Studies of Capsaicinoids Contents of Locally Grown and Commercial Chilies

Using Reversed-Phase High Performance Liquid Chromatography

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by

John Kailemia Muchena

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Dr. Chu-Ngi Ho, chair

Dr. Peng Sun

Dr. Yu-Lin Jiang

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ABSTRACT

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Capsaicinoids are a class of compounds responsible for the "heat" of hot peppers. Capsaicin and dihydrocapsaicin have the highest burning effect. The aim of this work is to separate and quantify the two major capsaicinoids in fruits harvested at different stages of development and at different seasons. Simple and rapid HPLC method involves 73:27% methanol water mobile phase with C₁₈ stationary phase and UV-Vis detector set at 210 nm. The method showed good reproducibility with 1.74% - 4.72% relative standard deviations, a linear response within 0.65 – 45.5 and 0.25-17.5 µg/mL for capsaicin and dihydrocapsaicin, respectively. The method achieved average recovery of 106% for capsaicin and 102% dihydrocapsaicin. Determination of capsaicinoids in four naturally grown chili and commercial source habanero were analyzed. The amount in the sample ranged from 1184-8156 µg/g for capsaicin and 430-3299 µg/g for dihydrocapsaicin.
DEDICATION

I dedicate this work to my wife Pamela Mukami, my parents George Muchena and Rael Kandebe, my brothers Kinoti and Mwenda, and my sisters Kagwiria, Gatumwa, Karimi, Kathure, and Kagendo. Your support and encouragements have been a huge resource to me.
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INTRODUCTION

The hot pepper is a popular plant found in most parts of the world. Due to its sensory attributes, chili fruit has been widely used as a food flavoring, a coloring agent, and feed additive in livestock. Capsaicinoids is a family of natural products extracted from hot pepper fruits. Capsaicinoids are used worldwide in consumer products, medicinal preparations, and pharmaceuticals. They are the main constituents of riot and self-defense pepper spray, and they are found in several over the counter pain treatments and also in many dietary supplements (1, 2). Because of these applications, there has been a lot of research about hot pepper and capsaicinoids.

Chili varieties differ in terms of size, shape, color, flavor, and pungency level (2). The concentration of capsaicinoids in hot pepper varies from one variety to another due to genetic make up, and even in the same variety depending on the environmental conditions in which the pepper grows (3).

Historical Background

The chili pepper is one of the very first domesticated plants in the Middle America. The archeological evidence for the consumption of chili pepper dates back to the seventh millennium BCE, long before the cultivation of maize and beans. At this period pepper were harvested in the wild (4). The botanical name of chili is a Latin name capsaicin, the word has a Greek based derivative of Latin “Kapto” meaning to “bite” which has some relation to hotness (4). The people who cultivated chili were aware of the highly potent and stimulating action of
chili leading to increased food intake as a result of increased salivation, warmth, and glow in the
mouth and possibly the postingestional comfort (5).

Christopher Columbus is believed to be the first European to discover chili in one of his
legendary travels to America around 1493. Within a century this hot pepper had attained a
worldwide distribution. Native Americans used the smoke from burning pepper as a form of
weapon against invaders (6). Portuguese introduced chili into India, Africa, and tropical
America where the plant develop greater pungency than in colder regions (4).

**Capsaicinoids**

This is a family of related compounds from capsicum fruits. Depending on the variety of
hot pepper the concentration of capsaicinoids ranges from 0.003% to 1% of the total weight (7).

Bennett and Kirby (8) applied mass and nuclear magnetic resonance spectral technique on 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 1 shows the parent structure of
the capsaicinoids, while Table 1 shows the carbon chains attached to the parent structure to
give the different capsaicinoids.

![Figure 1. The parent structure of capsaicinoids](image)
The structure of R is shown below.

Capsaicin and dihydrocapsaicin make up 80% to 90% of all capsaicinoids in the fruit in the ratio of generally around 1:1 and 2:1, respectively. It has been established that, less spicy varieties have capsaicinoids concentration range 0.003%-0.01%. Mildly hot varieties have capsaicinoids range from 0.01% to 0.3%, while the hot spicy ones have concentration range is 0.3% to 1% capsaicinoids of the total dry weight (7).

Table 1. Straight and branched chain of homologous series that attach to the parent structure of capsaicinoids

<table>
<thead>
<tr>
<th>Name</th>
<th>Systematic name</th>
<th>symbol</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin</td>
<td>Trans-8-methyl-N-Vanillyl-6-nonamide</td>
<td>C</td>
<td><a href="#">Structure</a></td>
</tr>
<tr>
<td>Dihydrocapsaicin</td>
<td>8-methyl-N-nonamide</td>
<td>DHC</td>
<td><a href="#">Structure</a></td>
</tr>
<tr>
<td>Nordihydrocapsaicin</td>
<td>7-methyl-N-Vanillyl-octamide</td>
<td>n-DHC</td>
<td><a href="#">Structure</a></td>
</tr>
<tr>
<td>Norcapsaicin</td>
<td>Trans-7-methyl-N-vanillyl-5-octamide</td>
<td>n-C</td>
<td><a href="#">Structure</a></td>
</tr>
<tr>
<td>Homocapsaicin</td>
<td>Trans-9-methyl-N-vanillyl-7-Homodihydrocapsaicin decamide</td>
<td>h-C</td>
<td><a href="#">Structure</a></td>
</tr>
<tr>
<td>Homodihydrocapsaicin</td>
<td>9-methyl-N-vanillyl-decamide</td>
<td>h-DHC</td>
<td><a href="#">Structure</a></td>
</tr>
</tbody>
</table>

The hot pungent taste of chili peppers is a result of its main ingredient capsaicin. Hot peppers belong to the genus capsicum whose botanical family is Solanaceae. The term pepper
is a general term that encompasses black pepper which is *piper nigrum* and pimento which is *pimento dioica*. Chili pepper, red pepper, paprika, are all species of *capsicum* (6).

These plants (Solanaceae family) produce secondary metabolites meant for protection against phytopathogens. Potatoes produce the glycoalkaloids α-chakonine, α-solanine, and calystegine alkaloids. Tomatoes produce glycolkaloids dehydrotomatine and α-tomatine. Jimson weed seeds produce the alkaloids atropine and acopolamine. Hot pepper contains capsaicinoids, high level of antioxidants vitamin C, and carotenoids (9).

**Capsaicin (Trans-8-methyl-N-vanillyl-6-nonenamide)**

Capsaicin is an off-white solid with a melting point of 62-65°C. It is odorless and dissolves in fat. It exhibits a pungent taste. Some of the properties that make these spices so popular in foods and seasonings include their color, aroma, and flavor. Capsaicin occurs naturally in the fruits of *capsicum* plant. Capsaicin is located mainly in the cross walls of the hot peppers placenta and spreads throughout the pod during processing (10). The structural characteristics of capsainoids that determine their spicy properties are associated with the presence of an amide bond connecting a vanillyl ring and an acyl chain (11). Capsaicin has a benzene ring and a long hydrophobic carbon tail with polar amide group (12) and has a molecular weight of 305.4.

Capsaicin was first isolated in 1846, and its structure was determined in 1919 (12). Chemical studies regarding the active constituents began in the early part of the 19th century. Bucholz is credited with the first work showing that the pungency principles were extractable with organic solvents. The name capsaicin was given by Thresh in 1846 (12) who reported the
crystallization of the active components. Nelson and Dawson (6) clarified the structure of capsaicin. They identified the products of the hydrolysis of capsaicin as vanillyl amine and an unsaturated fatty acid.

Total synthesis of capsaicin was conducted by Spath and Darling, and they confirmed the earlier work of Nelson and Dawson (6) by indicating that vanilloids contains a vanillyl (4-hydroxy-3-methoxybenzyl) that explains their biological activity. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonenamide) represent around 90% of total capsaicinoids present in hot spicy varieties (11).

**Uses of Capsaicin**

**Nutritional Additive**

The spicy nature of chili is associated with the amide bond connecting a vanillyl ring with an acyl chain (11). Due to its pungency, aroma, and taste, capsaicin has been widely used as a food additive. Its use is characterized by sneezing, coughing, pain, burn-like sensation in the mouth and throat, flushing of the face and neck, and also sweating of the forehead. Due to all these, the first encounter with hot chili, especially for the young, is usually unpleasant. However, people get used to it with frequent use.

In some countries’ populations, the use of chili was considered as a tradition or a cultural identity. In less affluent and developing countries the high potency of chilies was effective for stimulating warmth and strong salivation responses in the mouth, just like other spices. This created an economical way of improving the appetite by making repetitive cereals more interesting.
Capsicum fruits contain a proportional amount of essential nutrients including vitamin C whose quantity is more than that contained in an orange fruit and pro-vitamins A, E, B₁ (thiamine), B₂ (riboflavin), and B₃ (niacin) (13). The preference of chili can also be attributed to the urge of meeting the challenge of the explosive sensory response and overcoming the initial negative effects to enjoy the high sense of relief.

**Physiological and Pharmacological Effects of Chili**

Apart from the hot chili being used as a food additive, chili has been found to have pharmacological and medicinal effects. As early as in the Columbus voyages, a physician accompanying him reported the medicinal effects of chili. Some varieties of chili with high potency that cause irritation/pain were reported to be used for medicinal purposes for centuries in Mexico and eastern parts of Africa. Different preparations were used as remedies for common cold and fever as well as disorders of the stomach such as colic, atonic, and alcoholic dyspepsia. The burning sensation as a result of contact of ground chili with the skin and the high irritation in the surface of the nose, throat, and eyes were also noted during those ancient days (5). Later when the active components in pure crystalline form became available, more definitive observations started to be made.

Capsaicinoids also account for many biological activities that affect human health. Capsaicin has been found to act as a powerful antioxidant, anti-mutagenic and anti-tumoral agent. Anti-inflammatory properties of capsaicin have also been found. They function as topical analgesic against pain. They stimulate the cardiovascular and respiratory systems (11). They also have antimicrobial activities against pathogenic bacterias (9). Capsaicinoids are also
used in defensive sprays against muggers and pest repellants in agriculture. There is interest in using them as synergists with organophosphates insecticides (14).

Substance P and Capsaicin

Substance P is a neuropeptide responsible for the transmission of pain impulses from the periphery to the central nervous system. Noxious stimuli bring about release of substance P from sensory neurons distally toward the skin and joints and centrally into the spinal cord and brain stem. Several events linked with neurogenic inflammation follow the release of substance P. Purified capsaicin has been found to deplete substance P stores in the sensory neurons and blocks further synthesis of this neuropeptide. Thus, the neuron’s ability to send signals is diminished (6). It has been reported that capsaicin relieves pain by depleting substance P in small fiber nociceptor neurons where transient receptors’ potential action channel (subfamily V), type 1 (TRPV1) is principally situated (12). The sensitivity of sensory nerves to noxious heat and chemical stimuli is lost making them unable to release mediators involved in neurotransmission. Due to this action, capsaicin has found several clinical uses.

Chronic Pain Conditions

Capsaicin has been used to treat neuropathic pain states and musculoskeletal pain disorders. Randomized trial (12) indicated that osteoarthritis pain can be moderately reduced with application of capsaicin and the effect can be improved with the addition of glyceryl trinitrate which reduces the discomfort of capsaicin application alone. Capsaicin plaster can also be used to treat chronic low back pain according to Helmut et al. (12). Compared with a
placebo, capsaicin was found to be clinically relevant and highly effective in treatment of
nonspecific low back pain with no systematic side effects.

Intravascular application of capsaicin and resiniferatoxin has been used to treat
hyperactivity-bladder by increasing the ability of the bladder to store urine and reduces urge
incontinence in patients with neurogenic and non-neurogenic detrusor hyper-reactivity (12).
Capsaicin also reduces bladder pain in hypersensitive bladder. It has been shown that capsaicin
has protective properties against gastropathy associated with indomethacin and ethanol.
There are speculations that ingestion of spicy foods containing capsaicin reduce the incidence

It has also been found that capsaicin relieves the symptoms of pruritus significantly.
Pruritus is an itch or a sensation that makes a person want to scratch. This causes discomfort
and can be frustrating. Administration of capsaicin was found to improve the upper respiratory
reflexes of the patients with cough and swallowing dysfunction. Capsaicin improves cough
reflex sensitivity and reduction in swallowing reflexes latency time.

Topically applied capsaicin is help in reducing pain associated with diabetic neuropathy
and chronic musculoskeletal pain. Capsaicin reduces post-operative discomfort and vomiting
when applied at acupressure points and protects the stomach against non-steroidal anti-
inflammatory drug induced gastritis (12)

Side Effects of Oleoresin Capsicum

Some studies (16) have shown that capsaicin has a dual effect on chemically induced
carcinogenesis and mutagenesis. Consumption of large amount of capsaicinoids has been
associated with necrosis, ulceration, and even carcinogenesis, though low levels of capsaicin displays little or no harmful effects. Topical application of capsaicin causes irritation and burning as reported by Mark et al. (12). In relation to the use of capsaicin containing sprays as riot control or personal defense agent, capsaicin is a highly irritating substance that causes burning and stinging pain to the skin. When it comes into contact with the eyes it causes intense tearing, pain conjunctivitis, and blepharospasm. Large amount of ingestion is characterized by nausea, abdominal pain, vomiting, and burning diarrhea (12). The pungency threshold of the capsaicinoids in chilies is quite low, 62.5 mg in 10^6 mL, but capsaicin pungency response in human changes rapidly with the increase in concentration of capsaicinoids.
CHAPTER 2

ANALYTICAL TECHNIQUES FOR ANALYSIS OF CAPSAICINOIDS

In the previous chapter we have seen the evolution of capsaicinoids since people started domesticating the plants. We have also explored the ancient uses of capsaicum fruits and numerous resent uses of both the hot pepper fruits and the extract from the fruits. Due to all this, researchers have studied extensively the methods used for extracting and quantifying the capsaicinoids. In this chapter, some of the methods that have been used to extract and quantify these active hot components of hot pepper fruits are discussed.

**Extraction Techniques**

Because of wide spread application of capsaicinoids, techniques have been developed with the objective of reducing extraction time, consumption of the solvent, pollution in analytical laboratories, and sample preparation costs (17). Some of these methods include; soxhlet, ultrasound assisted extraction, extraction by supercritical fluids, pressurized liquids, and microwave-assisted extraction (11)

**Soxhlet Method**

This is a common method for continuous extraction of a component from a solid mixture. Boiling solvent vapors from the flask rises up and then condenses into the porous cup known as thimble. The hot solvent dissolves out the desired component from a solid mixture. When the inner chamber holding the thimble fills up, siphoning action takes place. The solvent containing the dissolved component is siphoned into the boiler below. The residual solvent
then drains out of the porous cup as fresh solvent drops continue to fall into the porous cup. This cycle continues until complete extraction is achieved. The commonly used solvents include 2-propanol, ethanol, hydrocarbons, and even water (18). The advantages of soxhlet extraction include presence of fresh solvent in contact with the solid matrix throughout the extraction period, relatively high extraction temperature with heat from the distillation flask is maintained, and no filtration is required after extraction. The method is economical and simple.

The method is widely used in industries because it has better reproducibility and efficiency and less extract manipulation is needed. The drawbacks include, long extraction time because the set up of the soxhlet apparatus does not allow agitation to speed up the process. The process requires lengthy evaporation/concentration procedure due to large volume of the solvents used, and there is a possibility of thermal decomposition since extraction usually occurs at the boiling point of the solvent for a long time (19).

Margarita et al. (20) used soxhlet method to extract capsaicin, they grounded the tissue and mixed samples of 3 g with 30 mL of acetonitrile and kept it for 4 h at 80°C with constant shaking and without reflux, and then the extract was cooled and then filtered.

**Microwave-Assisted Extraction**

Microwave assisted extraction is another method used for extraction of capsaicinoids. Microwave heating brings about expansion and breakage of cell walls of the plants and this leads to release of extracts into the solvent (19). The advantages of microwave-assisted
extraction include: reduced extraction time and solvent usage, improved extraction yield, simple, and low cost. It also allows better control over the sample temperature and speed of heating than hotplates or other heating methods. Microwave assisted extraction of capsaicinoids recovers approximately 95% in 15 minutes (22). But compared to supercritical fluid extraction, the technique is disadvantageous because of the additional filtration or centrifugation that is required to remove the solid residue, also the efficiency of micro-waves is poor when either the target compound or the solvents are non-polar or volatile.

Opal et al (22) developed a microwave assisted extraction procedure for extraction of capsaicinoids. In this method, 2.00 g samples were irradiated in a closed-vessel in acetone at 30% power for 7 min followed by gas chromatography of capsaicinoid derivatives. Compared with traditional reflux and shaken flask methods, the yield of the compounds extracted was significantly greater ($P < 0.05$). Approximately 95% of the total capsaicinoids was recovered in a fraction of 15 min compared with 2 h for the reflux and 24 h for the shaken flask methods.

Barbero et al. (23) developed a method for the extraction of capsaicinoids using microwave-assisted technique. The study found the optimum conditions to be: power of 500W magnetic stirring, 125 °C extraction temperature, using 0.5 g of freshly triturated peppers an extraction time of 5 min, and employing 25 mL of 100% ethanol as solvent. The capsaicinoids obtained were stable under the optimized extraction conditions. The relative standard deviation for repeatability of the method was less than 6%.
Supercritical Fluid Extraction

When the temperature and pressure of a given substance is raised beyond its critical point, the supercritical state is achieved. Selective extractions and fractionations are possible by manipulating the solubility of a compound in a supercritical fluid by changing the pressure or temperature of the supercritical fluid. The main advantages of supercritical fluid extraction are the use of fluids such as CO\textsubscript{2} that is nontoxic and pose fewer difficulties with disposal (27) and the solubility of a solid in a liquid increases with the density of the fluid and this can be achieved at higher pressures. Using moderate extraction temperatures of CO\textsubscript{2} (\leq 30°C) makes supercritical CO\textsubscript{2} useful for more heat sensitive compounds. The drawbacks of this method are its difficult operating conditions and it is also expensive (19).

Daood et al. (24) developed a method for the extraction of capsaicinoids from pepper using supercritical carbon dioxide and subcritical propane; they reported that capsaicinoids were easily recovered with supercritical carbon dioxide with 89% recovery but very little was recovered (12%) when using subcritical propane.

Sonication-Assisted Extraction

Ultrasonic –assisted extraction is one of the important techniques for extracting the desired compounds from vegetative materials. As sound waves travel through matter they involve expansion and compression cycles during travel in the medium. Molecules are pulled apart by expansion and pushed together by compression. The expansion can cause formation of bubbles in a liquid and create negative pressure. The bubbles form, grow, and finally collapse when they reach a critical size. Cavity collapse near a solid boundary is asymmetric.
and produces high-speed jets of liquid breaking up the solid surface. There are two designs of ultrasound assisted extractors, ultrasonic baths and a closed extractors fitted with an ultrasonic horn transducer. The mechanical effect of ultrasound induces a greater entry of solvent into the cell and accelerates mass transfer. Ultrasonic extraction facilitates the release of the cell content by disrupting biological cell walls. Plant extract diffuse across cell walls due to ultrasound causing cell rapture over a short period. The efficiency of this method depends on the moisture content of the plant, particle size, solvent used, frequency, pressure, temperature, and sonication time (19).

Sonication is relatively economical and easy to operate compared with apparatus like microwave-assisted extraction. It is a simple and efficient alternative to conventional extraction techniques. There is an increase in extraction yield and faster kinetics. It is possible to extract thermolabile compounds because one can reduce the operating temperature. Furthermore, any solvent can be used for extracting a variety of natural products.

Sumate et al. (1) 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, 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 °C. 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. (11) developed an ultrasound-assisted method that extracted majority of the capsaicinoids. Methanol was used as solvent, at an optimum temperature of 50 °C and an extraction time of 10 min. The reproducibility of the method was found to have Relative standard deviation (R.S.D) < 3%.

Vincent et al. (29) 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%.

Quantitative Methods

A lot of research has been carried out with the aim of identifying the most efficient and reliable method of identification and quantitation of capsaicinoids. Some of the methods that have been used for identification and quantification of capsaicinoids are discussed below.

Spectrophotometric Methods

Spectrophotometric method involves reactions of capsaicinoids with either vanadium oxytrichloride or phosphomolybdic acid to produce a colored species. Although not specific for capsaicinoids, the test can give a result that is proportional to the amount of heat. The drawback of these spectrophotometric methods is failure to differentiate between capsaicin and the synthetic analogs. This method is thus rarely used (32)

Kosuge and Inagaki (26) extracted capsaicinoids in ether-extracted concentrates taken in carbon tetrachloride, washed with acetic acid, and reacted with a mixture of phosphomolybdate and phosphotungstate (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 was used to calculate the amount of capsaicinoids. The sensitivity, reproducibility, and repeatability of this method are not available. Vanadium oxytrichloride reagent had problems of stability both with the reagent itself and the blue color product formed.

North et al. (34) obtained 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 process minimized the colored pepper pigments but the number of steps involved in the clean up made the method time consuming, and the recovery and reproducibility were often reported to be poor.

Nowadays colorimetric method is used for direct and rapid method for detecting the presence or absence of capsaicinoids in cultivars requiring no pungency such as paprika and bell pepper. The vesicles along the placenta containing possible capsaicinoids are reacted with 1% solution of vanadium oxytrichloride in carbon tetrachloride. If a green color product is produced in the reaction, then capsaicinoids are present (26).

**Scoville Organoleptic Method (Taste Method)**

Various methods have been developed to replace traditional sensory method and the difficult colorimetric methods used to quantify capsaicinoids. One of these methods is scoville organoleptic method. This method was put forward by Scoville in 1912 (30). This was the first
reliable method for measuring capsaicinoids heat content and it is commonly used in food industry. The method involves a test panel of five individuals that validates a chili sample and then records the pungency level. A sample is then diluted until there is no more detectable pungency that can be orally detected. The dilution is referred to as Scoville Heat Unit.

For a long time this has been the most important and the only sensory method for assessment of heat in the chili. The main disadvantages of this method are lack of accuracy and precision. There is also rapid taste fatigue and increased taste threshold as a result of the five samples required for tasting. Ethanol bite interferes with the pungency. Above all, the organoleptic method cannot determine the amount of the individual capsaicinoids present in the sample. Because of this, organoleptic method has been replaced with instrumental methods. Because of variations among batches of capsicum, the concentration range cannot be expressed in percentage but must be calculated for each batch from quantitative analytical data (6). Historically, the organoleptic method is preferred by the food industry because it is a direct measure of levels of heat. Organoleptic technique requires extensive training of panelists and the monitoring of their sensitivity to environmental factors to get reliable results.

Thin Layer Chromatography (TLC)

TLC is another method that has been used for identification and quantification of capsaicinoids. TLC provides rapid analysis of the mixture and product purity or determines the components in a mixture.
Dohmann et al. (35) developed a simple method for separation and quantitation of capsaicinoids. Chloroform extracts of the powdered chili were applied on silica gel-G plates along with standard capsaicinoids solutions equivalent to 100, 150, and 200 µg/mL capsaicin. The plate was developed with chloroform-methanol-acetic acid to a distance of 14 cm. The marks were marked as dark spots under ultraviolet light. The capsaicinoids near the solvent front were clearly separated from the pigments near the starting line. The separated bands of the capsaicinoids from the sample and standards were scraped off carefully into individual tubes and reacted with a mixture of phosphomolybdate and phosphotungstate (Folin-Denis reagent). The blue color formed was cleared by centrifugation and its absorbance measured at 725 nm. The total capsaicinoids in the sample was calculated by reference to the standard curve of absorption versus micrograms of crystalline capsaicinoids run on the same plate and the dilutions in making the extracts.

TLC method has not been found to be satisfactory in repeatability. It requires skill in making the plates quantitative, spotting of micro liter amounts of samples and collection of separated component areas quantitatively for colorimetry. Permanently coated plates to improve performance are now available but costly. Paper chromatography can also be used for the separation of capsaicinoids; however, they registered low resolution when compared to TLC technique.

Gas Chromatography

In GC, an inert carrier gas serves as a mobile phase that elutes the components of a mixture from a column containing the stationary phase. Separation takes place due to
differences in the positions of adsorption equilibria between gaseous components of the sample and the solid surface of the stationary phase. GC method has been used for the separation and analysis of capsaicinoids.

Bretty et al. (14) 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. Fortified bell pepper was used for measuring recovery. Recovery of 90%-110% of both capsaicin and dihydrocapsaicin was attained.

Araceli et al. (21) 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 for the gas chromatography–mass spectrometry analysis. The method was reported to be linear in the range 0.109–1.323 mg/mL for capsaicin and 0.107–1.713 mg/mL for dihydrocapsaicin with correlation coefficient up to 0.9970 for both capsaicinoids. The precision of the method was better than 10%.

Suzuki et al. (36) used a combination of gas chromatography–mass spectrometer with a column (2 m x 3 mm I.D) packed with 3% SE-52 on chromosorb W. The column temperature was kept at 260°. The carrier gas flow rate was 30 mL/min. Quantitation of capsaicinoids were done with 0.1-1.0 µg of authentic capsaicinoids, together with 0.5 µg of 5-α-cholestane as the internal standard, was used to calibrate the method. The method was able to determine
microgram to nanogram amounts of capsaicinoids without any interference from other components of the sample.

**High Performance Liquid Chromatography**

The extracts cannot be directly analyzed using gas chromatography because of poor volatility of the capsaicinoids hence the requirement to derivatize in order to convert the analytes to more volatile compounds before analysis (17). Due to the derivatization requirement of the gas chromatography, HPLC methods have been developed that are based on underivatized direct injections of the samples.

HPLC technique has superior separation capabilities because it provides accurate and efficient analysis of content and type of capsaicinoids present in a chili sample (37, 46). The separation is performed directly on the extracts of the natural materials without any preliminary clean up. Due to its speed, the method can handle a larger number of samples with sensitivity at sub-microgram levels due to sensitive detection system. The method can also be used for preparative scale with less separation efficiency. HPLC has superior separation capabilities for closely related compounds and is operated at room temperature. When it is combined with additional operational parameters such as reverse-phase systems, silver-ion complexing of olefinic compounds, and optical detectors, separation efficiency and sensitivity is greatly improved.

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 (37).

Due to the usefulness of HPLC, several people have used it in the previous studies for both separation and quantification of capsaicinoids.

Veronika et al. (13) used electrochemical detection to optimize high performance liquid chromatography. The working electrode potential was set at 750 mV. 60% methanol with pH 4 acetate buffer was used as mobile phase. Pico-moles of capsaicin per injection was reported to be determined. The technique was used to determine capsaicin in various cultivars of pepper. The reported highest content of capsaicin using this method was 227 mg per 100 g of fresh weight found in Takanotsume’ cultivar.

Nazari et al. (17) developed a method for determination and quantization of capsaicinoids they used liquid chromatography – electrospray/time-of-flight mass spectrometer for the determination of capsaicin and dihydrocapsaicin in capsicum fruits. The quantification was made using a synthetic capsaicin analog (4,5-dimethoxybenzyl)-4-methyl octamide as an internal standard analyte were resolved in less than 16 min, and the limit of detection were 20 pmole for capsaicin and 4 pmole for dihydrocapsaicin. The analyte recoveries were found to be, 83% and 93% for capsaicin and dihydrocapsaicin, respectively.

Margarita et al. (20) used HPLC equipped with Novapak C18 reversed phase column of 3.9 x 150 mm, with 73% methanol and water used as a mobile phase at a flow rate of 1.0 mL/min. 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 chili pepper.

The study found that capsaicinoids accumulation increased during the development of the fruits and reached a maximum of 200 µg of capsaicinoids/g in green house grown fruit, and 120 µg of capsaicinoids/g 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, and 120 µg/g of capsaicinoids, respectively.

Woodbury et al. (37) developed HPLC method that allowed analysis of as many as 50 samples a day that varied from less than 300 to 13000 ppm in capsaicinoids. This was possible due to spectrofluorometric 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. Today to convert concentration in ppm to SHU one multiplies ppm by 15. HPLC method developed by Woodbury and co-workers is the foundation for all subsequent modifications to the HPLC method. It involved extraction of the chili powder at 60 °C for 5 hours using 95% methanol.

Krishnamurthy et al. (39) 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, 5 µm, 250 x 4 mm column. The mobile phase was acetonitrile: 1% acetic acid water (55:45) with the flow rate of 2 mL/min. The detection was carried out at 281 nm. From this study the percentage of capsaicin obtained in green chilies was very low owing
to the high water content in green chilies, although water level did not interfere with the analysis of capsaicin.

Robert et al. (40) determined eight naturally occurring capsaicinoids with, norcapsaicin used as an internal standard. In this method, the sensitivity of electro spray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and coordination ion-spray (CIS; with silver) toward the determination of capsaicinoids was measured and compared. Positive-ion ESI was found to have the highest sensitivity. The repeatability of the validated method was 4% with the detection limit of 5 pg. Linear range from 20 to 6 ng and the method recovery was 99% to 103%.

Rachanneewan et al. (41) 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 of 15 cm length and 4.6 mm diameter with a UV detector. The standard used was capsaicin (CAPS, 8-methyl-N-Vanillyl-6- nonenamide) and dihydrocapsaicin (DHC, 8-methyl-N-vanillyl nonenamide) and prepared using acetonitrile as solvent. The calibration solutions were made after appropriate dilutions. This study found that changing column temperature was more important than changing the mobile phase flow rate. The recovery for this method was, 93% and 89.6%, for capsaicin and dihydrocapsaicin, respectively.

Barbero et al. (42) developed an HPLC method with fluorescence detection for the determination and quantization of main capsaicinoids using a monolithic column. The type of
column employed was the RP-18 (100 mm x 4.6 mm) monolithic column. Gradient method was used with the solvent A being water with 0.1% acetic acid, and solvent B with methanol with 0.1% acetic acid. The flow rate was 6 mL/min. They used a fluorescence detector with 278 nm for excitation and monitor and emission at 310 nm. The reproducibility of the method had relative standard deviation of less than 2%. The separation of the 5 capsaicinoids took less than 8 min.
CHAPTER 3

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUE

High performance liquid chromatography (HPLC) is a popular method of analysis because it is easy to learn and use and is not affected negatively by the volatility or stability of the sample compound. Basically, HPLC instrument consists of the reservoir of mobile phase, the injector whose function is to introduce the sample, high pressure pumps to deliver the solvent through the injector and column that is packed with a stationary phase where the separation takes place and to the detector for qualitative/quantitative measurements, and a data system to capture and manipulate the data (43). Under optimum conditions, separation occurs because different components in the mixture exit the column at different rates due to differences in their partitioning behavior (34). The distribution and partitioning is due to the structures and intermolecular forces between the components of the mixture and the stationary phases employed (44). After separation, the separated components pass through a detector where they generate a signal that is recorded on the data system.

HPLC has a great potential to differentiate and purify molecules that are soluble in liquids. This has made the technique very useful in cosmetics, food, environmental, biotechnology, and pharmaceutical industries. HPLC is used in the preparation, isolation, and purification of compounds due to the fact that compounds have different kinds of interactions with the mobile and the stationary phases resulting in different migration rates through the column. HPLC is also used for the purification and extraction of the target compound from structurally related compound or contaminants to identify and quantify them (34)
Classification of Liquid Chromatography

Several factors can be used for classification of liquid chromatography. These include the overall physical structure of the stationary phase such as column chromatography and thin layer chromatography. Classification can also be based on performance such as high performance liquid chromatography and high-performance thin layer chromatography. Major classification is based on the interaction of the analyte between the mobile phase and the stationary phase. This category includes reversed phase, normal phase, ion exchange, and gel filtration chromatographies. Liquid chromatography can be classified into five main techniques based on the mechanism of separation. This includes adsorption that involves competition between liquid mobile phase and solid adsorbent and partition that involves interaction between liquid mobile phase and liquid stationary phase. Others include size exclusion, affinity, and ion exchange. HPLC can also be classified into normal phase or reversed phase based on the relative polarity of the mobile phase and the stationary phase.

Normal and Reversed Phase HPLC

Normal phase chromatography involves a polar stationary phase and a non-polar mobile phase. The stationary phase in normal phase chromatography is mostly inorganic polymers mainly silica and alumina (46). In normal phase chromatography, analytes that are more polar experience greater retention. Increasing the polarity of the mobile phase decreases the retention time. Normal phase is commonly applied to the analysis of samples that are soluble in non-polar solvents such as hexane.
The system is referred as reversed phase if the stationary phase is less polar than the mobile phase (44). Reversed phase liquid chromatograph (RP-LC) is the most widely used mode of chromatography. It separates components on the basis of the hydrophobicity and polarity of the analyte. Solutes elute in the increasing order of hydophobicity or in decreasing order of the net charge, degree of ionization, and the ability to participate in hydrogen bonding (34).

Stationary phases used are usually non-polar groups bonded onto silica mostly organic groups such as \(-\text{CH}_3\), \(-\text{C}_4\text{H}_9\), \(-\text{C}_8\text{H}_{17}\), \(-\text{C}_{18}\text{H}_{37}\) (octadecyl group), \((-\text{C}_6\text{H}_5\)) phenyl groups, \([(-\text{CH}_2)_3\text{CN}]\) cyano groups, and \([(-\text{CH}_2)_3\text{NH}_2\)] amino group. The longer the carbon chain, the more it interacts with the solutes that are non-polar and dissolve in an organic solvent, thus the longer their retention time (44). The most commonly used stationery phase is the 18-carbon chain (25). The performance of the bonded phases is determined by the base silica and its pretreatment, the choice of the bonded functional group, the amount of material bonded to the silica, and the secondary bonding reactions (end capping).

The polarity of the mobile phase solvent is a great factor in reversed phase liquid chromatography. A mixture of water and a less polar solvent such as acetonitrile or methanol is used. Acetonitrile allows low operating pressure, has a slightly higher solvent strength, and it is applicable for detection down to the 185-205 nm range. But, it is expensive and more toxic than methanol. It is possible and practical to separate ionic compounds on RP-HPLC by using secondary equilibria such as ion suppression where a buffer of appropriate pH is added to the mobile phase to render the analyte neutral or only partially charged. Acidic buffers are used for the separation of weak acids, and alkaline buffers are used for the separation of weak bases.
Addition of buffer to a mobile phase makes the eluted analytes come out as sharper zones than in a similar mobile phase alone. Due to the instability of silica based support stationary phases at pH higher than 8 and lower than 2, the buffer used should be within pH 3-8. When non-ionic solutes are being separated, surfactants can also be added to decrease the retention time and reduce the band width because of reduced solvent viscosity and surface tension (44).

Reversed phase HPLC now is more popular than other types because the analyte peaks tend to be narrow and symmetrical and the adsorption/desorption equilibrium reactions are fast. Also less polar solvents are more powerful eluents than normal phase HPLC using silica or alumina (25).

**High Performance Liquid Chromatography Detectors**

The function of the detector is to produce an electrical signal proportional to the concentration of the sample and this signal is then transferred to a recording device for display and storage. An ideal detector should not be affected by temperature, pressure, or cause band spreading. It should show little or no signal from the solvents used, buffering salts, organic modifiers detergents, or chaotropic agents. It should also be responsive to all analytes across the full concentration range. Basically, the choice of the detector should depend on its selectivity and sensitivity within mobile phase and chromatographic limitations (46). The normal mode of operation of a detector in HPLC is the monitoring of the analyte signal that appears as an increase above the background signal due to mobile phase alone (43).
Detectors usually fall into selective and universal detectors. A selective detector measures a physical or chemical property unique to the solute in the mixture and only components with that characteristic will be detected. The response is generally significantly greater than the response caused by the solvent, sample matrix, and other components in the mixture. Examples of such detectors include ultra violet–visible (UV-Vis), fluorescence, and electrochemical detectors. Another category, known as universal detectors, signals the presence of virtually all the compounds including the solvent and sample matrix. They compare an overall change in some physical property of the mobile phase with or without an eluted solute. These detectors have limited dynamic range, sensitivity, and detection limit are thus commonly used in preparative chromatography. Examples of this kind of detector are the refractive index (RI) detectors.

Another way to categorize the detectors is whether they are destructive or nondestructive. Destructive detectors include electrochemical and mass spectrometric detectors in which the analyte or sample, or at least part of the sample, is altered by the detector itself. Non-destructive detectors include UV-Vis, fluorescence, and RI. The sample is unaltered in the detection process.

Absorption detectors such as UV-VIS detectors measure the ability of an analyte to absorb light. This can be accomplished at one or several wavelength. Absorption detectors are the most commonly used detectors. Only compounds with appropriate spectral characteristics are detected. The light from the source is passed through a monochromating device, for instance a filter or a grating, and then to a cell through which the mobile phase containing the
analyte flows. The amount of light transmitted is measured using a suitable photo-detector. If the light of intensity $P_0$ is focused onto the cell and light of power $P$ is transmitted, then from Beer-Lamberts law we can write,

$$A = \log\left(\frac{P}{P_0}\right) = \varepsilon b C$$  \[3,1\]

where $A$ is the absorbance of the solution, $b$ is the optical path-length through the cell in cm, $\varepsilon$ is the molar absorptivity of the solute at a particular wavelength used in L/mol cm, and $C$ is the molar concentration of the solute. At a given wavelength, a linear relationship exists between absorbance and concentration.

**Ultra-Violet- visible (UV-Vis) Detectors**

These detectors operate in the wavelength range 190-700nm. UV-Vis can operate at a given fixed wavelength, variable selected wavelength, or simultaneously over a wide range of wavelengths or a complete spectrum of the molecule. These detectors are usually operated as solute property detectors wherein direct detection of the solute is achieved by selecting a wavelength at which the solute exhibit a high molar absorptivity.

The advantages of these detectors include high sensitivity ($10^{-10}$ to $10^{-11}$ g), good selectivity, almost a universal detector at wavelength ($\leq 200$), low background with many solvents, allowing gradient elution without excessive background drift, non-destructive to analytes and ease of operation. The detectors are not temperature sensitive, and they can be used with gradient elution. Their disadvantages are that they cannot be used with solvents that
have significant absorption in the UV or with sample component that do not absorb in the UV. Also, at a given wavelength, detector response varies between molecules based on their absorptivity (46).

**Refractive Index Detectors**

Refractive index (RI) detectors are universal and non-destructive and can only be used with isocratic elutions. The detector compares the refractive index (light bending) of the pure mobile phase with that containing the analyte. For most RI detectors, light passes through a two channel flow-cell to a photodetector. One channel of the flow-cell directs the sample passing through the column while the other directs only the reference mobile phase. Detection occurs when the light is bent due to samples eluting from the column, and this is read as a difference between the two channels.

An advantage of refractive index detectors is that they are non-destructive. The moderate detection limit makes it useful for preparative scale chromatography. The major disadvantages of this detector are the presence of drift and noise because of the refractive indexes of compounds including solvents change with temperature and back pressure affects the density of mobile phase.

**Fluorescence Detectors**

Fluorescence detectors are used for analytes with molecules that can absorb light energy at a specific wavelength, rise to the excited state, and return to the ground state by emitting light at a longer wavelength. The detectors operate by exciting the analyte at \( \lambda_{ex} \) and
then detecting the florescence emitted at $\lambda_{em}$. Fluorescence detection is characterized by good selectivity because few molecules fluorescence at the same selected wavelength. Fluorescence detectors have very high sensitivity because it is easy to detect a small signal against zero background. They can achieve very low detection limits ($\leq 1 \text{ pg}$), and there is virtually no interference from the mobile phase and most other sample components. The main drawback is that distinct wavelengths needed for excitation and emission requires optimization for each compound. Non-florescent compounds consume time and involve complicated derivatization so that they are fluorescence (46).

**Mass Spectrometric Detectors**

Mass spectrometry is a resourceful and great tool of chemical analysis. The mass spectrometer consists of a mass source, a mass analyzer, transducer/detector, and a recorder. The entire spectrometer is evacuated. Mass spectrometers are often combined with gas or high performance liquid chromatographic systems or capillary electrophoresis columns to allow the separation and determination of the components of complex mixtures. The sample is inserted either as gas, liquid, or solid, but when liquid or solid are used they must first be vaporized. The gaseous molecules are ionized in the space between two charged plates and then accelerated into a mass analyzer. The ions are separated according to mass to charge ratio. As the heavy and the lighter ions are deflected, the ions with the appropriate mass to charge ratio pass through to the detector and are then recorded. Mass spectrometers can be used to confirm the identity of a compound by giving the molecular weight and structural information for individual chromatographic peaks. It often provides sufficient data to
determine the structure of an unknown. However, a major difficulty arises when the different species present in a mixture sample are introduced simultaneously into the source (45). Other detectors that are used in reversed phase HPLC include electrochemical detector, conductivity detectors, and amperometric or coulombic detectors.

Research

From previous discussions, this study is geared towards extracting and establishing the quantities of two mayor components of capsicum plant; capsaicin and dihydrocapsaicin in different varieties of naturally grown chili at different stages of development. The varieties considered under this study involve three color changes during fruit development: green, yellow, and red. The main focus of this project is to evaluate the amount of capsaicin and dihydrocapsaicin at these three stages of fruit development and in the fruits of the same variety harvested in summer and winter seasons of the year. The research objectives are given below.

1. To develop an efficient RP-HPLC procedure for the determination and quantification of capsaicin and dihydrocapsaicin at different stages of fruit development.

2. To establish the figures of merits for this HPLC method including linear dynamic range, reproducibility, recovery, and accuracy.

3. To establish the ratios of capsaicin and dihydrocapsaicin at different stages of development of chili fruit.

4. To compare levels of the capsaicin and dihydrocapsaicin in fruits harvested in summer and winter seasons.
5. To compare percentage amounts of both components in fruits at different
development stages.
CHAPTER 4

EXPERIMENTAL PROCEDURE RESULTS AND DISCUSSION

Experimental Procedure

In the previous chapter, methods that have been used to extract and quantify capsaicinoids were discussed. This chapter includes detailed description of the procedure that was used for the analysis of the two main components of hot pepper. We shall begin with discussing the reagents that were used for in the study and how each one of them was prepared for the HPLC analysis. Thereafter, HPLC system used and how basic conditions for the analysis such as wavelength, mobile phase, and flow rate were optimized is described. Then we will explore into how each figure of merit: reproducibility, linear dynamic range, and recovery were studied and optimized. The application of the developed optimized method to the samples and the results obtained are also included in this chapter.

Reagents

The following reagents were used for the research.

1. Deionized water obtained from US Filter Company (Pittsburgh, PA)
3. Capsaicin natural containing 60.0% to 70.0% HPLC capsaicin and 20% to 30% dihydrocapsaicin from Spectrum Chemical MFG-CORP (New Brunswick, NJ).
4. Helium for degassing the mobile phase from Valley National Gases, Inc. Johnson City, TN.
5. Dodecyl sulfate from Sigma Chemical Company (St Louis, MO)

6. Acetic acid from Fisher Scientific (Fair Lawn, NJ)

7. Green, Yellow, and red hot pepper from local garden.

8. Habanero from local grocery store in Johnson City, TN.

**Stock Solution and Reagents Preparations**

The following reagents and the stock solutions were prepared.

Capsaicin standard stock solution (1.00 mg/mL) was prepared by weighing 100 mg of natural capsaicin and dissolved it in 50 mL of methanol in a beaker and then transferred the dissolved solution into a 100 -mL volumetric flask and then filled to the mark. This solution was then divided into four brown bottles and refrigerated until use.

Capsaicinoids sample stock solution was prepared by transferring the sonicated filtered chili extract to a 50-mL volumetric flask and then topped to the mark after which the solutions were refrigerated until time of analysis.

Mobile phase was prepared by mixing 730 mL of methanol and 270 mL of deionized water in a container and degassed for 20-25 minutes before use.

**Standard Solutions for Calibration Curve and Linearity Studies**

Linearity test was carried out using two set of concentrations, low and high concentration of both capsaicin and dihydrocapsaicin. Four standard solutions of lower concentrations were prepared, each in triplicate. For these, 5, 10, 20, and 40 µL of the stock natural capsaicin solution were pipetted into eight separate 5-mL volumetric flasks and then
filled up to the mark with methanol. These dilutions resulted in 0.65, 1.3, 2.6, and 5.2 µg/mL of capsaicin solutions, and 0.25, 0.5, 1.0, and 2.0 µg/mL of dihydrocapsaicin.

For the solutions with higher concentrations, four standard solutions (each in triplicates) were prepared. For these, 100, 150, 200, and 350 µL of the stock capsaicin solution were pipetted into eight separate 5-ml volumetric flasks and filled up to the mark with methanol. The solutions that resulted from this were: 13, 19.5, 26, and 45.5 µg/mL, and 5, 7.5, 10, 17.5, µg/mL, of capsaicin and dihydrocapsaicin, respectively. The calibration curve for the samples was prepared using the same procedure except that it was not done in triplicates. The pipette solutions were 20, 50, 100, 200, and 300 µL and diluted into 5-mL volumetric flasks. The resulting solutions contain 2.6 to 39.0 µg/mL and 1.0 to 15.0 µg/mL of capsaicin and dihydrocapsaicin, respectively.

**Preparation of the Samples for Analysis**

Chili pods were grown under normal Johnson City, TN weather conditions. Most of the varieties were grown during summer. The fruits were harvested in the summer and one variety was harvested both during summer and winter when the plants were taken indoors. Fresh harvested hot peppers were selected at three maturing stage: green, yellow, and red. The stems were removed making sure there was no placenta removed. The chilis were then cut open longitudinally to facilitate drying. The samples were placed in an oven set at 60-70 °C and left to dry for around 48 hours. The weight of the beakers with the chili was weighed during the drying process until a constant weight was attained.
The dry chili was then grounded together with the seeds using mortal and pestle into almost powder form. Then 1.00 g of this grounded chili was weighed and mixed with 25 mL of methanol into a 50-mL conical flask. This was based on the work that was done by Collins et al (47) that indicated that samples < 1 g shows statistically significant differences in capsaicin levels, thus could not be used. Sumate et al. (5) and Barbero et al. (11) both used sonication method but Sumate et al. (5) used ethanol instead of methanol as solvent. This mixture was sonicated for 25 minutes at a temperature of 40 °C. The mixture was then filtered using a Buchner vacuum filtration apparatus. The solution was then diluted to 50 mL in a 50 mL-volumetric flask as previously described.

**Solution for the Reproducibility Studies**

Reproducibility was done twice using two different chili varieties. For Habanero, 1.0 mL of the stock solution was pipetted into 8 different 5-mL volumetric flasks and diluted to the mark by methanol ready to be injected into the HPLC system. For Variety 1 red, 2 mL of the stock solution was pipetted into 8 different 5-mL volumetric flasks and diluted to the mark by methanol ready to be injected into the HPLC system.

**Solutions for the Recovery Study**

Two sets for recovery studies were prepared; 1 mL of Variety 1- red harvested during winter was pipette into 9 different 5-mL volumetric flasks then 20 µL of capsaicin standard was added into first 3 of the 9 flasks, 40 µL was added to the next three other and no standard was added to the rest of the 3 flasks. The same procedure as described above was repeated with variety 4 yellow samples.
Instrumentation

HPLC Parameters

The study was carried out at a constant mobile phase flow (isocratic) rate of 1.0 mL/min. Before injecting the sample solutions into the HPLC system, the mobile phase was run through the system for more than 45 minutes in order to equilibrate the column. The mobile phase was a mixture of methanol and water in the ration of 73:27. Each standard or sample used for this research was prepared and injected to the HPLC system in triplicate. Data were collected using Shimadzu LC solution system HPLC equipped with a system controller type SCL-10 Avp, Shimadzu pump model LC-10 AS, Shimadzu UV-Vis, model SPD-10 A detector (all from Shimadzu USA manufacturing Inc, Wood Drive Columbia, MD), Rheodyne Injector model 7125 with a 20 µL loop. The analytical column used in this study is Agilent. Eclipse X DB –C_{18} 5 µm particle. 4.6 x 150 mm. The computer system used is Dell Optiplex 745 with Windows XP operating system (Microsoft Corp, Redmond WA) and data collection software LC-Solution from Shimadzu. A schematic representation of the instrumental set-up is displayed in Figure 2.

Data Analysis

Statistical analysis of the data generated using LC-Solution was performed using Microsoft EXCEL software for Windows Vista (Microsoft Corp Redmond, WA). After collecting the data, a regression curve was plotted in order to get the correlation coefficient and the equation of the line. Microsoft EXCEL spreadsheet was also used to find the mean, standard deviation, and relative standard deviation.
Results and Discussion

The first step in the separation of the analyte using HPLC is to select the most promising method. Below is the discussion of how each factor was evaluated and optimized for this study. The proposed method was evaluated based upon selectivity, sensitivity, reproducibility, and accuracy. In order to achieve this, first the optimization of the wavelength, mobile phase, the stationary phase were investigated as follows.

Optimization of the Wavelength of Detection

To find the optimum wavelength for the study, a set of standards and one of the chili samples were prepared and analyzed using the HPLC with the detector set at different

Figure 2. Schematic diagram of the HPLC system used in the project
wavelengths. The UV spectrum of capsaicin shows the long absorption at wavelength 205 nm. It is commonly used for samples with the lowest concentration of capsaicinoids. However, 205 nm is the cut off point of methanol and thus this wavelength was not suitable for this study. Several researchers had used 280 nm for their capsaicinoid analysis: (5, 34). Other wavelength that have been used are 278, 235, 227 nm (36). In this study the wavelength evaluated was 280, 227, 208, 205, and 210 to 215 nm. Finally, 210 nm was selected for use because the absorbance of capsaicin and dihydrocapsaicin as well as other peaks were better resolved than using the other wavelengths.

**Optimization of the Mobile Phase**

To determine the best combination of solvents for the mobile phase, various ratios of methanol and water were evaluated. The goal was to identify the mobile phase that would give a good separation with shorter retention time for the analytes. This would reduce the run time and the amount of methanol used. Several methanol: water ratios were tried. The 60:40 methanol: water mobile phase separated the components of interest well enough but the retention time for capsaicin and dihydrocapsaicin were too long, 14 and 16 minutes, respectively. 80: 20 and 75:25 methanol: water ratios were able to reduce the retention time but resolution suffers. There was great overlap of the analyte peaks with other peaks present. Eventually, 70:30 methanol: water was found to give better separation with satisfactory retention time so this ratio was used to carry out further tests. Some past studies have reported the use of a buffer, (12, 38, 47).
Many other studies, however, have not used any buffer (2). In this study the pH was varied using 0.025% to 1% glacial acetic acid. One percent and 0.5% dodecyl sulfate was used as surfactant. The effect of the buffer and the surfactant were tested separately and then in combination. Optimization of the mobile phase was carried out by changing its composition.

The result of this test is shown in the Figures 3 and 4 which are plots of average area against the retention time when various composition of mobile phase were used. The retention times of the analytes were obtained on µ bondapak, C$_{18}$, 3.9 x 300 column (from Waters Corporation Maple Street, Milford Massachusetts USA) at a flow rate of 1.0 mL/min, detection wavelength of 210 nm. The mobile phase composition a, b, c, d, e, f, and g were studied.

![Figure 3. Average area against the retention time of capsaicin for various mobile phases evaluated.](image-url)
From Figure 3 and Figure 4, it is apparent that mobile phase d containing methanol with 0.5% dodecyl sulfate and 0.025% acetic acid gave the highest peak area while mobile phase c containing methanol with 0.005% acetic acid gave the shortest retention time. However there was overlap of the peaks when mobile phase d that was with methanol with 0.5% dodecyl sulfate and 0.025% acetic acid was used. Thus mobile phase d was not good for our analysis. Mobile phase c containing methanol with 0.005% acetic acid had very poor sensitivity thus also making it undesirable for analysis. As evidenced by the plots shown in Figure 3 and Figure 4; addition of buffer, surfactant, or both did not improve the separation. The mobile phase of 73:27 methanol water without buffer or surfactant added was finally chosen to be used for the rest of this study. This mobile phase gave the best separation of analytes of interest in both the

![Figure 4. Average area against the retention time of dihydrocapsaicin for various mobile phases evaluated](image)
standard and the chili samples. The analyte peaks using the mobile phase 73:27 methanol:
water were well resolved and the retention time was satisfactory. This mobile phase (73:27) was also used in earlier studies by Margarita et al. (20).

Figure 5 shows the chromatogram for Variety 1 red chili obtained using three different mobile phases running at 1.0 mL/min. with detection at 210. As the chromatogram show, the peak profile and retention time were more favorable in the case of 73:27 methanol: water used as mobile phase. This choice has the advantage of being simple and without the need of adding any surfactant or pH modification.

Figure 5. Chromatogram for Variety 1 red chili using different methanol: water mobile phases at a flow rate of 1.0 mL/min, and detection at 210 nm. (A) methanol: water (70:30) and 0.05% acetic acid, (B) methanol: water (70:30), 0.05% acetic acid and 1% dodecyl sulfate, while (C) methanol: water (73:27). The retention time of the compounds is given in minutes.
The retention time of capsaicin and dihydrocapsaicin from the proposed method was found to be 4.63±0.2 minutes and 6.41±0.2 minutes, respectively.

**Flow Rate Selection**

The optimum flow rate for the analysis was also evaluated. The range that was investigated was 0.8-1.2 mL/min. Higher flow rate could not be permitted by the instrument due to build up of pressure. Flow rate of 0.8 mL/min was found to give broad peaks and increase the retention time. For the 1.2 mL/min flow rate, the retention time was reduced but the separation was affected since the peaks were not well resolved as evidenced by peak overlap. The optimum flow rate was found to be 1.0 mL/min and this flow rate was applied throughout the study.

**Stationary Phase Selection**

Appropriate stationary phase for the sample of interest is a very important factor to consider to achieve optimal resolution. Two analytical columns were evaluated for this study. µ-Bondapak, C_{18}, 3.9 x 300 and Agilent Eclipse x DB C_{18} 5 µm particle 4.6 x 150 mm. To evaluate the suitability of the column for the proposed study, a standard and a selected sample solution were prepared and chromatographed on each column at a flow rate of 1.0 mL/ min with the mobile phase 73: 27 methanol: water. The column, µ Bondapak, C_{18}, 3.9 x 300 mm was found to give broader peaks and longer retention times for both capsaicin and dihydrocapsaicin compared to the Agilent Eclipse x DB; C_{18} 5 µm particle, 4.6 X 150 mm. For this reason, The Agilent Eclipse x DB; C_{18} 5 µm particle, 4.6 x 150 mm column was used for the rest of the study.
Linear Dynamic Range

To examine the linear relationship of peak area and concentration of the two capsaicinoids, two sets of solutions of the standard were prepared as described in the procedure section at the beginning of this chapter. The first set of solutions contain capsaicin concentration ranging from 0.65 to 5.2 µg/mL, and the second set ranging from, 13.0 to 45.5 µg/mL. The two sets of solutions of standard contain dihydrocapsaicin with concentration ranging from 0.25 to 2.0 µg/mL and 5.0 to 17.5 µg/mL. Each concentration was prepared and injected in triplicates.

Figure 6 shows some of the chromatograms obtained at different natural capsaicin standard concentrations used in the linearity studies. Chromatogram A was that of 19.5 µg/mL capsaicin and 7.5 µg/mL dihydrocapsaicin, chromatogram B was that of 26.0 µg/mL capsaicin and 10 µg/mL dihydrocapsaicin, while chromatogram C was that of 45.5 µg/mL of capsaicin and 17.5 µg/mL of dihydrocapsaicin.

The results of the linearity studies are shown in Table 2 for the set of solutions using lower concentrations of the standard solutions of capsaicin and Table 3 for the set of solutions using higher concentration of the standard solutions of capsaicin. The values of the peak areas do not vary as much for the triplicate injections of the higher concentrations of the standards. The RSD values progressively decrease with increasing concentrations of capsaicin. This trend is to be expected.
Figure 6. Chromatograms of different concentrations of natural capsaicin standard used for linearity studies: Mobile phase used was 73:27 methanol: water. At a flow rate of 1.0 mL/min, with detection at 210 nm. The retention time of the compounds is given in minutes.
Table 2. Results of linear dynamic studies of low capsaicin concentration: Areas of the analyte peak \( t_r = 4.62 \text{ min} \) using mobile phase of 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm. Each reported area is an average of three injections.

<table>
<thead>
<tr>
<th>Capsaicin (µg/ml)</th>
<th>Average Area</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65</td>
<td>1.45E+05</td>
<td>10.6</td>
</tr>
<tr>
<td>1.3</td>
<td>2.40E+05</td>
<td>3.2</td>
</tr>
<tr>
<td>2.6</td>
<td>4.11E+05</td>
<td>3.6</td>
</tr>
<tr>
<td>5.2</td>
<td>7.98E+05</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 3. Linearity studies data for higher concentration of capsaicin: areas of the analyte peak \( t_r = 4.62 \text{ min} \) using mobile phase of 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm. Each reported area is an average of three injections.

<table>
<thead>
<tr>
<th>Capsaicin (µg/ml)</th>
<th>Average Area</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.0</td>
<td>2.04E+06</td>
<td>1.5</td>
</tr>
<tr>
<td>19.5</td>
<td>3.13E+06</td>
<td>2.7</td>
</tr>
<tr>
<td>26.0</td>
<td>3.99E+06</td>
<td>1.2</td>
</tr>
<tr>
<td>45.5</td>
<td>6.90E+06</td>
<td>0.3</td>
</tr>
</tbody>
</table>
The calibration curve using low concentration of capsaicin standard was graphed and the regression equation determined using Microsoft EXCEL. The plot is shown in Figure 7. The calibration plot was linear with the regression equation $y=143224 x + 49377$ for the concentration range of 0.65 to 5.2 µg/mL. The correlation coefficient of 0.9994 indicates good linearity.

![Figure 7. Calibration curve for the capsaicin standard solution at low concentrations: The mobile phase used was 73:27 methanol: water at a flow rate of 1.0 mL/min with detection at 210 nm.](image)

The data from the linearity studies in Table 3 were graphed using Microsoft EXCEL. The plot is shown in Figure 8. The peak areas is linear with concentration range studied as evidenced by the regression equation of $y=14835 x + 15870$ with a correlation coefficient of 0.9994. Combining the data of peak areas at low and high concentration solutions together, one can conclude that the procedure used shows a good and wide linear dynamic range.
The results of linearity studies for dihydrocapsaicin are presented in Tables 4 and 5, respectively, for a set of solutions with lower concentration and another set of solutions of higher concentrations. Just like in the case of capsaicin, the RSD values decrease with an increase in the concentration of the dihydrocapsaicin. Even though the variance in the triplicate values at each concentration is longer for the solutions with lower concentrations of the analyte, the results are still satisfactorily linear with the correlation coefficient of 0.9958 and 0.9993, for low and higher concentration of dihydrocapsaicin, respectively.
Table 4. Data for linearity studies at higher concentration of dihydrocapsaicin standard: Areas of the analyte peak ($t_r$ = 6.42 min) using mobile phase of 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm. Each reported area is an average of three injections.

<table>
<thead>
<tr>
<th>Dihydrocapsaicin (µg/mL)</th>
<th>Average Area</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>7.47E+05</td>
<td>3.0</td>
</tr>
<tr>
<td>7.5</td>
<td>1.16E+06</td>
<td>2.7</td>
</tr>
<tr>
<td>10.0</td>
<td>1.50E+06</td>
<td>1.5</td>
</tr>
<tr>
<td>17.5</td>
<td>2.58E+06</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 5. Data for the low concentration of dihydrocapsaicin: Areas of the analyte peak ($t_r$ = 6.42 min) using mobile phase of 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm. Each reported area is an average of three injections.

<table>
<thead>
<tr>
<th>Dihydrocapsaicin (µg/mL)</th>
<th>Average Area</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>3.02E+04</td>
<td>20.11</td>
</tr>
<tr>
<td>0.50</td>
<td>7.73E+04</td>
<td>8.75</td>
</tr>
<tr>
<td>1.00</td>
<td>1.30E+05</td>
<td>19.72</td>
</tr>
<tr>
<td>2.00</td>
<td>2.75E+05</td>
<td>11.19</td>
</tr>
</tbody>
</table>
Figure 9 and 10 are plots of the data from Table 4 and Table 5, respectively. These plots show that the peak areas are linear with concentrations for a wider range, i.e. from 0.25 µg/mL to 17.5 µg/mL. The equations for the straight lines are shown in the plots and their correlation coefficients are both better than 0.99 indicating good linearity.

From the presented data, one can conclude that there is a strong linear relationship between the area and the concentration of both capsaicin and dihydrocapsaicin over quite a large concentration range. However, at low concentration of both analytes, the relative standard deviation of triplicates were higher showing that there is a bigger fluctuation of the area from one run to the next.

![Figure 9](image)

**Figure 9.** Linearity curve at low concentration of dihydrocapsaicin: concentration range 0.25 to 2.0 µg/mL dihydrocapsaicin standard. Mobile phase used was 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210.
Reproducibility Studies

Reproducibility of the method using the actual samples was evaluated next, with the objective of checking the precision of the RP-HPLC method. Two different samples were selected for this study, habanero at yellow fruit stage and Variety 1 at red fruit stage. Eight samples of each variety were prepared according to the procedures outlined earlier in this chapter. Each sample solution was then injected into the HPLC in triplicate. The average mean area from each sample was used along with the regression line equation from the calibration curve to calculate the corresponding capsaicin and dihydrocapsaicin concentration in µg/mL.

Figure 10. Linearity curve at high concentration of dihydrocapsaicin: concentration range 5-17.5 µg/mL dihydrocapsaicin standard. Mobile phase used was 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm.
The concentrations in $\mu$g/mL were further used in the calculation of the actual amount of capsaicin and dihydrocapsaicin in 1.00 g of the sample.

A set of capsaicin standards were prepared as previously described and also injected in triplicate. Calibration curves used for calculation of capsaicin and dihydrocapsaicin in the chili samples for reproducibility study are shown in the Figure 11 and Figure 12. Figure 11 is the calibration curve for capsaicin with the regression line equation $y = 150950x + 17719$ and a correlation coefficient of 0.9998, while Figure 12 is the calibration curve for dihydrocapsaicin with the regression line equation $y = 144945 x + 86.4$ and a correlation coefficient of 0.9999.

![Figure 11](image.png)

Figure 11. Calibration curve of capsaicin standard for reproducibility studies: Concentration range 2.6-39.0 $\mu$g/mL. Mobile phase used was 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm.
The method of calculation of the actual amounts of capsaicin and dihydrocapsaicin in the sample is outlined below:

i. The concentration of both capsaicin and dihydrocapsaicin in µg/mL was multiplied by 5 mL, which was the final volume after dilution before injection.

ii. The amount in µg obtained above is then divided by the volume pipette into the 5.0 mL volumetric flask in this case 1 mL for Habanero yellow and 2 mL for Variety 1 red.

iii. The result obtained above is multiplied by 50 mL which was the final volume of the stock solution prepared from the samples.

The results of the reproducibility studies of Variety 1 red are shown in Table 6. The relative standard deviation for this study was 2.51% for capsaicin and 4.72% for dihydrocapsaicin.
The results of the reproducibility studies of habanero yellow stage fruits are shown in Table 7. The relative standard deviation for this study was 1.74 for capsaicin and 2.45 for dihydrocapsaicin. These values show that the precision of the method is acceptable and within experimental errors.

Table 6. Reproducibility data for capsaicin and dihydrocapsaicin using Variety 1 red: the amount (µg/mL) reported is obtained by using the average peak area of three injections. Mobile phase used was 73:27 methanol: water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Capsaicin</th>
<th>Dihydrocapsaicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (µg/g)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>1</td>
<td>610</td>
<td>1.30</td>
</tr>
<tr>
<td>2</td>
<td>629</td>
<td>1.87</td>
</tr>
<tr>
<td>3</td>
<td>613</td>
<td>3.22</td>
</tr>
<tr>
<td>4</td>
<td>629</td>
<td>0.37</td>
</tr>
<tr>
<td>5</td>
<td>635</td>
<td>1.22</td>
</tr>
<tr>
<td>6</td>
<td>619</td>
<td>1.31</td>
</tr>
<tr>
<td>7</td>
<td>643</td>
<td>3.11</td>
</tr>
<tr>
<td>8</td>
<td>659</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td>Data RSD</td>
<td>2.51</td>
</tr>
</tbody>
</table>
Another aspect of reproducibility tested was the retention time for both capsaicin and dihydrocapsaicin. This was evaluated using the habanero fruit at yellow stage during the reproducibility studies. The retention time for every run was recorded. The retention time was highly reproducible. Average retention time for capsaicin was 4.62 minutes with the relative standard deviation (RSD) of 1.74.

Table 7. Reproducibility data of capsaicin and dihydrocapsaicin for habanero yellow variety: The amount in µg/mL and relative standard deviation (RSD) reported is obtained by using the average peak area of three injections. Mobile phase used was methanol water 73:27 at a flow rate of 1.0 mL/min. Detection wavelength was 210 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Capsaicin</th>
<th>Dihydrocapsaicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (µg/g)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>1</td>
<td>8535</td>
<td>0.64</td>
</tr>
<tr>
<td>2</td>
<td>8749</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>8642</td>
<td>1.90</td>
</tr>
<tr>
<td>4</td>
<td>8404</td>
<td>1.09</td>
</tr>
<tr>
<td>5</td>
<td>8363</td>
<td>0.56</td>
</tr>
<tr>
<td>6</td>
<td>8338</td>
<td>1.11</td>
</tr>
<tr>
<td>7</td>
<td>8330</td>
<td>0.36</td>
</tr>
<tr>
<td>8</td>
<td>8374</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>Data RSD</td>
<td>1.74</td>
</tr>
</tbody>
</table>
standard deviation of 0.33 %. The average retention time of dihydrocapsaicin was found to be 6.40 with the relative standard deviation of 0.30 %.

Recovery Studies

To evaluate the accuracy of the proposed RP-HPLC method, recovery studies were carried out using Variety 1 red that had relatively high levels of both analytes and Variety 4 yellow with lower concentration of the analytes from earlier analysis. Because this was done separately, a set of capsaicin standard solution was prepared and injected in triplicates. The chromatograms of the samples used in this study are shown in Figure 13. Plots of average peak area against concentration give correlation coefficients of 0.996 and 0.999, for capsaicin and dihydrocapsaicin, respectively. These calibration curves are shown in Figure 14 and Figure 15 and were used to calculate the amount of capsaicin and dihydrocapsaicin recovered in µg/mL.

The sample solutions for the recovery studies were prepared as described earlier in this chapter. All the solutions were prepared in triplicate and injected in triplicate. Recovery of the analytes was calculated using this formula.

\[
\%\text{ Recovery} = \frac{\mu g_1 - \mu g_0}{\mu g_{\text{added}}} \times 100
\]  

[4,1]

where; \(\mu g_1\) is the total amount detected in the sample when a given amount of capsaicin standard is added to the original sample, \(\mu g_0\) represents the amount originally present in the sample detected when there is no capsaicin standard added to it, while \(\mu g_{\text{added}}\) represents the amount of capsaicin standard added.
Chromatograms of the Variety 4 yellow and Variety 1 red samples used in the study are shown in Figure 13. The retention time for capsaicin was $4.63 \pm 0.01$ min, while that of dihydrocapsaicin was $6.42 \pm 0.01$ min. As seen from the chromatograms shown in Figure 13, the two components were well resolved.

Figure 13. HPLC chromatograms of Variety 4 yellow (A) and Variety 1 red stage (B) used for recovery studies: The mobile phase used was 73 : 27 methanol: water. The flow rate was 1.0 mL/min with detection wavelength at 210 nm. The retention time of the compounds is given in minutes.
Figure 14. Calibration curve of capsaicin standard for recovery studies: Concentrations range 2.6-39 µg/ml. Mobile phase used was 73:27 methanol; water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm.

Figure 15. Calibration curve of dihydrocapsaicin standard for recovery studies: Concentrations range 1-10 µg/ml. Mobile phase used was 73:27 methanol; water at a flow rate of 1.0 mL/min. with detection wavelength at 210 nm.
The results of the recovery studies of capsaicin and dihydrocapsaicin are summarized in Table 8 and Table 9, respectively. The amount of both capsaicin and dihydrocapsaicin standard added, the amount that was recovered, percent recovery, and the relative standard deviation of the sample triplicates are shown.

Both percent recovery and RSD data for Variety 4 yellow were satisfactory. Variety 1 red high percent recovery of (117%) might have resulted from errors during the preparation of the sample solutions or the experienced area fluctuations noted when the analysis of this sample was carried out.

The observed large RSD value of 12.04 reported when 4.59 µg of capsaicin standard was added to Variety I red can be explained as resulting from inconsistencies in the areas of the peaks from one run to the next as this sample was run through the HPLC. This was the first experiment to be done before any other on that day; one could attribute these observed irregularities to the non-equilibrium in the column, though the instrument had been running for more than half an hour. In all the preceding analysis, the HPLC system was left to equilibrate for one hour in order to minimize such inconsistencies.

On the other hand, recovery of dihydrocapsaicin showed good percent recovery. All the reported recovery for both Variety 1 red and Variety 4 yellow were within 97% to 106% with all the RSD being lower than 9.00 as seen in Table 9. The percent recovery of all tested samples yielded values that are acceptable. The conclusion is that the proposed RP-HPLC method is reliable and accurate.
Table 8. Average amount of capsaicin recovered from recovery study: The mobile phase was 73: 27 methanol: water, at a flow rate of 1.0 mL/Min. with detection wavelength at 210. Retention time for capsaicin was 4.64 ±0.2 min. Each reported amount was an average of triplicate injections, and each relative standard deviation was obtained within each concentration of the standard solution.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Amount Added (µg)</th>
<th>Average Amount Recovered (µg)</th>
<th>% Recovery</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V 1 Red</td>
<td>2.58</td>
<td>3.02</td>
<td>117.11</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>4.59</td>
<td>4.93</td>
<td>107.41</td>
<td>12.04</td>
</tr>
<tr>
<td>V 4 Yellow</td>
<td>2.58</td>
<td>2.53</td>
<td>98.26</td>
<td>4.05</td>
</tr>
<tr>
<td></td>
<td>4.59</td>
<td>4.70</td>
<td>102.27</td>
<td>4.50</td>
</tr>
</tbody>
</table>

Table 9. Average amount of dihydrocapsaicin recovered: the mobile phase was 73: 27 methanol: water, at a flow rate of 1.0 mL/Min. with detection wavelength at 210. The retention time for dihydrocapsaicin was 6.43 ±0.2 min. Each reported amount is an average of triplicate injections, and each relative standard deviation was obtained within each concentration of the standard solution.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Amount Added (µg)</th>
<th>Average Amount Recovered (µg)</th>
<th>% Recovery</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V 1 Red</td>
<td>0.85</td>
<td>0.90</td>
<td>106.19</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>2.12</td>
<td>2.06</td>
<td>97.43</td>
<td>4.51</td>
</tr>
<tr>
<td>V 4 Yellow</td>
<td>0.85</td>
<td>0.85</td>
<td>99.97</td>
<td>8.62</td>
</tr>
<tr>
<td></td>
<td>2.12</td>
<td>2.23</td>
<td>105.03</td>
<td>6.89</td>
</tr>
</tbody>
</table>
Application of The Proposed HPLC Method to other Samples

The developed RP-HPLC method was applied to the determination of capsaicin and dihydrocapsaicin in four varieties of naturally grown hot pepper at green, yellow, and red stages of development and one habanero variety bought from a local shop in Johnson City, Tennessee. The level of both capsaicin and dihydrocapsaicin of Variety 1 in the fruits harvested during summer and winter season for seasonal variation comparison was also evaluated.

Each stock sample solution was prepared as described in the experimental procedure section. Each solution was prepared in triplicate and injected in triplicates into the HPLC. The average of the 9 injections was used to calculate the amount of capsaicin and dihydrocapsaicin in the samples. A set of standards were also prepared and used to construct a calibration curve for the analysis of the desired components. Capsaicin standard concentrations 2.6, 13.0, 26.0, and 39.0 µg/mL were used to construct a calibration curve. The equation of the regression line for capsaicin standard was $y = 167703 \times + 149933$ with a correlation coefficient of 0.9999. Dihydrocapsaicin standard concentrations were, 1.0, 5.0, 10.0, and 15.0 µg/mL were used for the calibration curve. The equation of regression line for dihydrocapsaicin was $y = 160629 \times + 7092.9$ with the correlation coefficient of 1.00.

The overall data that were collected from all the samples are tabulated in Table 10. The amount of capsaicin and dihydrocapsaicin is in µg/g of dry weight of the samples that were used in this study. Each sample was prepared in triplicate and injected into the HPLC in triplicates. The data shown are an average of these injections and triplicate samples.
Table 10. Average amount of capsaicin and dihydrocapsaicin in the samples at different stages: The mobile phase was 73: 27 methanol: water, at a flow rate of 1.0 mL/min, and detection wavelength set at 210. The retention time for capsaicin was 4.64 ± 0.2 min while that of dihydrocapsaicin was 6.43 ± 0.2.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Stage</th>
<th>µg of capsaicin per g dry sample, (RSD %)</th>
<th>µg of dihydrocapsaicin per g dry sample,(RSD %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety 1</td>
<td>Green</td>
<td>642 (7.4)</td>
<td>565 (3.8)</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>718 (5.4)</td>
<td>646 (3.9)</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1832 (5.5)</td>
<td>1172 (2.8)</td>
</tr>
<tr>
<td>Variety 1</td>
<td>Green</td>
<td>1437 (2.2)</td>
<td>558 (8.0)</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>1131 (5.2)</td>
<td>339 (6.3)</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1331 (4.7)</td>
<td>393 (7.8)</td>
</tr>
<tr>
<td>Variety 2</td>
<td>Green</td>
<td>834 (3.4)</td>
<td>329 (6.1)</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>1237 (5.8)</td>
<td>696 (3.3)</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1487 (5.1)</td>
<td>802 (7.5)</td>
</tr>
<tr>
<td>Variety 4</td>
<td>Yellow</td>
<td>1201 (6.1)</td>
<td>657 (3.6)</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1420 (6.4)</td>
<td>673 (3.8)</td>
</tr>
<tr>
<td>Habanero</td>
<td>Green</td>
<td>7845 (3.2)</td>
<td>2966 (2.5)</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>8467 (3.5)</td>
<td>3632 (3.1)</td>
</tr>
</tbody>
</table>
Stages of Growth

The data collected are further broken down into categories depending on the information to be discussed. In terms of the stages of development, the amount found in the different varieties at the different stages is shown in Figure 16 for the green stage, Figure 17 for the yellow stage, and in Figure 18 for the red stage. The graph that summarizes the complete results of the different stages for all the varieties and the amount of capsaicinoids found is also shown in Figure 19. Data for the fruits harvested during summer and winter are also included.

The amount of both capsaicin and dihydrocapsaicin increased from the green stage to red stage in the varieties tested except for the Variety 2 that was noted to have higher capsaicinoids in green stage than yellow stage. As noted earlier, the amount of capsaicinoids in the hot pepper depend on two main factors, the genetics of the plant and how it interacts with the environment. This observation might have resulted from changes in weather conditions during the fruit growth. It has been noted in earlier studies (48) that any kind of stress to the plant changes the amount of capsaicinoids in the fruits considerably. Increase in temperature, light, water, and fertilizers result in increase of the level of capsaicinoids (48). In this case the weather conditions that favor increase in capsaicinoids were prevalent at the green stage of Variety 2 growth and this might have caused this increase. The reported data for yellow and red stage of the same Variety 2 were consistent with the data in the other varieties. Probably, weather conditions might have returned to normal during yellow, and red stage of Variety 2.
Figure 16. Relative amount of capsaicin and dihydrocapsaicin at green stage of chili fruits development in different varieties. The amount is in µg of capsaicin and dihydrocapsaicin/g of dry weight of samples that were used in this study.

Figure 17. Average amount of capsaicin and dihydrocapsaicin found in the yellow stage of different varieties: The amount is in µg of capsaicin and dihydrocapsaicin /g of dry weight of the samples that were used in this study.
The summary of the amount of both capsaicin and dihydrocapsaicin in the three stages for all the varieties used in this research is also shown in Figure 19.

Figure 18. Average amount of capsaicin and dihydrocapsaicin in red stage of the tested varieties used: The amount is in µg of capsaicin and dihydrocapsaicin /g of dry weight of the samples that were used in this study.

Figure 19. Average amount of capsaicin and dihydrocapsaicin, found in three stages of development in all the varieties used for the study: The amount is in µg of capsaicin and dihydrocapsaicin/g of dry weight of the samples that were used in this study.
Capsaicinoids in Different Varieties

In the previous section, we have seen how the amount of capsaicin and dihydrocapsaicin differed from green, yellow, and red stages of chili. In this section, the amount of capsaicin and dihydrocapsaicin found in the varieties used for this project, either individually or combined will be evaluated. The total amounts of both capsaicin and dihydrocapsaicin combined found in different varieties used in this study are shown in Table 11 and Figure 20.

The amounts of capsaicin and dihydrocapsaicin reported in this table are found by adding the amount of capsaicin or dihydrocapsaicin in the three or two stages of chili tested, and then the total is divided by either three or two depending on the number of stages tested for each variety. To get the value in the total capsaicinoids column, average amount of capsaicin and dihydrocapsaicin in a given variety are added together. The amount 8156 ppm capsaicin and 3299 ppm dihydrocapsaicin found in Habanero variety were close to the amount 8840 ppm capsaicin and 3940 ppm dihydrocapsaicin reported earlier by Kurian et al. (47). The difference in these amounts is expected as these varieties were grown in different locations and under different weather conditions.

The last column on the right in Table 11 shows the hotness of each of the variety used in this project based on the two capsaicinoids tested. The hotness is given in terms of Scoville Heat Units (SHU). A Scoville Heat Unit is found by multiplying parts per million by a factor 15 as indicated in earlier studies carried out by Todd et al. (34). Habanero had the highest SHU while Variety 2 had the lowest SHU. The order of hotness for the varieties tested is shown below:
Table 11. The total amount of capsaicin and dihydrocapsaicin in different varieties used in this project: Each amount is an average of the amount found in the three or two stages of chili fruit development.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Capsaicin (µg/g)</th>
<th>Dihydrocapsaicin (µg/g)</th>
<th>Total capsaicinoids (µg/g)</th>
<th>Scoville Heat Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1 winter</td>
<td>1064</td>
<td>794</td>
<td>1858</td>
<td>27870</td>
</tr>
<tr>
<td>V1 summer</td>
<td>1468</td>
<td>430</td>
<td>1898</td>
<td>28470</td>
</tr>
<tr>
<td>Variety 2</td>
<td>1184</td>
<td>609</td>
<td>1793</td>
<td>26895</td>
</tr>
<tr>
<td>Variety 4</td>
<td>1311</td>
<td>665</td>
<td>1976</td>
<td>29640</td>
</tr>
<tr>
<td>Habanero</td>
<td>8156</td>
<td>3299</td>
<td>11455</td>
<td>171,825</td>
</tr>
</tbody>
</table>

Figure 20. The overall amount of capsaicin and dihydrocapsaicin of the tested varieties: The amount µg capsaicin and dihydrocapsaicin/g of dry weight was found by adding the amount of capsaicin and dihydrocapsaicin in the 3 stages or 2 and then dividing by 3 or 2 depending on the number of tested stages.
In this project, the percent of capsaicin was noted to increase while that of dihydrocapsaicin decreasing from yellow to the red stage in all the varieties tested. The reason for this could be that as the fruit changes from yellow to red the internal processes favor the production of capsaicin that is hotter than dihydrocapsaicin. The percentage amount of both capsaicin and dihydrocapsain in yellow and red stage of the varieties used is shown in Table 12.

Table 12. The percent of capsaicin and dihydrocapsaicin in yellow and red stages of different varieties: The average relative amount of both components in all our samples was 67.3% capsaicin and 32.7% dihydrocapsaicin.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Stage</th>
<th>Capsaicin (%)</th>
<th>Dihydrocapsaicin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety 1 winter</td>
<td>yellow</td>
<td>52.6</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>61.0</td>
<td>39.0</td>
</tr>
<tr>
<td>Variety 1 summer</td>
<td>yellow</td>
<td>76.9</td>
<td>23.1</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>77.7</td>
<td>22.3</td>
</tr>
<tr>
<td>Variety 2</td>
<td>yellow</td>
<td>64.0</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>65.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Variety 4</td>
<td>yellow</td>
<td>64.6</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>67.8</td>
<td>32.2</td>
</tr>
</tbody>
</table>
The main focus for the samples tested in summer and winter was to find if there was a
difference in terms of the amount of capsaicin and dihydrocapsaicin based on season. It was
noted that the level of both capsaicin and dihydrocapsaicin was higher in summer fruits than in
winter fruits. The percent of dihydrocapsaicin was higher in winter compared to percent found
in summer. Summer fruits were found to have higher amount of the total overall amount of
both capsaicin and dihydrocapsaicin. Table 13 and Figure 21 show the relative percentages of
both capsaicin and dihydrocapsaicin and their ratio in winter and summer fruits. It can be seen
that the amount of capsaicin and dihydrocapsaicin in the winter fruits is almost the same, while
in summer, the amount of capsaicin is much higher than dihydrocapsaicin.

Table 13. The ration of capsaicin and dihydrocapsaicin in three stages of winter and summer fruits

<table>
<thead>
<tr>
<th>Variety</th>
<th>Stage</th>
<th>Capsaicin (%)</th>
<th>Dihydrocapsaicin (%)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety 1 winter</td>
<td>green</td>
<td>53.2</td>
<td>46.8</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>yellow</td>
<td>52.6</td>
<td>47.4</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>61.0</td>
<td>39.0</td>
<td>1.56</td>
</tr>
<tr>
<td>Variety 1 summer</td>
<td>green</td>
<td>77.2</td>
<td>22.8</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td>yellow</td>
<td>76.9</td>
<td>23.1</td>
<td>3.34</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>77.7</td>
<td>22.3</td>
<td>3.48</td>
</tr>
</tbody>
</table>
As seen in Figure 21, the ratio between capsaicin and dihydrocapsaicin in the fruits harvested during winter is close to one compared to the higher ratio of both capsaicinoids in fruits harvested in the summer season.

Figure 21. Average amount of capsaicin and dihydrocapsaicin in summer and winter fruits: The amount is in µg of capsaicin and dihydrocapsaicin /g of dry weight of the samples that were used in this study.
CHAPTER 5

CONCLUSIONS

In the pepper industry, pungency is one of the key quality factors. Capsaaicin and dihydrocapsaicin are two mayor capsaicinoids responsible for the pungency of capsicum fruits. Reliable methods for analysis and quantification of the capsaicinoids that brings about pungency are needed. This chapter gives a summary of the method used and a brief summary of the results obtained.

The extraction method selected for this project was simple and easy. It essentially involved drying, grinding the pepper, and then transferring the grounded pepper to a container where capsaicinoids were extracted with methanol. The mixture was sonicated for 25 minutes, filtered, and diluted for HPLC analysis. The solvent used in this study was methanol. Methanol had been used previously by other authors, for example Barbero et al. (11) who observed that both methanol and ethanol gave equal recoveries during capsaicin extraction through sonication method. Methanol was used for this study because it was less expensive than other solvents like acetonitrile used for extraction of capasicinoids. Another reason why methanol was chosen is because it was mixed with water and used as the HPLC mobile phase for this study. Water is the least expensive of all and more convenient to use but it could not be used for capsaicinoid extraction because capsaicinoids are almost insoluble in it.

The HPLC method was validated with respect to linear calibration curves, good reproducibility, and accurate capsaicinoids recovery. The method was found to be linear within 0.65 to 45.5 µg/g range for capsaicin, and 0.25 to 17.5 µg/g for dihydrocapsaicin according to
the linearity studies that were conducted. The method was also found to be reproducible with the average RSD for eight aliquots being equal to 2.12, and that of dihydrocapsaicin was 3.58. The method also showed good recovery with the average capsaicin recovery of 106%, and that of dihydrocapsaicin being 102%.

This method was rapid for the HPLC analysis, as shown by the results acquired from linearity, reproducibility, and recovery studies. All these tests show that the method is precise and accurate and it can be applied for the determination of capsaicinoids in normal laboratories.

Future investigation would be to carry out similar studies for other nutritive components of hot pepper for instance carotenoids, anti-oxidants, phenolics, etc. This would give an idea of the stage at which these components are at the highest level in pepper fruits.
1. Sumate, B; Chada, P; Muenduen, P. Ultrasound assisted extraction of capsaicinoids from capsicum frutescens on a lab- and pilot-plant scale, *ultrasonic sonochemistry*. 2008, 15, pp 1075-1079.


19. Wang, L.; Curtis, L. W. Recent advances in extraction of nutraceuticals from plants, Trends in food science $&$ technology. 2006, 17, 300-312.


35. David, L.; Bente, W.; George, C. Instrumental methods in food and beverage analysis. 

   four analogues by using high-performance liquid chromatography and gas 

37. Woodbury, J.E. Determination of *Capsicum* pungency by high pressure liquid 
   pp 556-558.

   Rene, K. Study of capsaicin content in various parts of pepper fruit by liquid 

39. Krishnamurthy, R.; Marve, M.K.; Shinde, B.M. Evaluation of capsaicin content in red and 

40. Robert, Q. T.; Karen, W. P.; Michael, J. W.; Edward, W. V. Quantitative determination of 
   capsaicinoids by liquid chromatography–electrospray mass spectrometry, *Anal Bioanal 

41. Rachaneewan, K.; Mongkon, R.; Surasak, W.; Yuthsak, V. Optimization of High-
   performance liquid chromatographic parameters for the determination of capsaicinoid 

42. Barbero, G. F.; Liazid, A.; Palma, M.; Barroso, C.G. Fast determination of capsaicinoids 
   from peppers by high – performance liquid chromatography using a reversed phase 

43. Robards, K.; Haddard, R. P.; Jackson, E.P. *Principles and practice of modern 

44. Andrea, W; Phyllis, R. B. *HPLC and CE principles and practice*, Academic Press, San Diego, 
   CA, 1997, pp 35-44.


VITA

JOHN KAILEMIA MUCHENA

Personal Data: Date of Birth: March 10, 1978
Place of Birth: Meru, Kenya

Marital Status: Married

Education: Maseno University, Kenya, B.ED Science, 2002
East Tennessee State University (ETSU), Johnson City, Tennessee, USA; Chemistry, M.S. 2009

Professional Experience: Chemistry and Mathematics High School Teacher,
Meru, Kenya, 2002-2007
Graduate Teaching Assistant, ETSU, Johnson City, TN, USA, 2007-2009