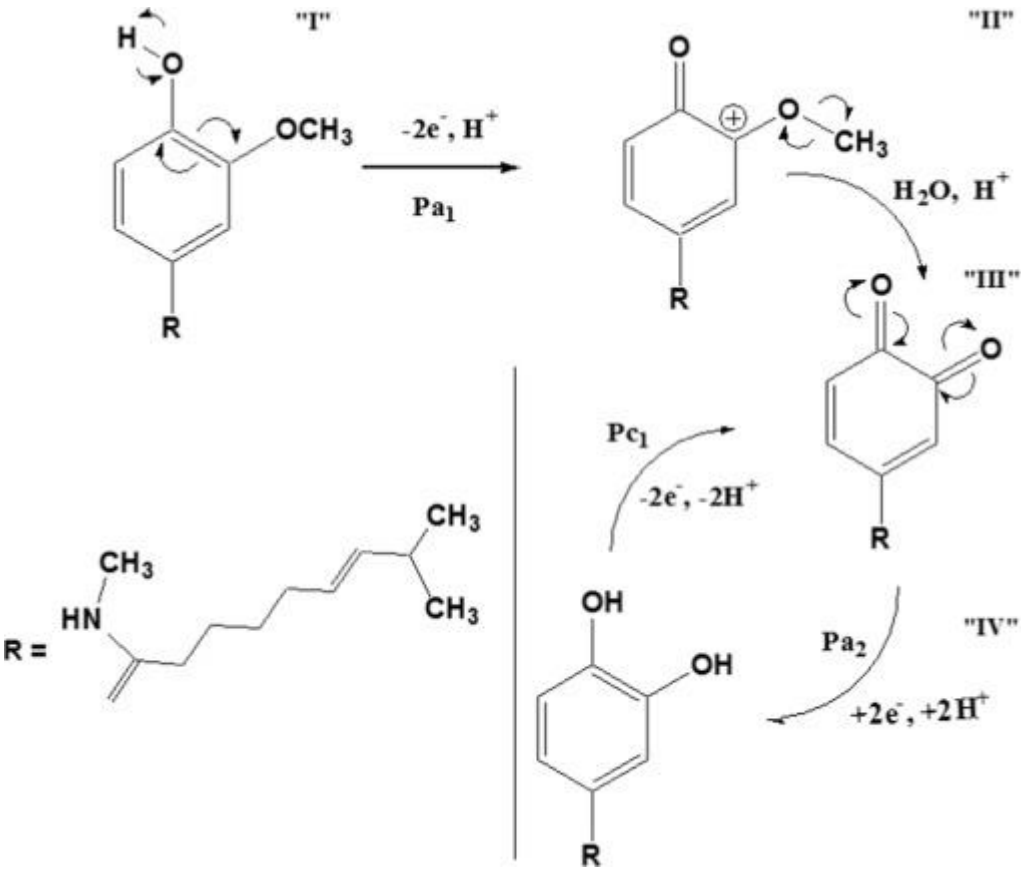


Figure 3.10 Mechanism for the electrochemical oxidation/reduction of capsaicin. Pa and Pc refers to the anodic and cathodic peaks respectively. [14]

3.3 Extraction method for capsaicinoids

Several solvents have been used for the extraction of capsaicinoids from capsicums. Isopropanol was used in ground fresh serranos [64]. Carbon tetrachloride was used in green paprika [31]

Bajaj *et al*. [65] and Suzuki *et al*. [66] used ethyl acetate to produce a nearly colorless extract from dried immature green peppers.

Govindarajan [32] found that acetone extraction of fresh fruit resulted in poor extraction and messy layer formation. However, drying of cut sections of green fruits allowed a clean solvent extraction.

Similary, Iwai *et al*. [55] used acetone and/or ethyl acetate in dried capsicums.

Subsequently, ethanol saturated with sodium acetate was used by Woodbury *et al.* [41]. Capsaicinoids from ground red pepper were extracted with 95% ethanol [67], however other solvents were examined in order to determine the most suitable medium for isolating the capsaicinoids. The solvents tested included acetonitrile, acetone, methylene chloride, and ethanol. Acetonitrile required a lengthy extraction time and carried with it extraneous interfering compounds. Acetone, although an excellent solvent for removing the capsaicinoids, some undesirable components which adversely affected the resolution of the individual compounds during the HPLC analysis also are extracted. Methylene chloride extracted the materials in a reasonable time but it was impractical as it carried residues not totally miscible with the mobile phase (acetonitrile-water (1% acetic acid), 40:60 v/v). Ethanol extracted greater than 98% of the capsaicinoids after 5 hours.

Likewise, Chiang *et al*. [68] used methanol to extract capsaicinoids from different samples such as spicy cheese sauce, salsa, picante sauce, spicy mix, and spicy tomato sauce.

Attuquayefio *et al.* [69] extracted capsaicin by blending 1.00 g of dry ground capsicum with 10 mL of acetonitrile and then sonicating it for 5 min. Sep-pak silica and C18 cartridges were used to clean up the extracts. The reported recovery for this method was 98%.

Weaver *et al*. [70] refluxed ground pepper sample for 1 h with acetone, followed by drying and redissolving in denatured ethanol.

Ogbadu *et al*. [71] used methanol to extract capsaicinoids from ground, fresh capsicums.

Capsaicinoid extracts were obtained by Soxhlet extraction of freeze-dried capsicums in acetone [72, 73]. Sauces were extracted with acetone as well [74].

Yao *et al*. [75] reported that supercritical CO_2 extraction was a superior solvent to extract capsaicin and dehydrocapsaicin from Scotch Bonnet peppers.

Collins *et al.* [76] extracted oven-dried capsicum fruits with acetonitrile. These authors also evaluated other solvents, including 95% ethanol, 95% ethanol saturated with sodium acetate, chloroform, and ethyl acetate, for the extraction of capsaicinoids from the same capsicum sample for varied lengths of time. They found

that acetonitrile yielded the highest amount most quickly, although they did not report any data.

Boonkird *et al*. [77] studied the influence of solvent type, powder to solvent ratio, and temperature on ultrasonic assisted extraction of capsaicinoids from dried capsicum fruit. From the study, the suitable condition for capsaicinoid extraction by sonication in an ultrasonic bath was at a ratio of 1.00 g of a solid material to 5 mL of 95% ethanol, at the temperature of 45 ºC. Under these conditions, 85% of the capsaicinoids were extracted from the raw material in 3 h. It was shown that the ultrasonic extraction allowed a significant reduction in extraction time at lower operation temperatures than using a conventional industrial hot maceration process.

Barbero *et al*. [78] developed an ultrasound-assisted method that extracted majority of the capsaicinoids. Methanol was used as solvent, at an optimum temperature of 50 ºC and an extraction time of 10 min. The reproducibility of the method was <3% RSD.

Perucka *et al*. [79] extracted capsaicinoids from hot pepper fruit with the petroleum ether:acetone mixture, and then separated using thin layer chromatography on silica gel and evaluated quantitatively using the HPLC method and spectrophotometrically.

Santos *et al*. [80] extracted capsaicinoids from malagueta pepper using supercritical fluid extraction (SFE) assisted by ultrasound with carbon dioxide as solvent at 15 MPa and 40 °C. Supercritical extraction assisted by ultrasound (SFE + US) increased the global yield of malagueta pepper oleoresin up to 30% when compared to SFE, without changing the total capsaicinoids and phenolics profile.

Recently, Sweat *et al*. [81] used toluene for capsaicinoids extraction in commercially grown Jalapeño, Habanero and Bhut Jolokia peppers.

3.4 Paper-based analytical devices (PAD)

Paper is a thin material fabricated by pressing moist fibers and drying them into flexible sheets. The first paper known to history was made in around 3000 BC by the ancient Egyptians who used strips from the papyrus reeds which they dampened,

made into a criss-cross pattern and pressed into sheets. Our word paper comes from "papyrus" [82]. Now it has been widely used in almost all aspects of human life, for example, writing, printing, packaging, and cleaning. Paper made from cellulose is a renewable material with annual production of 1.5×10^{12} tons [83]. It is also one of the cheapest materials all over the world. Paper is a porous hydrophilic material so that it wicks fluid, which amenities of liquid handing without using a pump. Due to improved techniques of printing paper, it is easier to imitate some other materials to make the mass production of paper-based analytical devices. Paper is a biocompatible material so that biomolecule probes are more stable and less likely to denature on paper. Paper is also chemically inert so that it does not cause interference with most analytical processes.

The simplest and earliest paper-based analytical devices is probably litmus paper, which was invented in the 1800s by Partington *et al*. [84]. The first paper based analytical device for the semi-quantitative analysis was proposed in 1656 by Comer *et al*. [85]. That research reported the detection of glucose in urine sample.

3.4.1 Fabrication of paper based analytical devices

Ten fabrication approaches were reported as shown in Table 3.2 including advantages and drawbacks of each method.

Table 3.2 Comparison of the ten published techniques for patterning hydrophilic-hydrophobic contrast on paper to create paper based analytical devices

Fabrication methods	Advantages	Drawbacks	Reference
Photolithography	High resolution of microfluidic channels	1. Requires expensive equipment 2. Requires an extra washing step to remove un-crosslinked polymer 3. Devices are vulnerable to bending	[86-88]
Plotting	1. Patterning agent (PDMS) is cheap 2. Devices are flexible	1. Deteriorated barrier definition 2. Cannot be readily applied to high throughput production	[89]
Ink jet etching	Requires only a single printing apparatus to create microfluidic channels by etching and to print bio/chemical sensing reagents	1. Creation of microfluidic channels requires 10 times of printing 2. The printing apparatus must be customized 3. Not suitable for mass fabrication	[90, 91]
Plasma treatment	1. Uses very cheap patterning agent (AKD) 2. Dramatically reduces the material cost	Requires different masks for creating different microfluidic patterns on paper	[92, 93]
Paper cutting	Requires only a cutter apparatus to create paper-based device	1. Not suitable for mass fabrication 2. Low resolution of microfluidic channels (rough barrier)	[94, 95]

Table 3.2 Comparison of the ten published techniques for patterning hydrophilic-hydrophobic contrast on paper to create paper based analytical devices (cont.)

Fabrication methods	Advantages	Drawbacks	Reference
Wax printing	Produces massive devices with simple and fast (5-10 min) fabrication process	1. Requires expensive wax printers 2. Requires an extra heating step after wax deposition	[96-98]
Ink jet printing	1. Uses very cheap AKD 2. Produces massive devices rapidly (<10min) and simply 3.Requires only a desktop printer to produce devices and to print sensing reagent	1. Requires an extra heating step for AKD deposition 2. Requires modified ink jet printers	[93, 99, 100]
Flexography printing	1. Allows direct roll-to-roll production in existing printing houses 2.Avoids the heat treatment of printed patterns	1. Requires two prints of polystyrene solution 2. Requires different printing plates 3. Print quality relies on the smoothness of paper surface	[101]
Screen printing	Produces devices with simple process	1. Low resolution of microfluidic channels (rough barrier) 2. Requires different printing screens for creating different patterns	[23]
Laser treatment	High resolution	Microfluidic channels do not allow lateral flow of fluids; requires extra coating for liquid flow	[102]

3.4.2 Paper cutting technique

Paper cutting technique use a removal paper to create PAD. The advantages of this fabrication technique are that no chemicals are needed to define flow boundaries, and the equipment is generally widely available and low cost. Because fabrication does not rely on the flow of wax, polymers, or solvents within paper for definition, a greater precision in manufactured device barriers was observed by which the measured standard deviation of fabricated channel widths as opposed to wax printing [96], inkjet etching [91], and laser-cutting fabrication [103] by which were ±45 μm, 50 μm, and 10 μm, respectively). However, because much of the material is removed, these devices suffer from low mechanical stability and rely on solid supports, increasing cost [104]. Besides using hand-held blades and hole punches to create devices [24], craft knife cutting [94] and CO_2 laser cutting [105] have been used to improve precision, speed, and production volume. Recently, Satarpai *et al*. used paper cutting technique for create adsorption filter paper disc [106].

CHAPTER IV
MATERIALS AND METHODS

The instrument set-up, operating conditions, chemical reagents, and experimental procedures are described in this chapter.

4.1 Instrumentation

4.1.1 High Performance Liquid Chromatography (HPLC)

An Agilent 1200 series high performance liquid chromatography (HPLC) was used in this research with the details given in Table 4.1. The chromatographic conditions are given in Table 4.2.

Table 4.1 HPLC instruments.

Instrument	Model	Manufacturer
Injector	7725i with 20 μL injection loop	Rheodyne (Washington, USA)
Delivery system	Quaternary pump 1200 (G1311A)	Agilent (California, USA)
Vacuum degasser		Agilent (California, USA)
Column	Capcell Pak C18 UG80 S5 (250 mm x 4.6 mm, 5 μm)	Shiseido (Tokyo, Japan).
Security guard column	Capcell Pak C18 UG80 S5 (10 mm x 4.0 mm, 5 μm)	Agilent (California, USA)
Detector	Diode array detector (G1315B)	Agilent (California, USA)
Data system	ChemStation software (version B.02.01)	Agilent (California, USA)

Table 4.2 HPLC operating conditions.

Parameter	Model
Mobile phase	A: Acetonitrile B: DI water
Gradient	Isocratic, A:B 50:50
Flow rate	1.5 mL min^{-1}
Detector	UV-vis at 222 nm
Run time	15 min
Column temperature	Ambient

4.2 Equipment

4.2.1 Thin-layer chromatography

The silica gel 60 F 254 precoated thin-layer chromatography (TLC) plates (20 cm x 20 cm, 0.25 μm) from Merck (Darmstadt, Germany) were used for capsaicinoids analysis.

4.2.2 Ultrasonic bath

An ultrasonic bath (Bandelin, model DT510, Berlin, Germany) was used for aiding sample extraction.

4.2.3 Water bath

The Water bath (Memmert, model WB22, Schwabach, Germany) was used with agitator (Memmert, model SV 1422, Schwabach, Germany) for Othman *et al.* extraction.

4.2.4 Portable flatbed scanner

All image result was scanned by portable flatbed scanner (Canon, model LiDE100, New York, USA).

4.2.5 Analytical balance

The AG-135 analytical electronic balance (Mettler, Zuric, Switzerland) was used to weigh chemicals.

4.2.6 Water purification system

Deionized water with 18.2 MΩ cm^{-1} from a Barnstead Easypure II water purification system (Barnstead, Iowa, USA) was used throughout this work.

4.3 Chemical reagents

All chemical reagents used in experiments were of analytical grade and HPLC grade which are listed in Table 4.3.

Table 4.3 The list of chemical reagents

Chemical reagents	**Suppliers**
Capsaicin	Sigma-Aldrich (Missouri, USA)
Folin-Ciocalteu (FC) reagent	Sigma-Aldrich (Missouri, USA)
Potassium carbonate	Sigma-Aldrich (Missouri, USA)
Sodium carbonate anhydrous	Sigma-Aldrich (Missouri, USA)
Potassium permanganate	Ajax Finechem (New South Wales, Australia)
Ethanol	Merck (Darmstadt, Germany)
Sodium hydroxide	Merck (Darmstadt, Germany)
Hexane	RCI Labscan (Bangkok, Thailand)
Ethyl acetate	RCI Labscan (Bangkok, Thailand)
Toluene	RCI Labscan (Bangkok, Thailand)
Acetone	RCI Labscan (Bangkok, Thailand)
Chloroform	RCI Labscan (Bangkok, Thailand)
Ethyl ether	RCI Labscan (Bangkok, Thailand)

4.4 Preparation of standard solution and reagents

4.4.1 Capsaicinoids standard solution

A stock of capsaicinoids solution was prepared at 2000 mg L^{-1} in ethanol and diluted with ethanol to 10 - 1000 mg L^{-1} of capsaicinoids solution as standard solutions.

4.4.2 Sodium carbonate solution of 2, 4, 6, 8, 10, 12, 14, and 16% (w/v)

A 0.20, 0.40, 0.60, 0.80, 1.00, 1.20, 1.40, and 1.60 g of sodium carbonate was dissolved in deionized water and adjusted volume to 10 mL for preparation of 2, 4, 6, 8, 10, 12, 14, and 16% (w/v) respectively.

4.4.3 5% (w/v) Sodium hydroxide

A 5.00 g of sodium hydroxide was dissolved in deionized water and adjusted volume to 100 mL.

4.4.4 Irradiation treatment solution

A 3.00 g of potassium permanganate and 20 g of potassium carbonate was dissolved in deionized water and added with 5 mL of 5% (w/v) sodium hydroxide from 4.4.3 and made up volume to 300 mL.

4.5 Experimental procedures

4.5.1 Preparation of TLC separation

The silica gel precoated thin-layer chromatography (TLC) plate was cut to a size of 4.8 cm x 0.5 cm. After spotting of standard solution or chilli extract on to the starting point of TLC, the TLC plate was developed with a mobile phase in a pre-saturated chromatographic chamber. In order to determine the retention factor or retardation factor (R_f) of the compounds, the TLC was visualized by irradiation after treatment with an irradiation treatment solution.

4.5.1 Preparation of PAD

A puncher was used to cut a circular paper with a diameter of 6.0 mm from Whatman® No. 4 filter paper (GE, Buckinghamshire, UK). 1.0 μL of FC reagent and 2.0 μL of Na_2CO_3 solution were freshly prepared and pre-deposited onto this circular paper of paper based device (PAD).

4.5.3 Preparation of TLC-PAD connector

A TLC-PAD connector was made from polyvinyl chloride (PVC) sheet (thickness = 140 μm). The PVC sheet was cut to act as a belt to allow the insertion of the PAD and the TLC plate as shown in Figure 4.1.

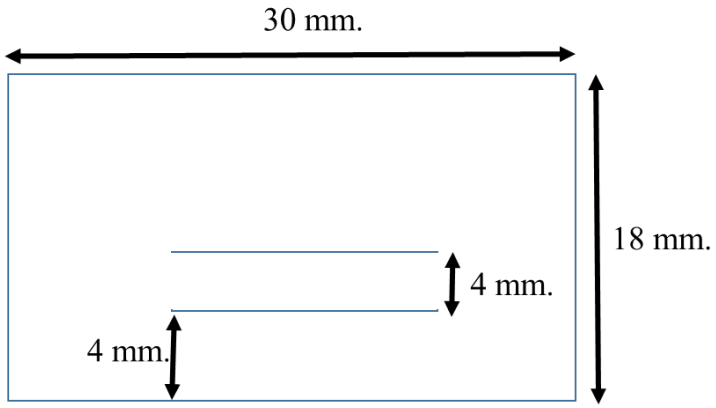


Figure 4.1 Illustration of TLC-PAD connector

4.5.4 Extraction of capsaicinoids from chilli samples

4.5.4.1 Othman's extraction method [49]

For Othman's capsaicinoid extraction method, each dried chilli (DC) and seasoning powder (SP) (5 g) was placed in ethanol (5 mL). Bottles were capped and placed in a water bath at 80 °C for 4 hours, then swirled manually every hour. Samples were removed from the water bath and cooled to room temperature. The supernatant layer of each sample (5 mL) was filtered through 0.45 μm cellulose acetate membrane filter into a sample vial using a 5 mL disposable syringe. The vial was capped and stored at 5 °C in a refrigerator until analysis.

4.5.4.2 Modified extraction method

The method reported by Othman *et al*. [49] was slightly modified and used for capsaicinoids extraction. Two types of samples were extracted including dried chilli (DC) and seasoning powder (SP) samples. For capsaicinoids extraction, 2 g of samples were placed in ethanol (5 mL) in a 10 mL beaker. The beaker was placed in an ultrasonic bath for 15 min. The supernatant layer of each sample (5 mL) was filtered through 0.45 μm cellulose acetate membrane filter into a sample vial using a 5 mL disposable syringe. The vial was capped and stored at 5 °C in a refrigerator until analysis.

4.5.5 TLC separation and colorimetric detection of capsaicinoids

The proposed method consisted of two steps including TLC separation and colorimetric detection of capsaicinoids as illustrated in Figures 4.2. In the first step, a 2 μL of standard or extracted sample was pipetted onto TLC at the starting line. Then, it was developed with a mobile phase: hexane-ethyl acetate (50:50, v/v) in a pre-saturated chromatographic chamber. Subsequently the spot of capsaicinoids at retention factor (R_f) of 0.41 was cut (as shown in Figure 4.2 a) and connected with PAD by using the PVC sheet connector. In the second step, the cut TLC was immersed in ethanol, by which capsaicinoids were eluted from the TLC plate to the pre-deposited reagents on the PAD (Figure 4.2 b). The blue color appeared when FC reagent reacted with phenol groups of capsaicinoids. For quantitative analysis, the PAD was imaged with a portable flatbed scanner and the grayscale intensity determined by using ImageJ software (NIH, USA).

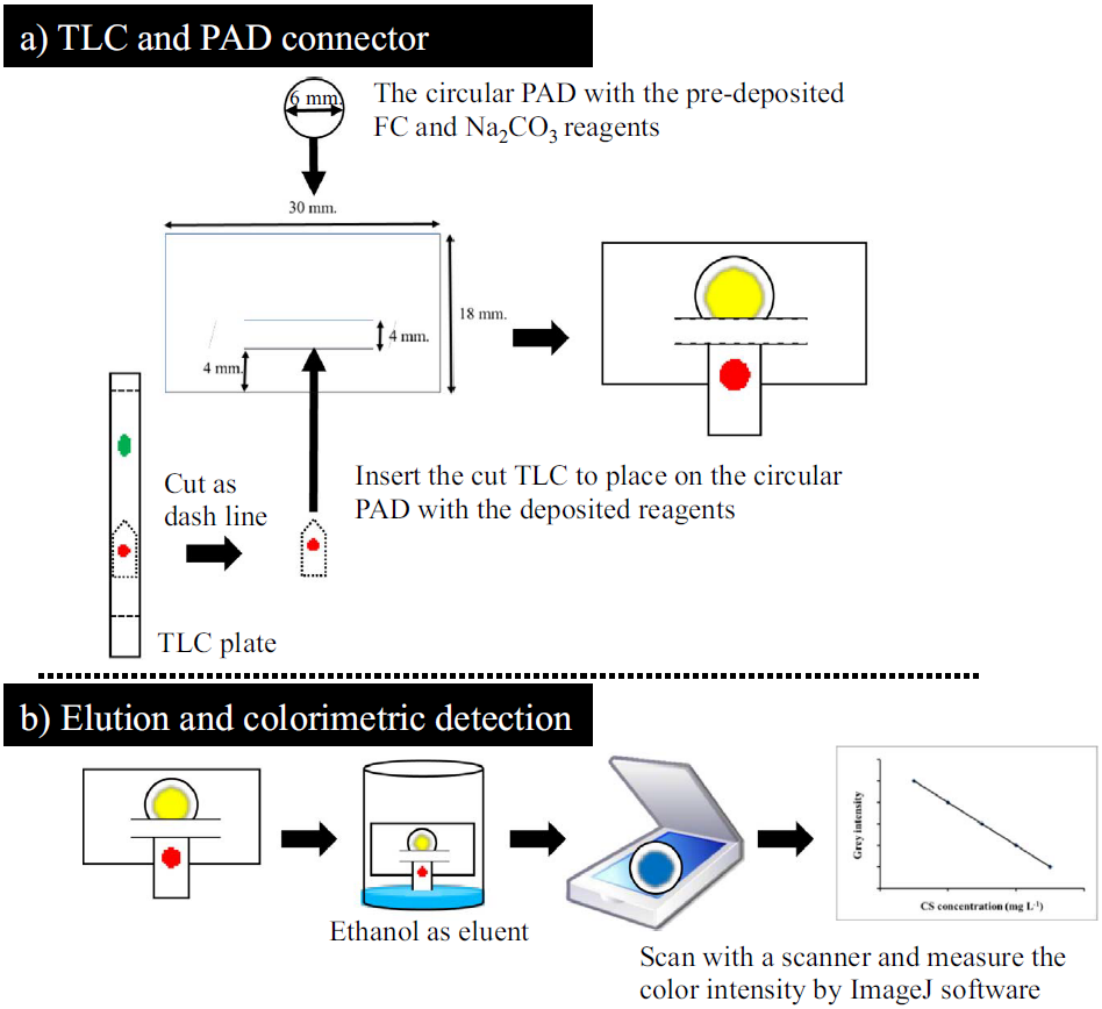


Figure 4.2 Illustration of the proposed method which consisted of two steps: a) TLC separation of capsaicinoids from chilli samples; and b) elution and colorimetric detection of capsaicinoids.

4.5.6 Calculation of resolution in TLC

$$R = \frac{d}{(W_1 + W_2)/2}$$

Resolution (R) of two chromatographic zones is defined as the distance between zones centers (d) divided by the average of the widths (W) of the zones.

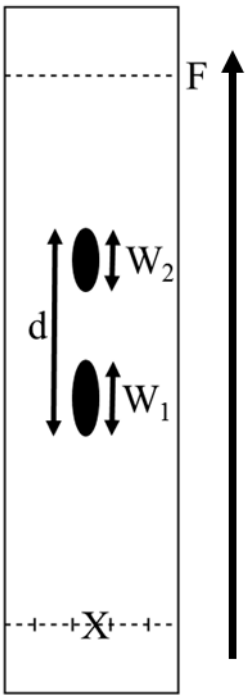


Figure 4.3 Chromatographic resolution determined from spots as the ratio of the separation of zone centers to the average zone width. X = origin and F = solvent front of the TLC plate; the arrow shows the direction of solvent development.

CHAPTER V
RESULTS AND DISCUSSION

5.1 Extraction efficiency of capsaicinoids from chilli

The extraction procedure of capsaicinoids from chilli was modified from the method reported by Othman *et al*. [49]. The extract was subjected to further analysis by high-performance liquid chromatography (n=3). The extraction efficiencies obtained by the modified method was compared with those obtained by Othman *et al*. method as summarized in Table 5.1.

Table 5.1 Comparison of two methods for capsaicinoids extraction from chilli samples (n=3)

Samples	Modified extraction method ($\mu g\ g^{-1}$)	Othman.et.al method [49] ($\mu g\ g^{-1}$)
Dried chilli (DC1)	4000 ± 30	4140 ± 20
Dried chilli (DC2)	3810 ± 10	3880 ± 30
Dried chilli (DC3)	3890 ± 10	4030 ± 30
Seasoning powder (SP1)	415 ± 30	458 ± 20
Seasoning powder (SP2)	625 ± 10	661 ± 10

Although the concentrations of capsaicinoids obtained by the modified method were slightly lower than those by Othman *et al*. method, the results obtained by the two methods were not significantly different as analyzed by the paired t test (t_{stat} =

3.8301, p = 0.0186, $t_{critical}$ (two tail) = 4.6040, and df = 4). Therefore, the modified extraction method was selected for further use in this work as it could be carried out in a shorter extraction time (about 15 min) without requirement of heating process. As a result, sonication-assisted extraction can improve efficiency extraction and the resulting features of modified extraction method.

5.2 TLC for separation of capsaicinoids: Selection of mobile phase

TLC was used for separation of capsaicinoids from other components in chilli. Three mobile phase systems were examined including (50:50, v/v) hexane-ethyl acetate [107], (40:45:15, v/v) ethyl ether-chloroform-acetonitrile [108], and (45:30:25, v/v) toluene-acetone-chloroform [109]. The choices of mobile phase were selected from the literatures. Visualization of the spot was performed after treatment with an irradiation treatment solution. The retention factors or retardation factors (R_f) obtained with these three types of mobile phase are summarized in Table 2.

Table 5.2 TLC separation of capsaicinoids from chilli extract with different mobile phase systems

Mobile phase systems	R_f (CS)	R_f (mixture components)	Resolution
(50:50, v/v) hexane-ethyl acetate	0.41 ± 0.01	0.88 ± 0.06	3.10
(40:45:15, v/v) ethyl ether- chloroform-acetonitrile	0.68 ± 0.03	0.91 ± 0.04	1.82
(45:30:25, v/v) toluene-acetone-chloroform	0.56 ± 0.03	0.86 ± 0.01	1.65

Capsaicinoids could be separated from other components in chilli using either one of the three mobile phase systems. However, with hexane:ethyl acetate

(50:50, v/v), the best resolution (3.10) was obtained , as calculated by equation in section 4.5.5. Therefore, hexane:ethyl acetate (50:50, v/v) was selected for further experiment.

5.3 Confirmation of spots on TLC with HPLC

To confirm the efficiency of capsaicinoids separation on TLC, each spot on TLC was eluted by using ethanol and ultrasonic bath. Then it was injected to HPLC for identifying compounds in each spots. The chromatogram of the compounds eluted of capsaicinoids spot at R_f 0.41 from TLC is shown in Figure 5.2.

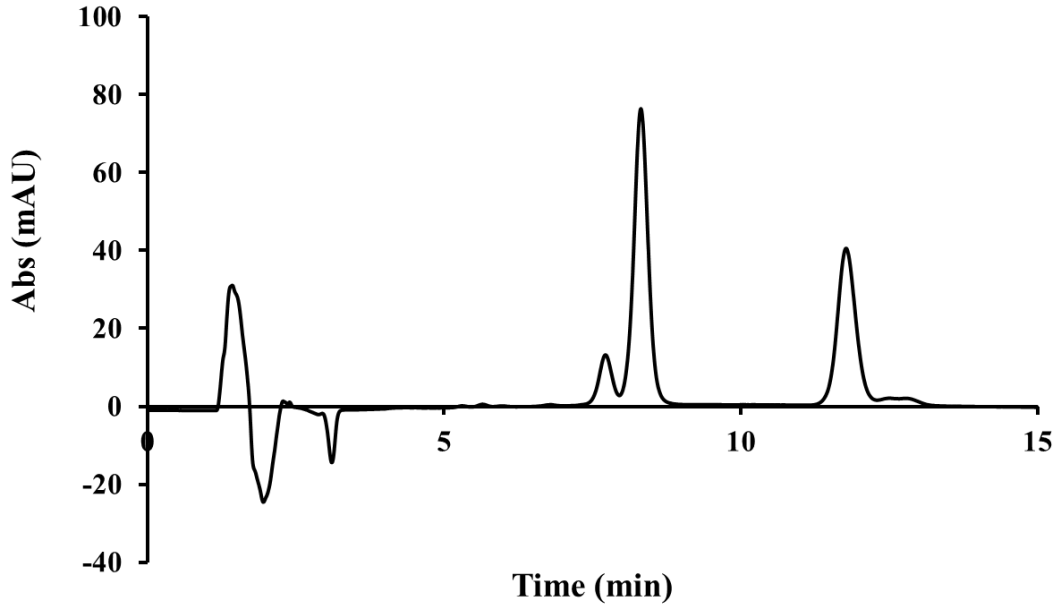


Figure 5.1 HPLC chromatogram of the standard solution corresponding to 100 mg L^{-1} of capsaicinoids

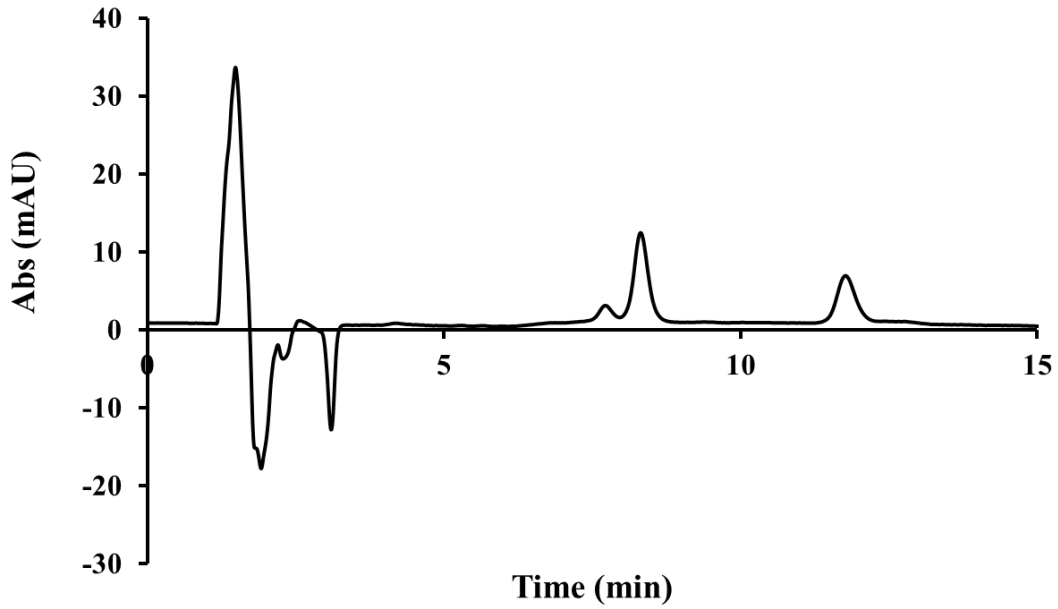


Figure 5.2 HPLC chromatogram of the compound eluted of capsaicinoids spot at R_f 0.41 from TLC

The nordihydrocapsaicin, capsaicin and dihydrocapsaicin are eluted at 7.70, 8.43, and 11.58 minute, respectively when compared with chromatogram of 100 mg L^{-1} standard capsaicinoids solution shown in Figure 5.1.. Similary, other spot at R_f 0.88 was eluted and injected to HPLC. The chromatogram of the compound eluted of other spot from TLC shown in Figure 5.3.

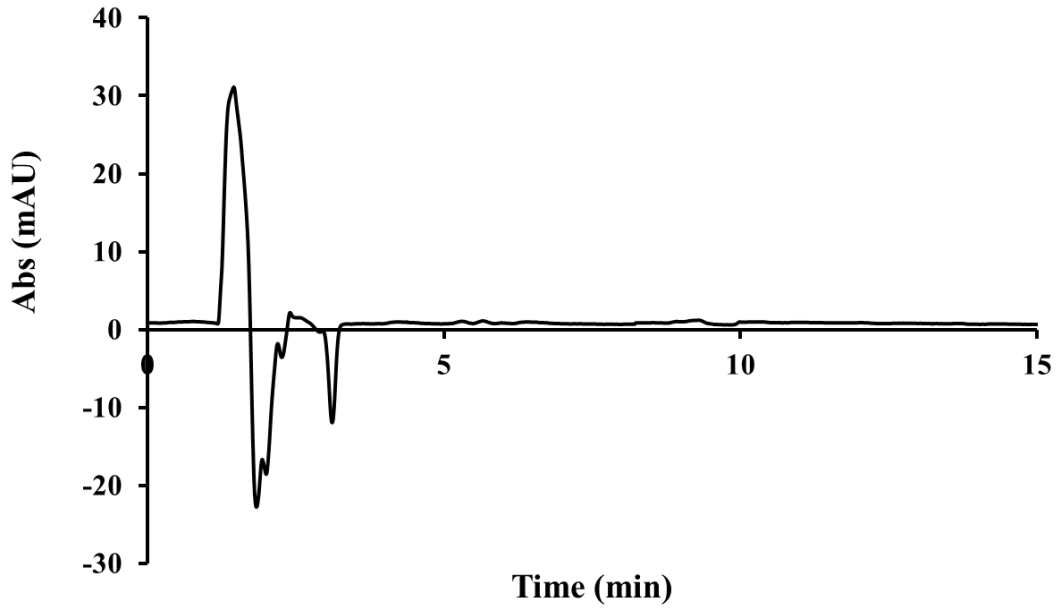


Figure 5.3 HPLC chromatogram of the compound eluted of other spots at R_f 0.88 from TLC

The characteristic peaks of capsaicinoids do not appear in the chromatogram as shown in Figure 5.3. Therefore it can be concluded that TLC with hexane:ethyl acetate (50:50, v/v) as a mobile phase is efficient to separate capsaicinoids in real sample.

5.4 Colorimetric detection of capsaicinoids: Optimization

5.4.1 Sequence of reagents dropping

TLC was used for separation of capsaicinoids from other components in chilli. Three mobile phase systems were examined including (50:50, v/v) hexane-ethyl acetate. Considering from the Folin-Ciocalteu assay, the sequence of reagents dropping is as follows: capsaicinoids; FC; and Na_2CO_3, subsequently [66], as shown in Figure 5.4a. Nonetheless, to facilitate the analysis on the PAD in this work, the sequence of reagent dropping on the PAD was adjusted to be as follows: FC; Na_2CO_3; and capsaicinoids, subsequently, as shown in Figure 5.4b. The effect of reagent dropping sequence was tested by using 1000 mg L^{-1} CS. The blue color in the solution and on the

PAD were further analyzed and showed the grayscale intensity of 207.9 ± 0.4 for solution based and 208.0 ± 0.5 for PAD (n=3). As the grayscale intensities obtained from both methods were not different, the sequence of reagent dropping on the PAD was suitable for use.

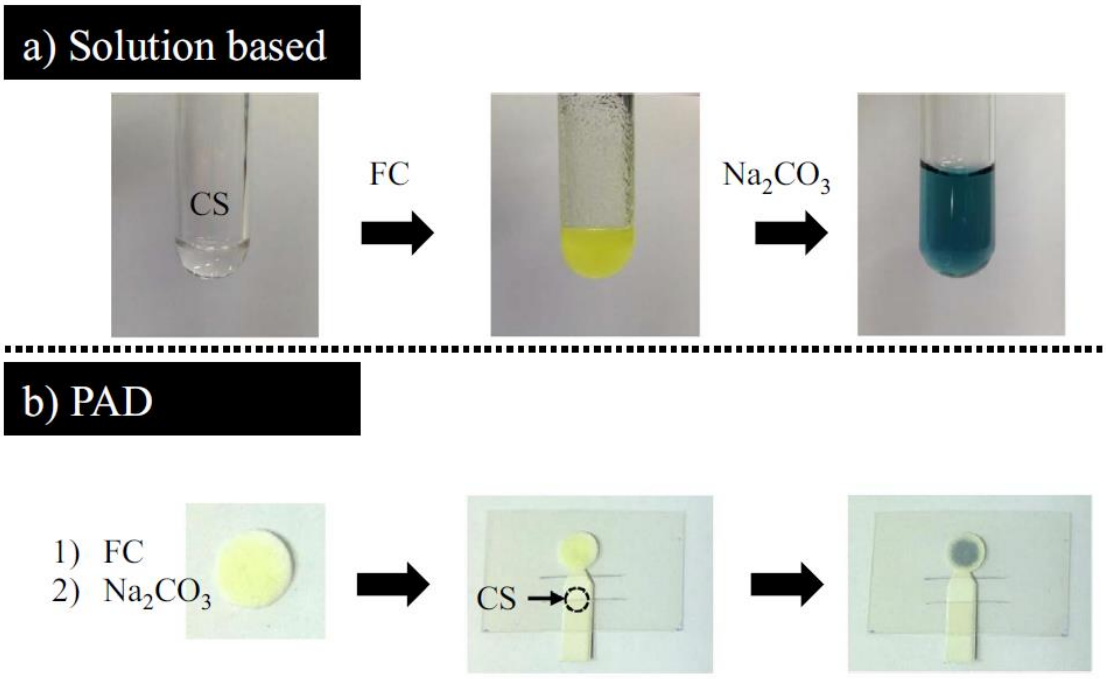


Figure 5.4 Colorimetric detection of 1000 mg L^{-1} capsaicinoids by: a) the solution based system; and b) PAD system.

5.4.2 Effect of Na_2CO_3 concentration

Furthermore, the concentration of Na_2CO_3 was also studied in the range of 2-16 % (w/v). For the standard capsaicinoids of 200 mg L^{-1}, the effect of Na_2CO_3 concentration on color development is illustrated in Figure 5.5. Increasing Na_2CO_3 from 2 to 10 %, the blue color gradually increased while the yellow background color decreased. At Na_2CO_3 concentrations higher than 10% (w/v), the blue color gradually decreased and the yellow background color disappeared. Considering from the grayscale intensity obtained from the ImageJ program, however, the lowest grayscale intensity value was obtained at 10% Na_2CO_3 and therefore this concentration was selected for further use. The lower grayscale intensity represents the more intense color as the scale is set as the black color is equal to “0” and the white color is equal to “255”.

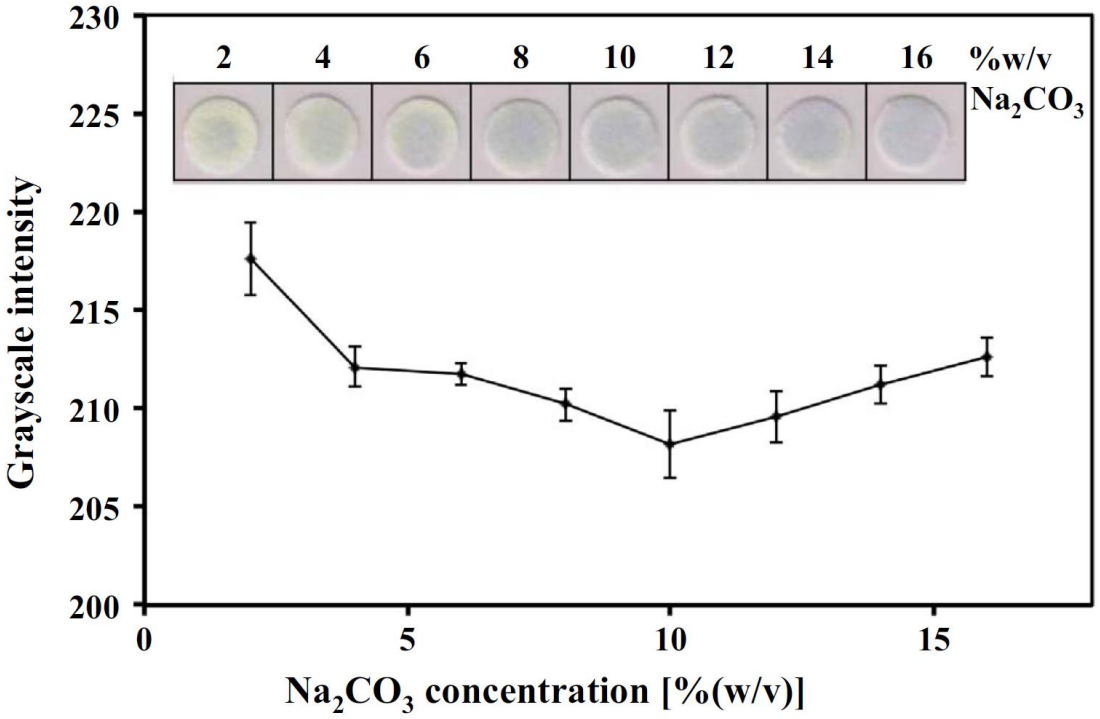


Figure 5.5 Effect of Na_2CO_3 concentration on colorimetric detection of 200 mg L^{-1} capsaicinoids (n=3).

5.4.3 Ratios of FC and Na_2CO_3

To obtain highest sensitivity for colorimetric detection of capsaicinoids on the PAD, the ratio of FC and Na_2CO_3 was also optimized. The results are shown in Figure 5.6 for CS concentration of 200 mg L^{-1}. At the ratio of FC and Na_2CO_3 at 1:1, yellow color slightly appeared while the lowest grayscale intensity value was obtained. Increasing the volume of Na_2CO_3 to be higher than FC, the yellow color completely disappeared, however, the blue color intensity also decreased. With the volume of FC higher than Na_2CO_3, the colorimetric reaction was not completed and the yellow color still appeared. Therefore, the ratio of FC and Na_2CO_3 at 1:2 was used for further experiment as the standard deviation of the gray intensities was also lowest.

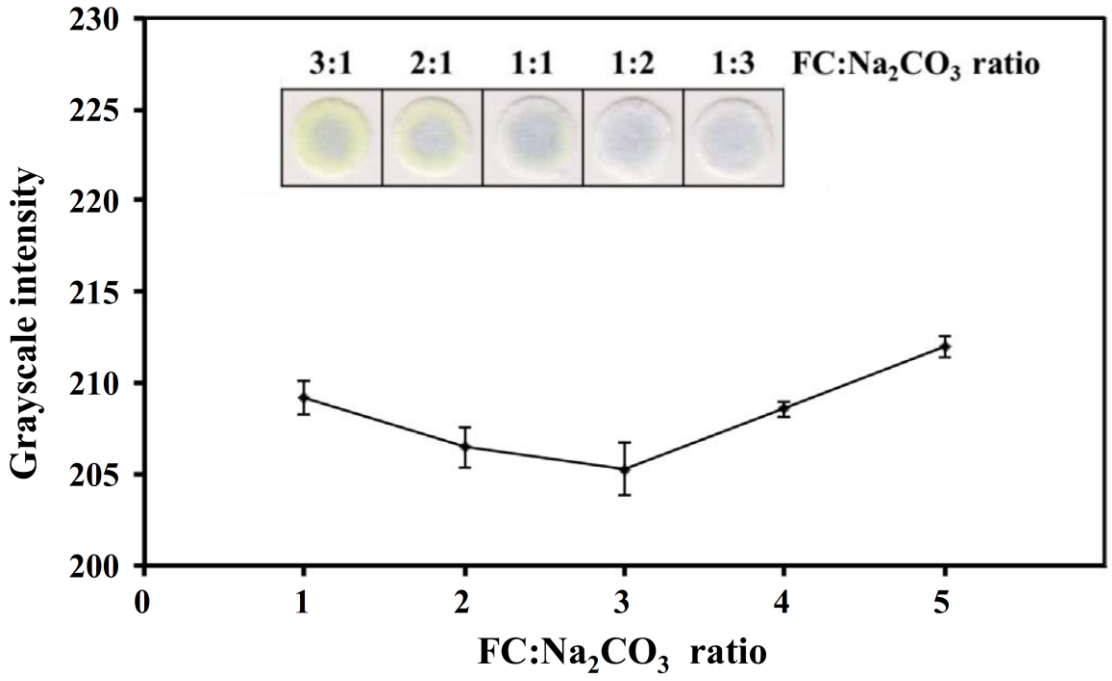


Figure 5.6 Effect of FC and Na_2CO_3 volume ratio on colorimetric detection of 200 mg L^{-1} capsaicinoids (n=3).

5.5 Analytical performance

5.5.1 Working range and limit of detection (LOD)

The working range of the method was obtained from calibration curve between the capsaicinoids concentrations and grayscale intensity of standard. The results are shown in Figure 5.7.

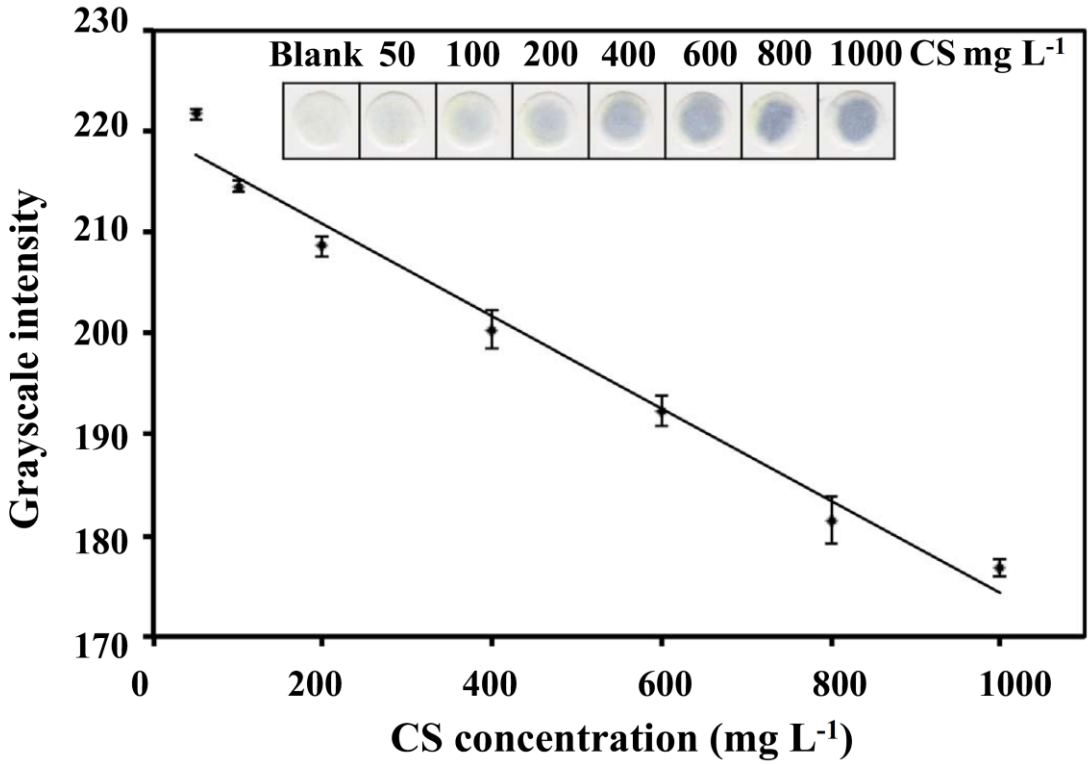


Figure 5.7 Visual color change of various CS concentrations on PAD and its calibration curve. The linear equation was y = -0.0483x + 221.84 with the correlation coefficient of 0.9721.

With the optimum condition, the proposed method was applied to capsaicinoids detection in the range of 50 to 1000 mg L^{-1} capsaicinoids. Linear calibration curve was obtained with the calibration equation as y = -0.0483x + 221.84 with a good correlation coefficient of 0.9721 (n=3). The limit of detection (LOD) was found to be 50 mg L^{-1} capsaicinoids.

5.5.2 Comparison with HPLC

The accuracy was checked by comparing result from modified method and conventional method (HPLC) (n = 3) with the results shown in Table 5.3.

Table 5.3 Analytical applications for chilli samples, comparison between the proposed method and HPLC method

Samples	By proposed method (μg g^{-1})	By HPLC (μg g^{-1})	Percent difference
Dried chilli 1 (DC1)	4030 ± 20	4000 ± 30	0.8
Dried chilli 2 (DC2)	3790 ± 20	3810 ± 10	-0.5
Dried chilli 3 (DC3)	3830 ± 8	3890 ± 10	-1.6
Seasoning powder 1 (SP1)	426 ± 8	415 ± 30	2.5
Seasoning powder 2 (SP2)	590 ± 10	625 ± 10	-5.6

The analytical results obtained from the proposed TLC-PAD method were in good agreement with those from HPLC method as shown in Table 5.3. This was proven by the paired *t* test that the results were not significantly different between the two methods (t_{stat} = 0.8594, p = 0.4385, $t_{critical}$ (two tail) = 2.7764, and df = 4). Therefore, the proposed method is reliable and can be considered as a promising method for capsaicinoids determination in chilli samples.

5.6 Analytical performance of HPLC for capsaicinoids determination

HPLC is an important technique in modern separation chemistry. Especially, the conventional method for capsaicinoids determination is a HPLC. In this work HPLC was used for qualitative and quantitative analysis of capsaicinoids.

5.6.1 Retention time

The retention time depends on the interaction between functional group of analytes and functional group of stationary phase in the column. Thus, the retention time of nordihydrocapsaicin, capsaicin and dihydrocapsaicin are eluted at 7.70, 8.43, and 11.58 minutes, respectively (see Figure 5.8)

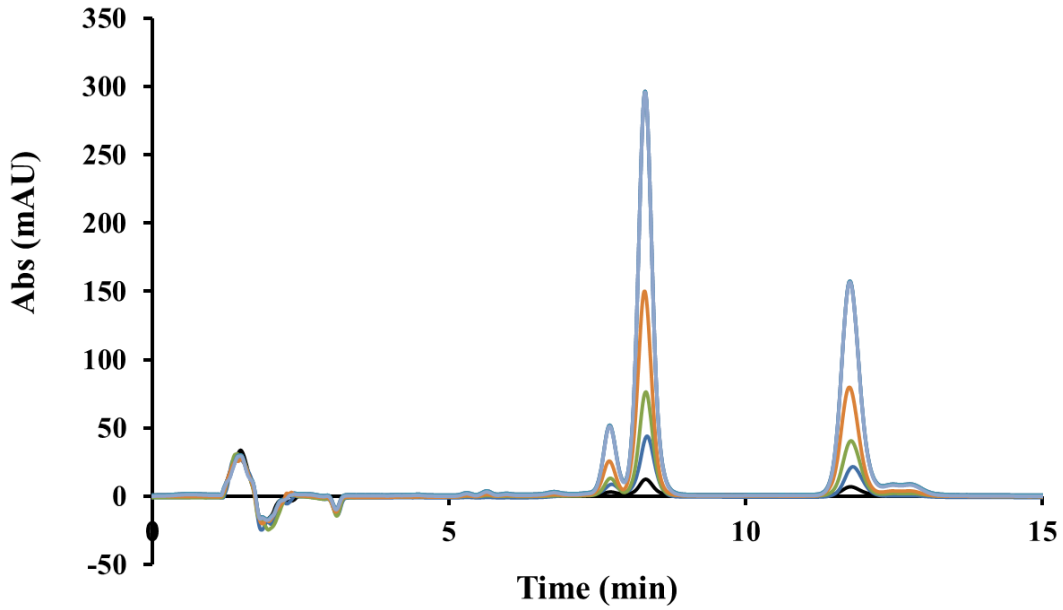


Figure 5.8 HPLC chromatogram of the standard solution corresponding to 10 - 400 mg L^{-1} of capsaicinoids

5.6.2 Linearity range

The linearity of the HPLC method was obtained from calibration curve plotting between the concentrations and the sum of peak area of the three peak of standards. The results are shown in Figure 5.9.

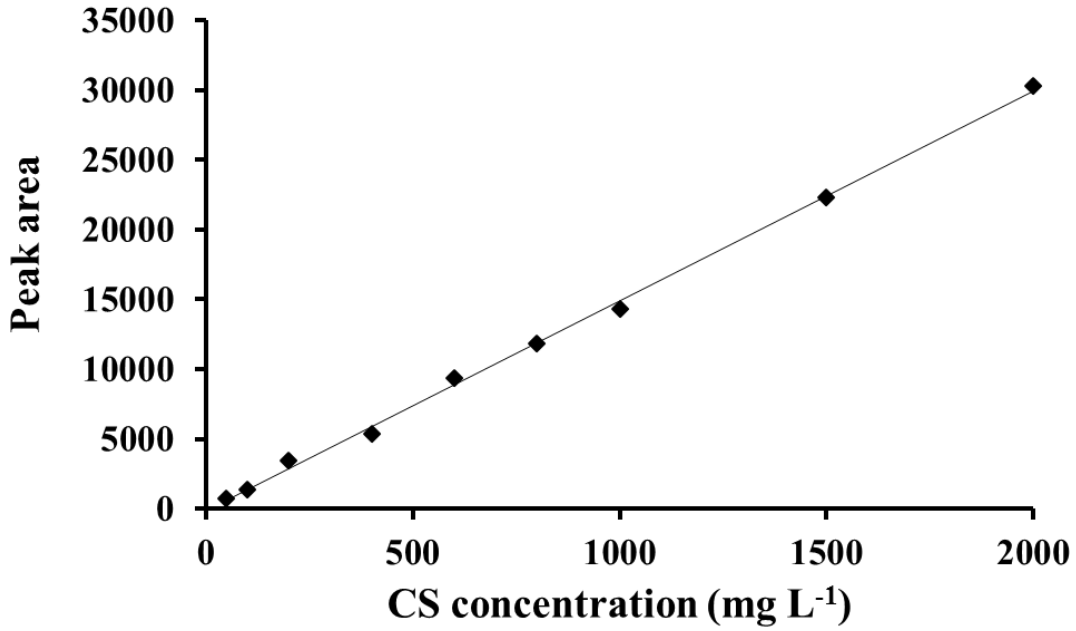


Figure 5.9 The calibration curve of standard solution corresponding to 10-2000 mg L^{-1} of capsaicinoids.

The linearity of capsaicinoids was found in the range of 10 – 2000 mg L^{-1} showing a wide concentration range of the analysis. The linear equation was y = -15.015x – 111.54 with the correlation coefficient of 0.9983.

5.7 Application to chilli samples

The standard addition method was used for recovery study. Two concentrations of standard capsaicinoids (200 and 1000 mg L^{-1}) were spiked into all five samples studied herein (n = 3) with the results shown in Table 5.4. Two types of samples including dried chilli and seasoning powder were determined for their capsaicinoids concentrations using the proposed method. Capsaicinoids in three dried chilli samples were 3790 - 40304 ± 30 µg g^{-1} and 426 - 590 ± 20 µg g^{-1} were found in two seasoning powder samples. The recoveries were found to be in the range of 90.3 -109% indicating that the proposed method was reliable.

Table 5.4 Recovery results for standard addition of capsaicinoids

Samples	**Initial CS concentration ($\mu g\ g^{-1}$)**	**Standard added ($\mu g\ g^{-1}$)**	**Found ($\mu g\ g^{-1}$)**	**Recovery (%)**
Dried chilli 1 (DC1)	4030 ± 20	0.0	4030 ± 20	100
		200	4240 ± 20	105
		1000	4930 ± 20	90
Dried chilli 2 (DC2)	3790 ± 20	0.0	3790 ± 20	100
		200	3970 ± 8	90
		1000	4760 ± 5	97
Dried chilli 3 (DC3)	3830 ± 8	0.0	3830 ± 8	100
		200	4030 ± 30	102
		1000	4810 ± 20	98
Seasoning powder 1 (SP1)	426 ± 8	0.0	426 ± 8	100
		200	608 ± 20	91
		1000	1350 ± 10	92
Seasoning powder 2 (SP2)	590 ±10	0.0	590 ± 10	100
		200	783 ± 10	96
		1000	1680 ± 2	109

CHAPTER VI
CONCLUSION

A simple, easy to operate, and low-cost method has been developed for capsaicinoids determination in chilli by using TLC-PAD based on Folin-Ciocalteu colorimetric detection. The samples could be effectively extracted by ethanol with ultrasonic assistance within a very short period (15 min), and separated using a TLC with (50:50, v/v) hexane-ethyl acetate as the mobile phase. Under the optimal conditions, the proposed method offered a linear detection range of capsaicinoids from 50 to 1000 mg L^{-1}. The proposed method also provided high recovery (90–109%) with the detection limit as low as 50 mg L^{-1}. The results obtained by the proposed method were in good agreement with those from the HPLC method. This developed method provides advantages of rapidity, simplicity, cost effectiveness and accuracy, and hence suitable for use as a screening test of capsaicinoids in raw chilli and its products.

Suggestions for future work

As the new method by using thin-layer chromatography (TLC) coupled with paper-based analytical device (PAD). This approach can be further applied for other analytes determination when it requires separation method for sample preparation.

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APPENDICES

APPENDIX A
IMAGEJ PROGRAM FOR IMAGE ANALYSIS

This is the photo of ImageJ program used for reading the grayscale intensity from the captured image.

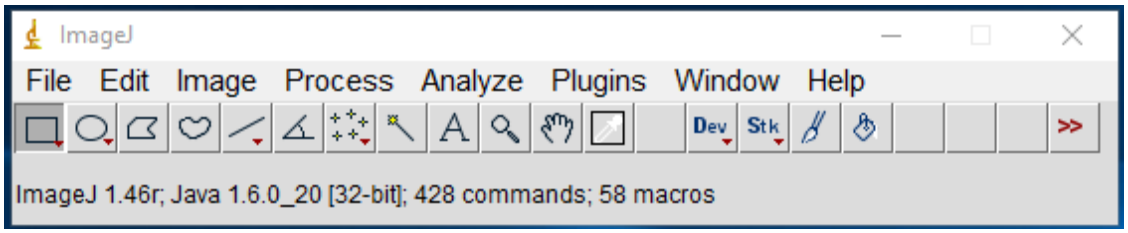
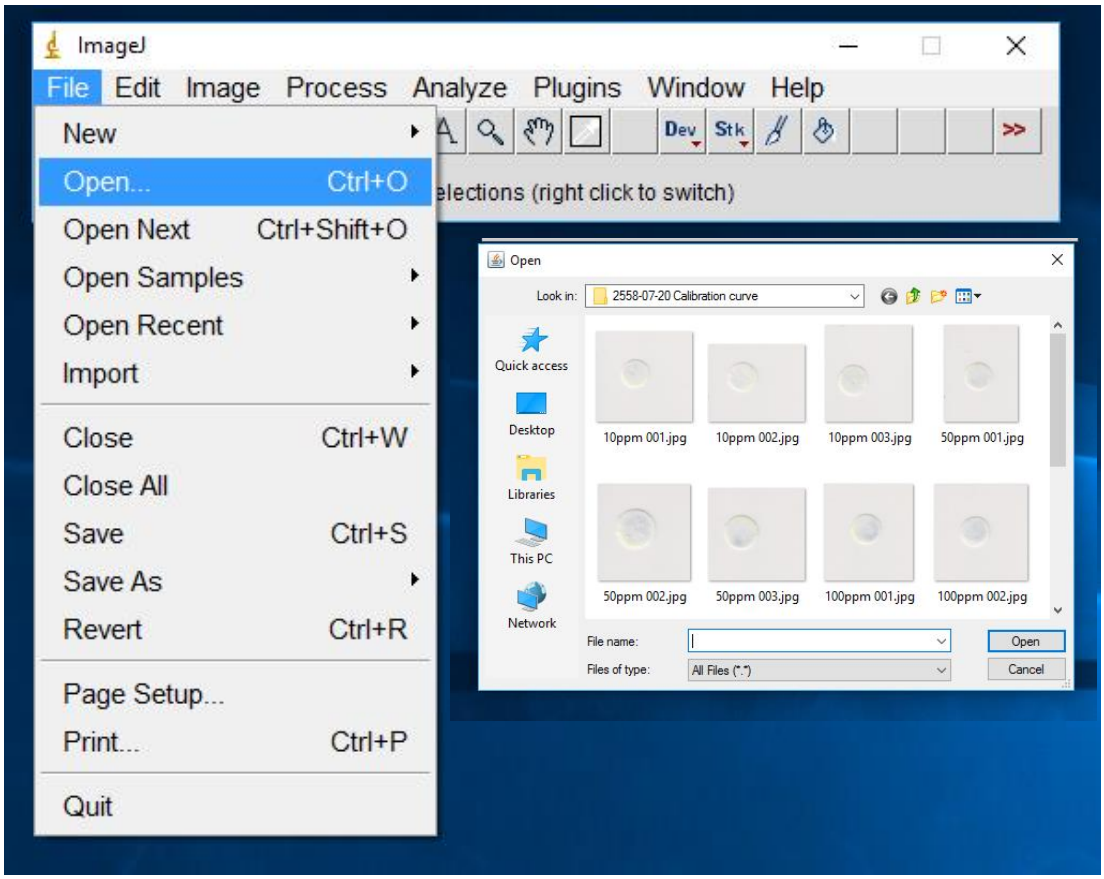


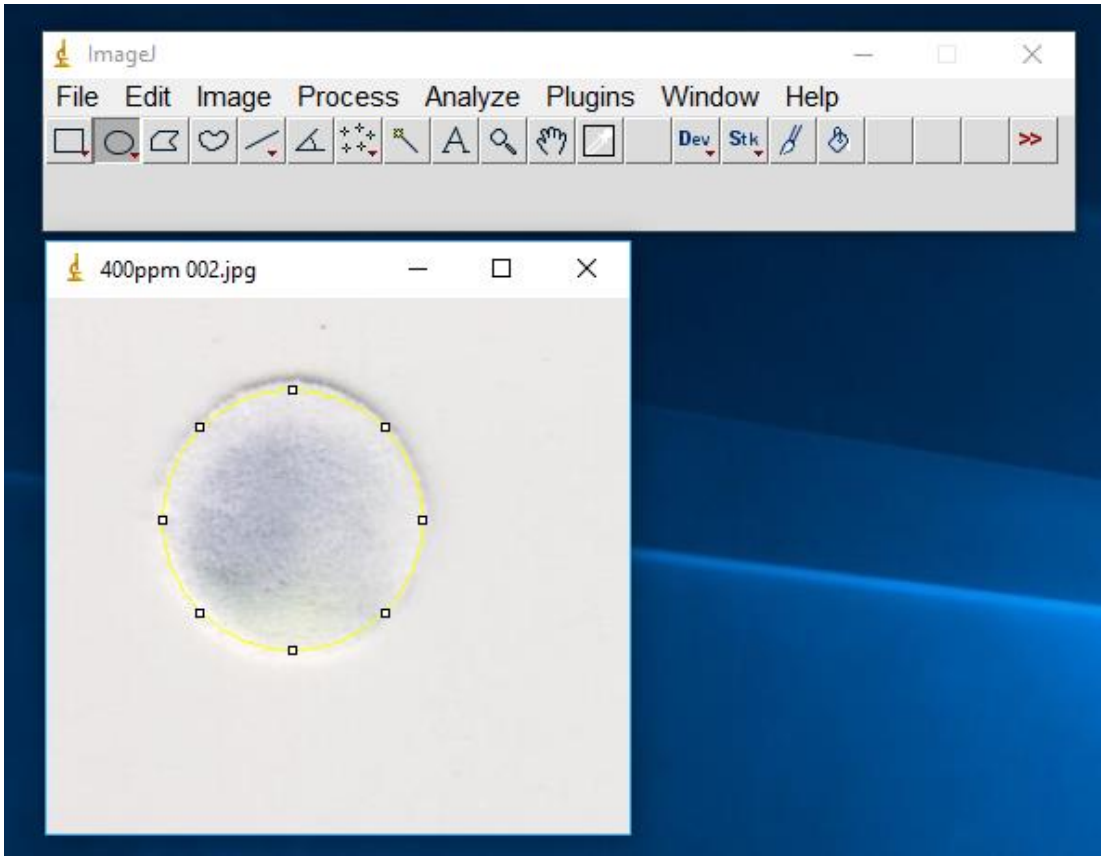
Figure A.1 ImageJ program used in this work.

This is an example of the instruction of how to read the grayscale intensity from the ImageJ program.

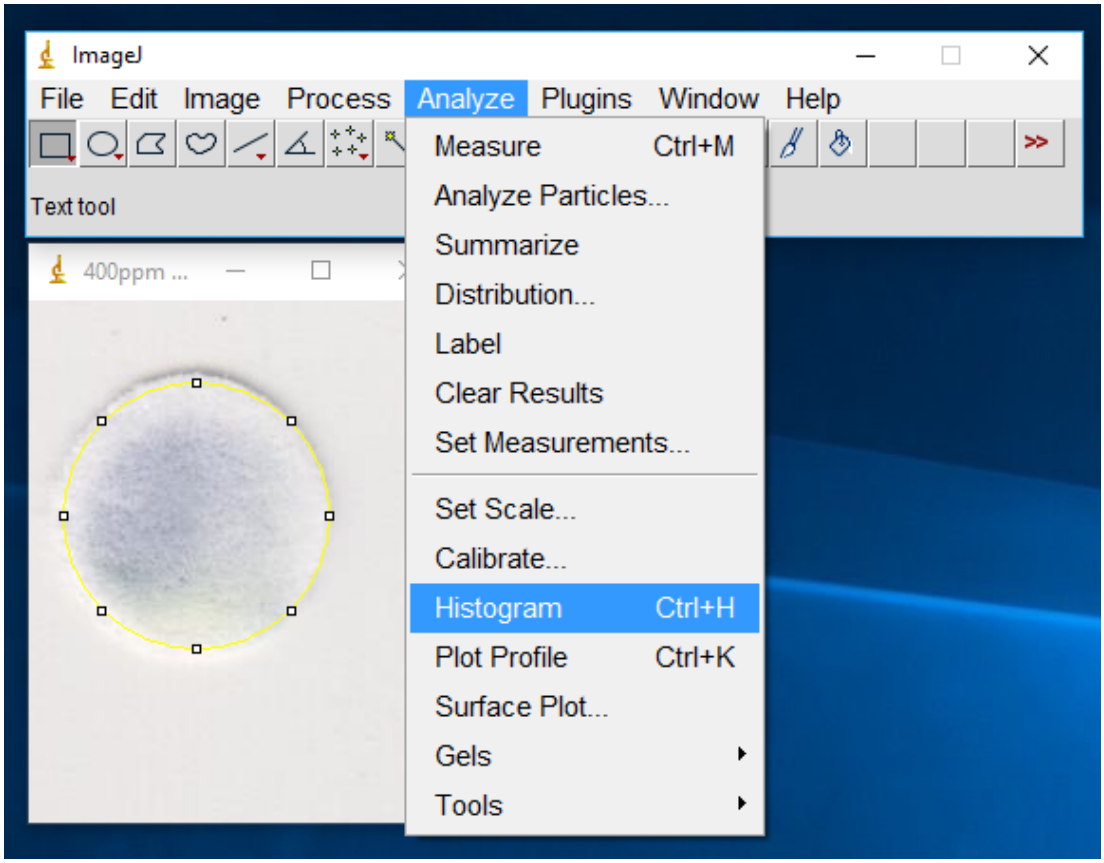
1. Open the captured image of the paper-based analytical device



2. Select the area with circle that would like to read the grayscale intensity



3. Then select the "Analyze" menu item and inside of it, click on "Histogram".



4. Record Count and Mean.

Note: Please observed, count value must always equal.

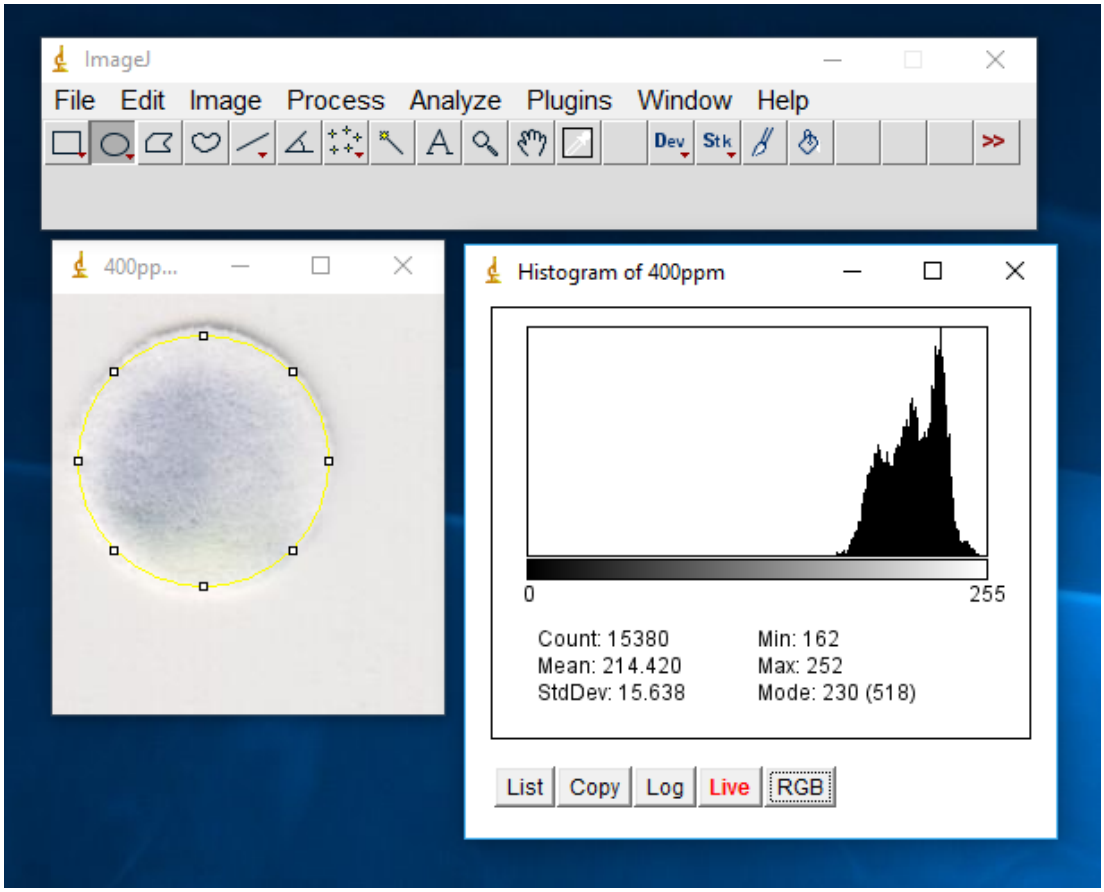


Table A.1 Data of grayscale intensity for calibration curve

CS concentration (mg L^{-1})	**Grayscale intensity**			**Average grayscale intensity**	**S.D.**
	1	2	3		
10	226.665	225.867	225.665	226.065	0.53
50	221.462	221.331	222.425	221.739	0.60
100	213.681	214.269	215.764	214.571	1.07
200	206.946	208.281	210.697	208.641	1.90
400	200.034	199.034	202.034	200.367	1.53
600	192.278	190.113	194.765	192.385	2.33
800	181.084	182.539	180.863	181.495	0.91
1000	176.627	178.145	175.764	176.845	1.21
1500	175.876	172.929	174.187	174.330	1.48
2000	171.486	172.658	172.956	172.366	0.78

Table A.2 Data of grayscale intensity for effect of Na_2CO_3 concentration on colorimetric detection of 200 mg L^{-1} capsaicinoids

Na2CO3 concentration (mg L^{-1})	**Grayscale intensity**			**Average grayscale intensity**	**S.D.**
	1	2	3		
2	215.442	218.774	218.502	217.573	1.850
4	213.013	210.935	212.444	212.131	1.074
6	211.117	211.869	212.236	211.741	0.570
8	209.381	210.152	211.059	210.197	0.840
10	209.587	208.647	206.287	208.174	1.700
12	209.766	208.151	210.718	209.545	1.298
14	211.732	211.779	210.121	211.211	0.944
16	212.893	211.535	213.465	212.631	0.991

Table A.3 Data of grayscale intensity for effect of FC and Na_2CO_3 volume ratios on colorimetric detection of 200 mg L^{-1} capsaicinoids

Ratio	**Grayscale intensity**			**Average grayscale intensity**	**S.D.**
	1	2	3		
3:1	209.825	208.139	209.651	209.205	0.927
2:1	207.089	205.207	207.108	206.468	1.092
1:1	206.932	204.34	204.584	205.285	1.431
1:2	208.264	209.073	208.418	208.585	0.430
1:3	211.879	211.497	212.68	212.019	0.604

Table A.4 Data of grayscale intensity for samples determination

Sample	Weight of sample (g)	Grayscale intensity			Aver. grayscale intensities	Conc ug g^{-1}	Average Conc ug g^{-1}	S.D.
		1	2	3				
	2.0058	182.466	182.894	182.667	182.676	4043		
DC1	2.0106	182.697	183.016	182.964	182.892	4011	4029	17
	2.0085	182.568	182.784	182.754	182.702	4034		
	1.9915	185.31	185.227	185.224	185.254	3804		
DC2	2.0076	184.876	185.145	184.976	184.999	3799	3787	24
	2.0045	184.942	185.742	185.648	185.444	3759		
	2.0076	185.082	184.785	184.387	184.751	3825		
DC3	2.0037	184.964	184.654	185.036	184.885	3819	3826	8
	2.0085	184.482	184.356	185.106	184.648	3834		
	1.9905	213.548	213.942	213.627	213.706	423		
SP1	2.0095	213.456	213.974	213.654	213.695	420	426	8
	2.0065	213.146	213.485	213.654	213.428	434		
	2.0065	210.245	210.679	210.893	210.606	580		
SP2	2.0074	209.945	210.456	210.974	210.458	587	590	12
	1.9985	209.365	210.486	210.693	210.181	604		

Table A.5 Data of grayscale intensity for samples determination with standard addition at 200 mg L^{-1} capsaicinoids

Sample	Weight of sample (g)	Grayscale intensity			Average grayscale intensities	Conc ug g^{-1}	Average Conc ug g^{-1}	S.D.
		1	2	3				
	1.0035	180.365	180.965	180.978	180.769	4237		
DC1	1.0096	180.615	181.006	180.489	180.703	4218	4237	19
	1.0042	180.514	180.784	180.354	180.551	4256		
	1.0041	183.318	182.958	183.741	183.339	3969		
DC2	1.0065	183.954	183.456	182.256	183.222	3972	3975	7
	0.9972	183.695	183.456	183.264	183.472	3983		
	0.9971	182.589	183.047	182.645	182.760	4057		
DC3	1.0096	183.005	182.975	182.445	182.808	4002	4029	28
	1.0008	182.753	182.948	183.012	182.904	4027		
	1.0074	210.343	209.945	209.786	210.025	607		
SP1	0.9965	210.065	210.645	210.612	210.441	592	608	16
	0.9981	209.687	209.487	210.213	209.796	625		
	1.0035	206.865	206.564	206.345	206.591	787		
SP2	1.0062	206.485	206.548	206.351	206.461	791	783	11
	1.0089	206.987	207.005	206.485	206.826	770		

Table A.6 Data of grayscale intensity for samples determination with standard addition at 1000 mg L^{-1} capsaicinoids

Sample	Weight of sample (g)	Grayscale intensity			Average grayscale intensities	Conc ug g^{-1}	Average Conc ug g^{-1}	S.D.
		1	2	3				
	1.0035	174.012	173.987	173.854	173.951	4940		
DC1	1.0096	173.879	174.364	173.658	173.967	4909	4933	21
	1.0042	173.546	174.106	173.849	173.834	4949		
	1.0041	175.972	175.671	175.452	175.698	4757		
DC2	1.0065	175.592	175.655	175.458	175.568	4759	4761	4
	0.9972	176.094	176.112	175.592	175.933	4766		
	0.9971	174.929	175.529	175.5214	175.326	4829		
DC3	1.0096	175.179	175.047	175.125	175.117	4791	4814	21
	1.0008	175.07154	175.654	174.918	175.215	4823		
	1.0074	195.99	195.689	195.47	195.716	1342		
SP1	0.9965	195.61	195.673	195.476	195.586	1364	1349	12
	0.9981	196.112	196.13	195.61	195.951	1343		
	1.0035	188.913	189.513	189.526	189.317	1677		
SP2	1.0062	189.163	189.554	189.037	189.251	1676	1678	2
	1.0089	189.062	189.332	188.902	189.099	1680		

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