12-2011

Survey of Flavonoids and Their Distribution in Different Kinds of Onions Using High Performance Liquid Chromatography and Gas Chromatography Mass Spectrometry.

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Survey of Flavonoids and Their Distribution in Different Kinds of Onions Using High Performance Liquid Chromatography and Gas Chromatography – Mass Spectrometry

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A thesis

presented to

the faculty of the Department of the Chemistry

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Chemistry

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by

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December 2011

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Keywords: Onion, High Performance Liquid Chromatography, Gas Chromatography Mass Spectrometry, Flavonoid, Alliin.

ABSTRACT

Survey of Flavonoids and Their Distribution in Different Kinds of Onions Using High Performance Liquid Chromatography and Gas Chromatography – Mass Spectrometry

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This research is done to determine the distribution of flavonoids in diverse varieties of onions (commercial and wild onions). Reflux extraction method was done on dried onions and the abstracts were analyzed subsequently by high performance liquid chromatography (HPLC) and gas chromatography – mass spectrometry (GCMS). Statistical calculations were done to see if there are significant differences in the varieties of onions studied from the chromatographic profiles obtained. The chromatograms obtained were patterned using visual observations, scatter plot study, correlation co-efficient, and ANOVA to evaluate the significant difference of the distribution of flavonoids in varieties of onions. The organic white onions seem to have closer flavonoids profile to that of natural wild onions with a co-relation coefficient of 0.99 from HPLC data and 0.88 from the GCMS data. The ANOVA results also support these conclusions. However, natural wild onions tend to have more constituents that can be beneficial.

DEDICATION

I would like to dedicate this work to everyone who encouraged me and gave me the strength to achieve my goals. I dedicate this thesis to Racharla (mom, dad, and brother) and Samala (husband and in-laws) families. I dedicate this thesis to all professors in Department of Chemistry at ETSU for their support and guidance to do better.

ACKNOWLEDGMENTS

I would like to express my deepest and sincere gratitude to my supervisor Dr.Chu-Ngi Ho for his support, patience, guidance, and kindness throughout my studies. I am very thankful for having him as my advisor.

I wish to express my sincere thanks to Dr. Peng Sun and Dr. Ningfeng Zhao for being on my thesis committee and their detailed and constructive comments have made this work a success. I esteemly thank Dr. Ray Mohseni and Mr. Mark Klein who supported me in learning the instrumental techniques throughout my study. My special regards to the faculty for their skills and knowledge that I have acquired throughout my course work, and the staff (Tom Webster and Walt Witt) of chemistry department at ETSU for their support in my graduate education. I would like to acknowledge department of chemistry and department of public health for awarding graduate assistantship.

I indebtly thank my loving husband Dr. Mallikarjun Samala for his moral support, understanding and encouragement in every step of my education. I am grateful to my parents Mr. Shankaraiah and Ms. Madhulatha and my father-in-law Mr. Ashalu and my mother-in-law Ms. Amrutha and my sweet brother Avinash for their support and prayers.

Finally, I would like to thank my friends, my fellow graduate students, and undergraduate students for their support.

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CHAPTER 1

INTRODUCTION

Onion is the oldest edible bulb vegetable known to man. It has been cultivated since the distant past. It is grown in many countries all over the world and is used in different forms as food, species, and medicine. The word onion originates from the Latin word "unio" which means "single". The name also explains the union of many separate and concentrically arranged layers of the onion (1). The top 10 countries that grow onions are India, China, Australia, United States, Turkey, Pakistan, Russian, South Korea, Japan, and Spain (2). In 2006, Japan was importing onions from abroad; almost one-third of onions were consumed by them. Onion is also called Oignon (French), Zwiebel (German), Cebolla (Spanish), Cipolla (Italian), Choong (Chinese), Piaz (Indian), Nira (Japanese), and Hua horm (Thai) (3).

Botany of Onion

Onions are of many varieties that vary considerably in color, shape, and size. These are grown both wild and domestically. Wild onions are grown naturally rather than being specifically cultivated. Cultivated plants rise from seeds sown in the summer of the previous year and will form a good sized bulbs by the end of the season. The good time to plant the seed is in early spring. It is important to have firm surface and fine soil. If the soil is loose around the bulbs, they may get covered up. It needs sufficient air and light to ensure proper development and provide bulbs that keep well when stored. The bulbs are formed by swelling of the leaf wall at the base of the leaves. When the bulbs are developing, dilute liquid fertilizers are given weekly. Good care against thrips and other pests should be taken. In August, almost all the onions will finish growing and are ready to use (4). It is good to

store in well ventilated room. Fresh onions have a characteristic odor and are used as flavoring spice and also as a medicine.

Based on the growth characteristics, the onion can be divided into three different groups (5):

1) The common onion: One single plant is formed from the each bulb and bublets are not formed from the inflorescence. A wide range of environmental conditions are responsible for the shape of the bulb shapes, color of dry scale, pungency, and many other characteristics. These plants are first propagrated through seeds.
2) The aggregatum group: The characteristics in this group of plants are lateral bulbs or shoots. Fewer bublets are formed from the inflorescence. These plants are propagated by vegetative means, such as potato or multiplier onion and shallots.
3) The proliferous group: Bublets are formed from the inflorescence and poorly developed ground bulbs are also seen. Usually there is a lack of true seed and therefore vegetative inflorescence and bulbets are used for reproduction. These groups of plants are not grown for commercial production but belong to home gardening, such as Egyptian onions.

Taxonomy of Onion

Based on the Integrated Taxonomic Information System (ITIS), the latest review of 2010 places onion in the family Asparagales/ Alliaceae. This family contains approximately 700 species (6). It belongs to the genus Allium and the species cepa, usually refers to "Allium Cepa" and commonly known as "garden onion" or "bulb onion." Only in cultivation it is known as Allium cepa (7). In Central Asia there are other related wild species. Beside garden onion, there are 20 other species that are consumed by humans (8). Most of the species in this genus are perennial plants and all these plants have underground storage

organs that contains shoots, rhizomes or bulbs. Some of the species that are closely related to Allium cepa include Allium asarense and Allium vavilovii from Iran (9). All the Allium species contains sulfur containing compounds that are responsible for the different flavors, biological, and medicinal activities, and characteristic odor (6). The scientific classification of garden onion is shown in Table 1:

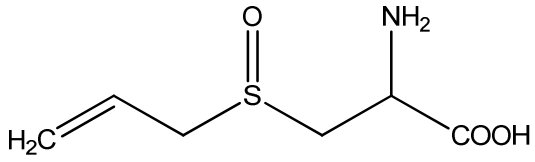
Table 1: Scientific classification of garden onion (10, 11)

Domain:	Eukarya
Kingdom:	Plantae
Subkingdom:	Tracheobionta
Division: (Angiosperms)	Magnoliophyta
Class:	Liliopsida (monocots)
Subclass:	Liliidae
Order:	Asparagales
Family:	Amaryllidaceae (Alliaceae)
Genus:	Allium L.
Species:	A.cepa

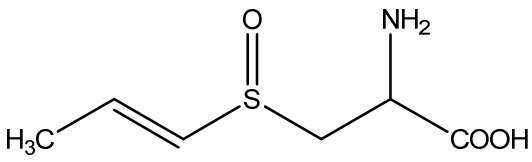
Chemical Composition and Chemistry of Allium Cepa L

Onion contains more than 85% of moisture, 1.5% of protein, 11.6% carbohydrates. It also contains selenium and vitamins (A, C, and B complex vitamins), and low levels of iron, zinc, calcium, sodium, phosphorous, and potassium. It also contains polysaccharides such as saccharose, fructosans and some peptides (alliceptin), phenolic acids (caffeic, p-coumaric), anthocyanins (cyanidin), essential oil, flavonoids (mostly quercetin), and numerous sulphur compounds (includes thiosulfinates, thiosulfonates , sulfoxides, etc.). Along with these they

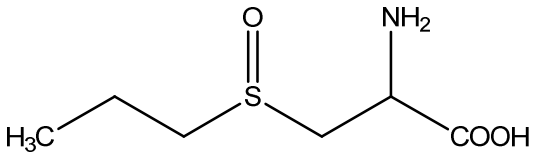
also contain more than 30 sulfur compounds and 17 amino acids (like glutanic acid and orginine are abundant) (12). Allium plants have one common constituent group S-Alk(en)yl-cysteine sulfoxide (ACSOs, or Alliin) that is responsible for its flavors and odor (13). Some other organic sulfur compounds include trans -S-(1-propenyl) cysteine sulfoxide (Isoallin), D-Propyl-L-Cysteine sulfoxide (propiin), and S-methyl-L-cysteine sulfoxide (Methiin). These compounds are shown in Figure 1.



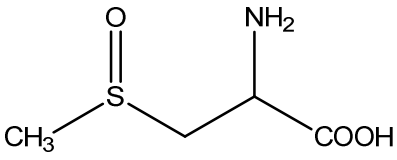
S-Alk(en)yl-L-cysteine sulfoxide
(Alliin)



S-Propenyl-L-cysteine sulfoxide
(Isoalliin)



S-Propyl-L-cysteine sulfoxide
(Propiin)



S-Methyl-L-cysteine sulfoxide
(Methiin)

Figure1: Chemical structures of some sulfoxides from Allium plants

In onion, all thioallyl compounds like S-Alk(en)yl-cysteine sulfoxides(Alliin) are the main components responsible for its flavor. Isoallin and methiin are usually found in low concentration (14). In 1948, Stroll and Seebrook first identified Alliin (14). It is a stable non-protein amino acid. In onion, alliin is located in cell cytoplasm of the bulb and allinase is located in the cell vacuole. When onions are crushed or cut, the alliin is converted to allicin by an enzymatic reaction using allinase and a byproduct is ammonia (15). This allicin breaks

down into sulfide compounds which give its distinctive smell. A typical enzymatic reaction in onion cell that produce allicin is shown in Figure 2.

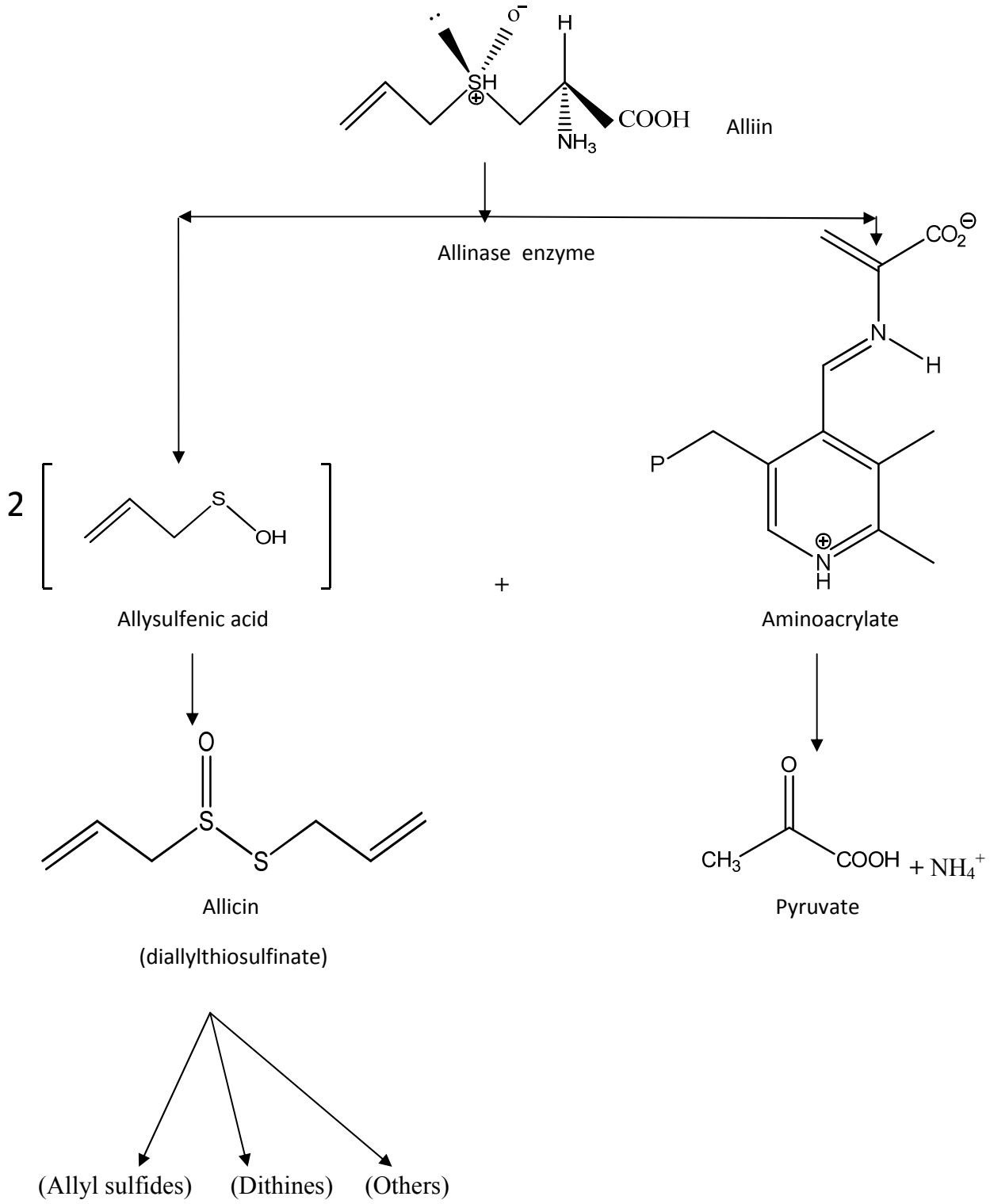
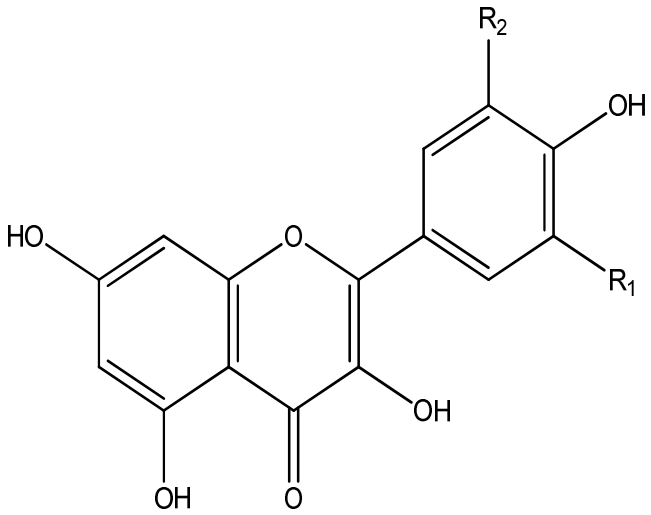


Figure 2: Ezymatic hydrolysis of Alliin to Allicin. Allicin is unsatable and further decomposes into sulfur compounds.

The sulphur compound that is responsible for lachrymatory factor is S-propenyl cysteine sulphoxide (16). When onion is crushed or cut this sulphur compound is converted to 1-propenesulfenic acid and rapidly converts to syn-propanethial-S-oxide (a volatile gas) in the presence of lachrymatory factor synthase (17). This volatile gas diffuses across the air and reaches the eyes where it activates the sensory neurons and creates a stinging sensation. Therefore, tear glands produce tears to get rid of irritation (18).

The major flavonoid that is present in the Allium cepa is quercetin. The major glycosides that constituents 80% of flavonoids in onion are quercetin 4 –O-β-glucoside and quercetin 3, 4-O-β-diglucoside. Some other flavonoids that are present in onion are kaempferol (19), isorharmnetin derivatives (20), and myricetin (21). The glycosyl unit in these is identified as glucose. The bulb color and the type depend on the amount of flavonoids present in them. These flavonoids compounds are shown in Figure 3.



Compound	R_2	R_1
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H
Myricetin	OH	OH

Figure 3: Examples of flavonoids from Allium plants

Medicinal Properties and Uses of Onion

In ancient time, the Egyptians believed and worshipped the onion because of its spherical shape and concentric rings which symbolized eternal life (22). In the eye sockets of Ramesses IV, the Egyptians found some onion traces, so it was even used in Egyptians burials (22). In ancient Greece, it was believed that consumption of large quantities of onion would lighten the balance of blood. The Roman gladiators firm up their muscles by rubbing with onions. In the middle ages, onions were used to pay rent and also given as gifts.

Onions were prescribed by doctors to ease bowel movements and erection. It also relieved coughs, snake bite, hair loss, and headaches. In the 1500s it was prescribed by doctors to help with infertility in women, cattle, dogs, and many other pets. From some recent evidences, pets like dogs, cattle, and other animals should not be given onions, due to toxicity during digestion (23-25).

Numerous clinical trials were done to confirm the medicinal and health effects of onions. Various studies have proved that high consumption of onions reduces neck and head cancer (26) and also lowers blood sugar, lipids, and cholesterol (27). Onions also act as expectorant, appetite, stimulant, and diuretic (27). To treat topical scars products that contain onion extract had been used. Some found that they were ineffective (28-30) while others found that they act as bacteriostatic or anti-inflammatory (31) and can develop collagen organization in rabbits (32). The juice that is obtained from the fresh onions has antibacterial properties due to the interactions and presence of allicin, disulphide, and cysteine compounds (33). Regular consumption of onion in human and animal blood shows the antiplatelet aggregation effect (33, 34). It has low risk of lung, esophageal, and stomach cancer (35). Other benefits of onion include antiseptic, rubefacient, digestant, antiparasitic, antifungal activity, anti-inflammatory, and antihistamine effects (36).

Several groups of substances are present in onions that promote health effects. Onions contain flavonoids, especially flavonols and anthocyanins as sources of antioxidants that play an important role in prevention of cardiovascular diseases and forms of cancer (37). Onions have the capacity to prevent sore throat because of the pleiomeric chemicals present (38). Onions also contain an essential oil that acts as heart stimulant and increases blood pulse (33). There are many benefits of onion that have given rise to the production of many onion food supplements based on quercetin content. Many other investigations were done to find out the pharmacological effects. All these investigations and medicinal value have resulted in the production of high quantity and good yield of fresh onion.

CHAPTER 2

METHODS OF FLAVONOID ANALYSIS

Sample Preparation Techniques For Flavonoid Analysis

For any analysis, a general prerequisite is isolation of flavonoids from the crude sample matrix. In the past there were many sample pre-treatment methods developed to determine flavonoids in various samples. Food, plants, and liquid samples (biological fluids and drinks) are three types of flavonoids containing matrices. All the solid samples are first homogenized, preceded by freezing with liquid nitrogen or drying and then followed by analyte isolation using solid-liquid extraction. All the liquid samples are first filtered, centrifuged, and then analytes are isolated using liquid-liquid extraction. In recent years the sample preparation methods have been improved for greater specificity, selectivity, and better reproducibility without the use of organic solvents and smaller sample size and volume. The methods include pressurized liquid extraction (PLE), matrix solid-phase dispersion (MSPD), supercritical-fluid extraction (SFE), microwave-assisted extraction (MAE), solid-phase extraction (SPE), solid-phase micro-extraction (SPME), and ultrasonication (39).

Pressurized Liquid Extraction (PLE)

It is also known as accelerated solvent extraction. This method uses a high temperature and pressure with a forced flow of solvent. To move this solvent under high pressure, a compressed nitrogen gas is used as a carrier. High temperature and pressure are used to enable rapid extraction. In this method the aqueous and organic solvents are used more efficiently (39). In corn and oats kernels the efficiency of extraction of polar and nonpolar lipids by using pressurized solvents such as ethanol or methylene chloride was examined (40). Isolation of tocopherols from several nuts and seeds using PLE was done and

shown that it yields very clean extracts and recoveries (41). Good efficiency in the determination of tocopherols and tocotrienols in oil residues from palm fiber and carotene was reported by using homemade PLE system (42). In the recent years, PLE was applied for extraction of 5 flavonoids from Lysimachia clethroide by Jiang (43). The extraction recoveries are found to be equivalent to soxhlet extraction.

Matrix Solid-Phase Dispersion (MSPD)

This method can be used for solid, semi-solid, and highly viscous biological samples. It has some major advantages such as reduced cost, low sample consumption, low solvent consumption, ability to perform simultaneous extraction, and easy to clean in single step. The isoflavonoids were identified and determined by LC-DAD-MS in Radix astragali (44). Conventional extraction methods like ultrasonic and soxhlet methods were compared to MSPD extraction and it was clear that MSPD extraction is comparatively a better method than the other two methods based on the extraction efficiency and solvent consumption (39). This extraction method was used to extract simultaneously 23 phenolic compounds including quercetin, and kaempferol from wine samples using GCMS in the selected ion monitoring mode. All the parameters like pH, sample ionic strength, and eluting solvent were optimized. This optimized MSPD requires a small amount of sample, commercial silica as solid phase and a small amount of ethyl acetate as eluting solvent for extraction of compounds (45).

Supercritical Fluid Extraction (SFE)

In recent years, this method has been applied in food and medical industries. This method has advantages that include the ability to perform rapid extraction (less than 30 minutes), and without the use of organic solvents because carbon dioxide is used as extraction solvent (39). Density of carbon dioxide is used to control the selectivity. The

compounds that are extracted by this method have high diffusion coefficients that lessen their degradation and enhance the rate of extraction. Another advantage is the low cost of carbon dioxide. It is also nonflammable and oxygen deficient which prevents degradation of sample caused by oxidation. However, the method has a disadvantage that the sample should not contain fat because it is extracted with the desired compounds. This method was employed to extract flavonoids in Pueraria lobata (46). Another three flavonoids were also identified from Patrinia villosa by Peng et al. with this method (47).

Microwave-Assisted Extraction (MAE)

This method involves heating of solid sample and solvent mixtures in a vessel that is closed completely. This vessel is kept in the microwave and extraction is done with microwave energy under controlled temperature and pressure conditions. Extraction of analyte increases due to high temperatures and pressures in closed vessel. Consumption of organic solvent was much less (39). It was found that at microwave extractions performed at 110^{o}C, for minutes using 30 mL toluene gave more than 80% recoveries for highly chlorinated congeners in spiked ash samples that contains relatively high content of carbon (48). Recently, this method was used in Herba Epimedii for extracting flavonoids (49). This method is fast and reliable for busy analytical laboratory that allows about 80 extractions and filtrations to be done within four hours. This method is more efficient than others by using about 90% less solvent than the soxhlet, making it much more friendly technique.

Solid Phase Extraction (SPE)

It is the simplest but most effective sample preparation method. This method is commonly used to extract flavonoids from the sample extracts and aqueous samples. It includes disposable cartridges, sample components of interest for appropriate solid sorbent,

and selective eluent for desired component. For a maximum efficiency, separation and recovery the sorbent that is used should be homogeneous and free of voids and channels. The extraction efficiency depends on the pH, sample volume, eluent volume, and the type of sorbent. In many experiments, C_{18}-bonded silica is used as the sorbent and slightly acidified sample and solvent solutions are used to prevent ionization of flavonoids (39). SPE shows high recoveries for polar compounds

Solid Phase Micro-Extraction (SPME)

SPME is used to isolate and pre-concentrate the organic molecules from solid, liquid, and gaseous samples (39). It is also used for polar and non-polar analytes. Non-volatile compounds are analyzed by using this method when coupled with liquid chromatography. Mechanism of this method is very similar to SPE, the only difference is the volume of sorbent. The sorbent that is used in this method is a piece of fused silica fiber coated with polymeric stationary phase that is placed on a syringe (39). Using this method also has another advantage that it can be selective (50). When compared with the similar extraction methods, SPME has better detection limit, low cost, time (5 minutes), solvent use (none), and precision (< 1-12% RSD). It has a limitation that it reduces concentration capacity due to the small volume of polymer coating on the fiber. Mitani et al. determined some compounds such as genistein and daidzein by using on-line in-tube SPME-LC-DAD UV in soybeans (51). It is a very simple method that almost anyone could perform.

Ultrasonic Liquid Processor

In this method the sample is transferred to a glass tube and required amount of solvent is added. To undergo extraction the microprobe is injected into the tube at room temperature for 30 seconds using an ultrasonic liquid processor (52). It is often used to disrupt the cell

membranes and release the contents from the cells. This process has some advantages include higher throughput, reduced processing time, and lower energy consumption.

Techinques for Separation and Identification of Flavonoids

In earlier times, thin layer chromatography (TLC) was used for flavonoids analysis. Separation, quantification, and identification of flavonoids can be done in a single step by coupling high performance liquid chromatography (HPLC) to mass, ultraviolet (UV), and nuclear magnetic resonance (NMR) detectors. Presence of phenyl ring in the structure has immense benefit for identification of flavonoids. Some of the common methods that are used for separation of flavonoids are chromatographic methods and electrophoretic methods.

Chromatographic Techniques

Thin Layer Chromatography

Thin layer chromatography (TLC) is commonly used for separation and determination of flavonoids because it is inexpensive, with short separation time, and highly convenient. It however lacks quantitative precision, sensitivity, and separation efficiency when compared with column separation. This technique involves movement of a mobile phase by capillary action through a thin layer of sorbent. The separation of sample results from the differences in migration of sample components in the direction of the mobile phase travelled. It is termed as retention factor or retention index (R_f), R_f is given as

$$R_f = \frac{\text{distance travelled by sample from origin}}{\text{distance travelled by solvent from origin}} \quad [1]$$

A number of samples and standards are analyzed parallel on a single plate by using this method. Harsh separation conditions that destroy analytical method can be done with this method because TLC plate can be used only once (53). Generally, the crude extracts

contain mixtures of many different compounds of flavonoids and isocratic separation cannot separate those compounds satisfactorily. To resolve this problem a mixture of mobile phases or double-development (2D) TLC are used for separation of flavonoids. UV light or densitometer is used to detect these compounds at 350-365 or 250-260 nm wavelength.

Based on the quality of absorbent layer and sample application, the conventional TLC technique has been improved and changed to high performance thin layer chromatography (HPTLC). This new technique is more rapid, sensitive, and efficient than conventional TLC (54). HPTLC has some advantages over HPLC. The mobile phase in HPTLC does not limit selection of detector because the solvent is evaporated between the measurement and development so that it does not affect the detection process. For HPLC, this does not apply because UV absorbing solvents cannot be used with UV detector (54). Analysis of samples in HPTLC is simultaneous, but in HPLC it is sequential due to the nature of method development (54). Both the aglycones and glycoside flavonoids were determined in Vaccinium myrtillus and V.vitisidaea (Ericaceae) by using TLC and densitometry at 254 nm (55). HPTLC method was recommended for use in routine quality control of onion and its formulation due to its specificity, sensitivity, good precision, and accuracy.

Gas Chromatography

Gas chromatography (GC) is used in the separation of volatile compounds. It separates the components by partitioning them between the mobile phase and a stationary phase. The mobile phase is an inert gas known as the carrier gas that flows through the column continuously. The stationary phase may be a liquid or solid (56). The commonly used carrier gases are helium, nitrogen, and hydrogen. The choice of the gas depends on the desired separation efficiency, speed, and detector (57).

The stationary phase is a high boiling phase. The liquid stationary phase is used in gas-liquid partition chromatography in which the liquid is non-volatile and is bonded to the inside of the column. If the stationary phase is solid, it is used in gas-solid adsorption chromatography in which the analyte is adsorbed on solid particles. The components that constitute a gas chromatographic instrument are carrier gas supplier, injector, a thermostatted oven, a detector and a data processor (54). When the volatile sample is injected into the heated port through the septum, the sample evaporates and passes through the hot column by carrier gas over the stationary phase that separates the components of the sample. The separated components flow through the detector and results are recorded by the recording device (57). Usually the columns are of packed or open tubular columns. Open tubular columns are widely used and give better separation efficiencies and good sample detection over packed columns (54). Other advantages include greater sensitivity and shorter analysis time (57). The disadvantages of this column include lower sample capacity and poor operation technique (54).

Gas chromatography gives excellent resolution. It also has mass identification capacity of a mass spectrometer that is used as a detector (58). Because of this reason gas chromatography-mass spectrometry (GC-MS) is widely used for characterization of volatiles in allium plants. To analyse flavonoids glycosides that are more polar needs high temperature-high resolution (HT-HR) gas chromatography with columns that can withstand temperatures to about 400^{o}C temperatures (59). This technique gives faster separation, easily coupled with a wide variety of detectors, high resolution, and does not require solvents. This high resolution gas chromatography (HRGC) is coupled with mass spectrometry to analyze the flavonoids and natural products also.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is used widely and is a powerful technique in separation and analysis of various natural compounds such as flavonoids (aglycones and glycosides) since 1970s. Its advantages include simplicity, sensitivity, specificity, high resolution, speed, precision, good reproducibility, recovery, accuracy, and sample preservation (non destructive) (59). This technique also allows simultaneous separation and quantification of flavonoids. To obtain adequate resolution, a wide range of stationary and mobile phase combinations have been used for the separation of flavonoids in a mixture. Silica gel columns are used in normal phases for appropriate separation of nonpolar or weakly polar flavonoid aglycones. Usually no gradient is employed and therefore different classes of flavonoids in plants are separated by using reversed phase HPLC (RP-HPLC) (59). The common HPLC instrument includes C18 column, a solvent delivery system, sample injection, detector, and recorder. The column is selected based on the class of flavonoids to be separated and the characteristics of the stationary phase that is capable of getting satisfactory retention, peak shapes, and selectivity. Guard columns are used to extend the life time and to protect the analytical column from sample impurities. Compounds are identified by using retention times or other means. The advantages of this method include speed, ease of use, and specificity.

Isoflavones found in Leguminosae plant kingdom are successfully analyzed by HPLC on C18 column (60). Berente et al, compared the reversed phase columns (C18, C12, and phenyl-bonded) to separate 20 wine anthrocyanins, which also include mono and di glucosides, acylated derivatives (61). Flavonols, flavones, flavanones, isoflavones, anthrocyanidins, catechins, and their respective glycosides were determined by using HPLC (60). Flavonoids like quercitin and isohamnetin glycosides were analyzed in onions by Lee

and Mitchell using LC-(ESI) MS/MS and HPLC (62). Rodriquez and co-workers analyzed the total flavonoids and phenol content in onion cultivars by using HPLC coupled with diode array detector (63). Zielinska et al. determined quercetin and its glycosides such as quercetin-3, 4′-diO-beta-glucoside, quercetin-3-O-beta-glucoside, and quercetin-4′-O-beta-glucoside from the onion bulbs by using HPLC with amperometric detection (64). In 2009, Malene employed extraction methods (like pressurized liquid extraction) on freeze dried onions and HPLC with UV detection to quantify seven flavonoids (65). Using the reversed phased HPLC for quantitative determination of flavonoids in onion reported the overall recoveries of 97.1%-102.3%, precision to be less than 6.14% with accuracy above 89.11%.

Electrophoretic Techniques

Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique that has high efficiency for separation. It has short analysis time, requires small sample, and uses much less reagents. Separation of flavonoids is due to the differences in electromigration between the analytes, at a given electric field. Severel modes of this technique are used for separation of analytes, but the commonly used methods are capillary zone electrophoresis and micellar electrokinetic chromatography (59).

In capillary zone electrophoresis (CZE), the separation is based on charge to mass ratio that determines the analyte electrophoretic migration time (59).

Micellar electrokinetic chromatography (MEKC) is the combination of electrophoresis and chromatography. In this method the distinction is made between the charged and neutral analytes (59). Electrophoretic mobility of flavonoids is determined by the phenolic hydroxyl group that is present in the flavonoids and that can be ionized (59).

Analysis of phytochemicals has been well documented by using CE (66, 67). CE seperation of flavonoids is possible as they are negatively charged at high pH values (66, 68). Method validation for CE in quantitative aspects had been reviewed by Suntornsuk (68). Petersson and his group had reported their analysis of anthocyanins in red onion by using capillary electrophoresis and mass spectrometry (69).

CHAPTER 3

EXPERIMENTAL METHODS AND PROCEDURES

In this chapter, the methods that were used in the project are presented. It focuses on the instruments that were used, the chromatographic process, and their advantages in determination of flavonoids.

High Performance Liquid Chromatography Process

High performance liquid chromatography (HPLC) has a liquid mobile phase and a stationary phase. It applies high pressure to move the solvent through a column. The solution moves through the column due to some specific physical and chemical interactions with the stationary phase that is present in the column occur (70). The column contains small size particles bonded with different stationary phases. These smaller size particles create high backpressure. Solvent delivery system, injection system, a column, a detector, a controller, and a recorder are used to run this process. Unlimited supply of solvent, a high resolution stationary phase, fine porous pack materials, desirable flow rate of solvent, and high pressure pump are required to get good chromatographic resolution. The solvent should have low viscosity (71). Solvents that are commonly used include any miscible combinations of various organic liquids (such as methanol and acetonitrile) or water. HPLC grade solvents are used to avoid degradation of column by impurities. Helium gas is used to eliminate gas bubbles by degassing the solvent (57, 58). In the detector the solvent pressure is reduced to the atmospheric pressure. So in the detector it is essential to prevent degassing to avoid spikes and baseline drifts in the chromatogram. Valve injection is better for injecting the samples instead of syringe injection (71). After the sample is injected, it is transported

through the stationary bed by the mobile phase. The separated components pass through the detector and record the chromatograms (71).

Isocratic Verses Gradient Elution

Isocratic elution was brought into existence by Csaba Horvath. It means that the composition of mobile phase remains constant throughout the separation (70). It is of low assay cost, with increased reliability, good reproducibility of chromatographic data, simple and convenient to use. As the mobile phase composition is constant throughout the run, the column remains in equilibrium during the sample separation, so the next sample can be injected continuously as soon as the previous sample has eluted. In isocratic elution the peaks elute in the same order and selectivity does not change even if the dimensions of the column changes. Isocratic elution is commonly used for routine work among many other assays (72).

Gradient elution is described as the change in the mobile phase composition during the separation process. The isocratic method is ineffective when the components in the sample have widely different polarities and results in poor resolution. Under these conditions gradient elution is preferred. The mobile phase in gradient elution has two components typically termed as “A” and “B”. Solvent “A” is the weak solvent that moves the solute slowly and solvent “B” is the strong solvent that moves the solute rapidly through the column. In reverse phase chromatography, most often water or an aqueous buffer is used as solvent “A”, and organic solvent (acetonitrile, methanol, isopropanol) miscible with water is used as solvent “B” (70). The retention of later-eluting components decreases so that they elute faster, giving narrower peaks for the components. In this assay the elution order may change if the dimension of the column or flow rate changes. Some of the problems affiliates with gradient assay include solvent demixing and drifting baselines. Artifactual peaks are

also observed due to UV-absorbing impurities in the mobile phase which results in appearance of peaks but do not confirm with the sample bands during separation. Longer time is required for re-equilibration of the column after gradient elution (72).

Normal Verses Reversed Phase HPLC

Normal phase HPLC (NP-HPLC) or adsorption chromatography separates sample components based on adsorption to a stationary surface chemistry and by polarity. It has a polar stationary phase and a non-polar, non aqueous mobile phase. This works more efficiently for separating analytes that are readily soluble in non-polar solvents. If the mobile phase solvents are more polar, the retention time of the analyte decreases. But if the solvents are hydrophobic, retention time increases. Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous polar mobile phase. It exactly refers to the reversal of the normal phase HPLC. In RP-HPLC the commonly used stationary phase is silica treated with RMe_2SiCl, where R is a straight chain alkyl group such as C_8H_{17} or $C_{18}H_{37}$. RP-HPLC can separate a broad spectrum of ionic and non-ionic compounds by the use of ion pairing (also known as ion interaction). This technique is known as reversed phase ion-pairing chromatography. RP-HPLC columns are stable and separations are reproducible because of the chemically bonded stationary phases. In addition, because of the weak surface energy of stationary phases, the re-equilibrium time decreases and analyses are rapid. It can also be used to determine the complexation constants, dissociation constants, and hydrophobicity. RP-HPLC technique has a limitation that silica based column packings limit the pH range between 2-7.5. The pH of the mobile phase also has an important role on the retention time of the analyte and also changes the selectivity of certain analytes. The retention mechanism is more complex than the other forms of chromatography and better understanding is needed to control it (54).

HPLC Columns

In HPLC the column is an important component in the separation of components in a sample. Reversed phase columns are composed of a stationary bed that is strongly non-polar in nature. Usually narrow columns are used because small size samples are analyzed. The column is packed with rigid and small particles with narrow particle size distribution (54). So far the most commonly used column particles in HPLC is silica (57). However, silica is ineffective at high pH because it is soluble in base (54). These silica based column are more compatible with organic and aqueous mobile phase solvents and can withstand high pressures. The term theoretical plate is used to express the column efficiency and it is defined as the measure of the broadening of a peak while passing through the column (73). As the particle size decreases, the efficiency of the packed column increases with an increase in number of the plates, decrease the run time, and better detection limit if the size of the particle is small. However, if particle size is small, it leads to high pressure.

Particle size that is commonly used today in HPLC columns is 3-5µm (57). The temperature of the column must be controlled in HPLC. If the column is heated, usually the viscosity of the solvent decreases and thus allows the solvent to flow faster which decreases the retention time, and improves the resolution by accelerating mass transport of the solutes. But, high temperatures degrade the stationary bed and therefore the life time of the column suffers (54).

Detector Used in HPLC

In HPLC detectors produce a signal that depends on the concentration of the sample. However, chromatogram includes noise as the detectors detect components over the background of signal and sample matrix signal (73). Another parameter of importance to the

detector is the minimum concentration of sample can be detected by the detector with a given confidence level and this is referred to as the detection limit. The noise of the detector may come from instrument electronics, the temperature fluctuation, changes in the flow rate, and changes in the output signal due to the pulse from the pump. Some other properties that are considered for a good detector are operation high sensitivity, low drift, fast response, simple and reliable operation, and minimal peak broadening (74).

Detectors are classified into two different categories: the general detectors and selective detectors. General detectors are also known as bulk property detectors, and selective detectors are also known as solute property detectors. Selective detectors are independent of the mobile phase and they respond to a chemical or physical property of the solute. Examples of selective detectors are electrochemical, fluorescence, and spectrophotometric detectors. General detectors are used to compare the difference in the mobile phase alone and some physical property of the mobile phase. Examples of these detectors include conductivity, refractive index, and dielectric constant detectors (57, 74).

Differential refractometers are used to measure the changes in the refractive index of the eluent. It is able to detect concentrations of 10^{-5} to 10^{-6} g/mL. These detectors are not used with gradient elution due to the change in the base line and also with the solvent that has a refractive index close to that of the solute. It is highly sensitive to temperature change.

UV detectors are not temperature sensitive. They have good sensitivity and are able to detect the concentrations down to 10^{-8} g/mL. These detectors can be used with gradient elution and when compared with other detectors they are relatively inexpensive. It has an advantage that it is sensitive to most of the organic compounds. However, it cannot be used to detect a sample component that do not absorb in the UV region, and a mobile phase solvent that absorbs in UV region.

Diode array detectors have an ability to perform spectroscopic scanning and also accurate absorbance readings at a variety of wavelengths. The source is dispersed by grating to a photodiode array for detection. Absorbance is measured at more than one wavelength and ratios are calculated for two selected wavelengths by using this detector. Absorbance ratio can be calculated by measuring the absorbance simultaneously at several wavelengths.

UV-Vis detectors are the most commonly used detectors in HPLC and they belong to the selective detector category. These detectors are based on ultraviolet and visible spectrophotometers. This detector follows the Beer Lambert Law that relates absorbance and the concentration of a component for its operation. Absorbance that is observed only depends on the concentration of the analyte because the absorptivity and the path length for a specific compound remain constant in a given detector. Good selectivity, high sensitivity, and easy operation are some of the advantages of UV-Vis absorbance detector. The detector is universal and has low background with different HPLC solvents, allowing gradient elution with low background drift. In using UV-Vis detector at a given wavelength, the absorbance measured varies based on the molar absorptivity of the molecules. Limitation of the UV-Vis detector is that below the UV wavelength cut off of the solvent the detector will not operate (69). It is more sensitive than refractive index detector but is less sensitive than the mass spectrometer.

Other detectors also used in HPLC are amperometric and fluorescence detectors. Amperometric detectors are useful for detecting eletroactive substances. The concentration of the species generating the current is directly proportional to the current. These detectors are sometimes used in HPLC for analyses of organic components and they also have wide biological applications (75). Fouling of the electrodes is the major problem with these

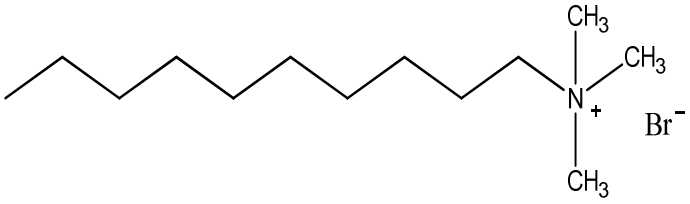
detectors and this can be solved by cleaning the electrodes and by submitting the working electrode to a series of strong anodic and cathodic pulses.

Fluorescence detectors are used exclusively in liquid chromatography. It is a specific detector that senses only substances that are fluorescent. It is the most sensitive HPLC detector among the existing modern detectors. These detectors have greater sensitivity to sample concentration but less sensitivity to instrument instability. They also provide good selectivity over the UV absorption detectors (73).

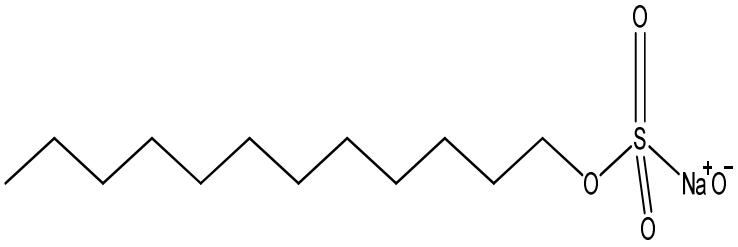
Use of Surfactants in HPLC

Surfactants are the compounds that reduce the surface tension of a liquid, the interfacial tension between a liquid and a solid, or between two liquids. They enhance the solubility of the sample and prevent aggregation (73). Usually surfactants are organic compounds that are amphiphilic in nature. It means that they contain a hydrophobic group that is on the non-polar end and a hydrophilic group that is on the polar end. Therefore, the molecule contains both a water soluble and a water insoluble component. Based on the charge that is present on the hydrophilic group the surfactants are categorized into anionic, cationic, amphoteric, and non-ionic compounds (76). At interfaces, surfactants have an ability to adsorb on to an aqueous solution. When the surfactant adsorb on to the hydrophobic surface, they normally expose the hydrophilic group towards the water that makes hydrophilic surface. The surface tension has been reduced between the surfaces (77). While separating the compounds by HPLC, the surfactants are added to the mobile phase. They reduce the surface tension and the viscosity of the mobile phase but increase the diffusion coefficient and eluent strength causing band width reduction (73). Deming and Tang (78) studied the effect of surfactants in reversed-phase chromatography. They reported that addition of surfactant to the mobile phase reduces the interfacial tension and therefore

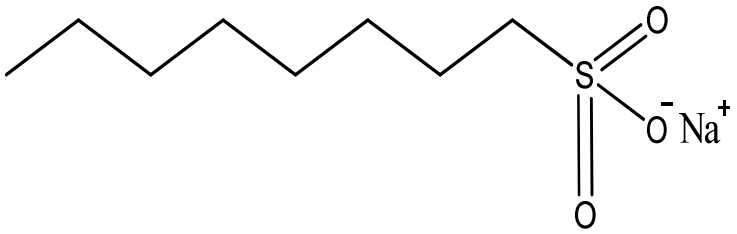
decreases the retention time of the injected sample. Surfactants should be used with caution because they bind strongly on the walls of the column and consequently may damage it (73). Some of the surfactants that are commonly used in high performance liquid chromatography are shown in Figure 4.



(Decyltrimethyl ammonium bromide)



(Sodium dodecyl sulfate)



(1-octanesulfonic acid)

Figure 4: Examples of surfactants that are commonly used in determination of compounds using HPLC.

Gas Chromatography Process

Gas chromatography (GC) consists of a carrier gas as the mobile phase and the microscopic layer of liquid or polymer on an inert solid support as its stationary phase. Helium or an unreactive gas such as nitrogen is used as the mobile phase. A specially designed instrument is used to run this process. Its basic units are a carrier gas flow controller, injection port, column, detector, and recorder. Small amount of sample (liquid mixture) is injected into the instrument. This mixture evaporates in the hot injection chamber. Then the stream of inert gas sweeps out the vapor into a heated column that has the stationary phase (high boiling liquid). In the column the components move back and forth between the gas phase and liquid phase at different rates and thus separate the pure components. These components are detected by the detector and thus send an electronic message to the recorder for recording the chromatogram. The carrier gas should have a desirable flow rate, because it influences the flow of components through the column. If the flow rate is faster then it reduces the retention time. Generally, the flow rate is kept constant throughout the run. This technique shows poor selectivity and poor sensitivity unless coupled with mass spectrometer. In GC, the efficiency of separation depends on the compounds travelling through the column at different rates. The rate depends on the volatility of the compounds, polarity of the compounds, column temperature, column packing polarity, flow rate of the gas, and the length of the column.

Detector Used in GC

Components of a mixture are separated and must be detected as they exist from the GC column. The detector interacts with the solutes and all the interactions are converted into an electronic signal. This signal is sent to the data system. To generate a chromatogram the magnitude of the signal is plotted against time. Flame ionization and thermal conductivity

detectors are the most commonly used detectors for commercial gas chromatographs. The selection of the detector depends on the separation application. GC detectors are far more sensitive than the LC detector because detection of vapors in gases is easier than the detection of solutes in a liquid. Different types of selectivity are given by using different detectors. A non-selective detector does not respond to the carrier gas but responds to all other compounds. A range of compounds with common chemical and physical properties are determined by selective detectors. Specific detector reacts to specific compounds. Concentration dependent detectors are the detectors where the signal is related to the concentration of solute in the detector and usually the sample is not destroyed. Mass flow dependent detectors are the detectors that usually destroy the sample and the signal depends on the rate of solute molecules entering the detector. Some of the commonly used detectors are mass spectrometer, flame-ionization detector, nitrogen-phosphorous detector, electron-capture detector, and thermal conductivity detector.

Mass spectrometry (MS) is used to determine the structural and chemical information of the molecules. It is also used for quantitation of molecules and atoms. The spectrometers separate the molecules or atoms from each other by using the difference in mass-to-charge ratio (m/e) of ionized molecules and atoms. It is generally operated by creating gas-phase ions from the eluent that exit from the column. These ions are separated in space or time based on the mass-to-charge ratios and then the quantity of ions are also measured. The ion separation power of MS is explained by resolution and it is defined as

$$R = m / \Delta m \qquad [2]$$

where, m is the mass of the ion and Δm is the difference in the mass between the two resolvable peaks.

GC-MS is a tool of choice for any experiment to confirm the identification of the components. It has good selectivity, good sensitivity, high degree of standardization, specificity, instrument ruggedness, and sample throughput. Generally, GC-MS runs in electron ionization –single ion monitoring (EI-SIMs) mode and sometimes in chemical ionization (CI) monitoring mode. EI-SIMs is usually used because it is a good compromise between sensitivity and selectivity (79). CI is more sensitive than EI, but EI is still preferred. Some of the advantages of GC-MS over other techniques include small sample size and the ability to determine multiple components during one run (80). GC-MS has one shortcoming in that it may require derivitization of the compounds that are extracted and introduce another step with the associated liabilities (81).

Flame-ionization detector (FID) is another common GC detector. Anlaysis with this detector involves the detection of ions formed in flame. From the GC column the eluent passes through the hydrogen/air flame. It produces ions by breaking down the organic molecules. All the ions are collected on a biased electrode and produce the signal (82). Within a dynamic range these detectors are extremely sensitive. They are insensitive to H_2O, SO_2, CO_2, CS_2, CO, NO_x, and noble gases because they cannot be ionized/oxidized by flame. It has a disadvantage that it destroys the sample completely. It does not detect compounds that contain carbon-hydrogen bonds.

Electron capture detector (ECD) is used to detect electron absorbing components via electron capture ionization in the output stream of gas chromatography. It uses a radioactive beta particle emitter to oxidize the carrier gas and produce a current. The current is produced between a biased pair of electrodes. If the organic molecules contain electronegative functional groups, such as nitro and phosphorous groups that pass through the detector, they reduce the current between the electrodes by capturing less number of electrons. Depending

on the concentration of the sample, ECD is 10-1000 times more sensitive than FID and million times more sensitive than TCD. It has a limited active range. It has great application in determining halogenated compounds (83).

Thermal conductivity detector (TCD) is a detector that is commonly used in gas-liquid chromatography. It senses the changes in the thermal conductivity of the column sample and compares the result to a reference flow of carrier gas. It consists of a thermistor or an electrically –heated filament in a temperature controlled cell. The sensing element temperature depends on the thermal conductivity of the carrier gas flowing around it. TCD is less sensitive than other detectors and also have a larger volume that does not provide good resolution. It is non-destructive and non-specific. TCD is known as an universal detector because it detects all compounds such as organic and inorganic compounds and also permanent gases such as oxygen, nitrogen, and carbon dioxide.

Columns Used in GC

In GC there are two major types of columns, conventional packed columns and open tubular columns or capillary column. Packed columns contain a solid support material that is finely divided and inert and is coated with a liquid stationary phase. These columns have an internal diameter of 2-4 mm and length of 1.5-10 m. These columns are commonly used for gas analysis. Capillary columns consist of a capillary tube, and the walls of the column are coated about 0.2 μm to 1 μm as a film with the liquid stationary phase. These columns have an internal diameter of 100 μm to 500 μm and length of 10 m to 100 m. The selection of the column depends on the polarity of the sample. Capillary columns provide better separation efficiency than packed columns, but they are easily overloaded by large amount of sample. For a better resolution, reduced run time, and good separation of components, the polarity of the sample should closely match to the polarity of the stationary phase. The temperature of

the column is precisely controlled electronically and it depends on the sample's boiling point. In some methods the temperature is maintained the same throughout the analysis and the method is known as isothermal. But, in most of the experiments the temperature of the column increases during the analysis, rate of temperature increase, and the final temperatures are varied. This method is known as a temperature program. If the column is exposed to higher temperature for longer time, its lifetime is reduced.

In GC, another important factor that is to be considered in columns is the material from which it is made. Typically these columns are made up of stainless steel, glass, or a silicate glass such as borosilicate glass or aluminium silicate glass. Some other factors considered are the operating pressure and flow rate of carrier gas in the column.

Resolution of Bands

The quality of chromatographic separation can be determined by the extent to which two bands are distinguished from each other. This is referred as resolution. Quantitatively it is defined as:

$$R_s = 2(t_2-t_1) /(W_2-W_1) \qquad [3]$$

where t_1 and t_2 are retention times, and W_1 and W_2 are the peak widths measured at their respective retention times. Bands that are totally separated have big R_s values. Resolution of the band depends on the selectivity, efficiency of separation, and retention. Selectivity refers to the discriminating power of the stationary phase that produces differential interaction for at least two compounds. Retention refers to the ability of a molecule to interact with the stationary bed or the affinity of the molecules for it. Efficiency refers to the movement of the sample through the column and generates the separation chromatogram. These factors are

$$R_s = \sqrt{N}/4 \, [(\alpha - 1)/\alpha] \, [(1+k_1)/k_1] \qquad [4]$$

where ($\alpha - 1$) is the selectivity term, N is the efficiency and k_1 is the retention factor of the solute (73). This retention factor term is used to describe the migration rate of an analyte on a column.

Method Development

In chromatography method development is to establish an analytical procedure that will be appropriate for the analysis of a particular sample. New methods may be developed due to poor existing procedures or a method to examine a new analyte. Most commonly new ones are developed from similar methods used in the literature or from any existing methods (73). It may be done by a trial and error process. This approach is time consuming and it is inefficient. Efficient method development needs extensive practical experience and good knowledge of chemistry of the analyte. All methods are developed based on the properties of the analyte and narrow down the optimum conditions suitable for a particular analyte determination (54). The first step that is done for developing a new method is varying the mobile phase composition with the aim of obtaining a reasonable run time, easily detecting narrow bands, and good resolution. The next steps include sample preparations, mobile phase pH, changing the column, adding any surfactants, and changing the wavelength of detection (84). The success of the new method is evaluated by confirming its parameters such as accuracy which gives the correct numerical answer for the analyte, and specificity which is the ability of the method to measure accurately the analyte response in the presence of all potential sample components, precision measurements, limits of detection, and robustness that measures the capacity of a method to remain unaffected by small but deliberate variations in method parameters (73).

Research

In Chapter 1, we discussed the history, botany, and taxonomy of onions from the literature. From the chemical composition of onions, mainly flavonoids have advanced throughout the history. Scientists and researchers have identified some of the flavonoids present in onions and their importance in health. These onions are grown commercially and in the wild also. The literature reviewed mentions that flavonoids present in these onions are extracted by using different extraction methods and identified by using different techniques. A simpler and reliable extraction method is required. The cost and availability of the materials should be within one's budget. Also a simple but efficient HPLC and GC methods for separation of flavonoids is desirable.

The objectives of this research project are outlined below:

1) To establish a simple but effective extraction procedure to extract flavonoids from different varieties of wild and commercial onions.

2) To find out the best HPLC mobile phase and GC-MS operating parameters for efficient separation of flavonoids in these onions.

3) To determine the distribution of flavonoids in different varieties of onions by using HPLC and GC-MS.

4) To use statistical methodologies to determine if there are significant differences among the varieties of onions.

CHAPTER 4

EXPERIMENTAL PROCEDURES

In this chapter all the reagents, samples, standard solutions, instrumentation, and data analysis used in this research work are presented. The preparation of different samples and experimental procedures for analyzing them are explained in detail. The parameters of HPLC and GCMS procedures along with the statistical methodologies are also given.

Reagents

1. Optimum grade methanol, hydrochloric acid, both ACS certified, were obtained from Fisher Scientific (Fair Lawn, NJ).
2. Teritary Butyl Hydroquinone (TBHQ) was obtained from Spectrum Chemical, Gardena, CA.
3. Sodium dodecyl sulfate (SDS), decyltrimethylammonium bromide (DTAB) and 1-octane sulfonic acid, quercetin-3-β-D glucoside were obtained from Sigma Chemical Company (St.Lousi, MO).
4. Myricetin and quercetin dyhrate (99%) were obtained from Acros Organics, NJ.
5. Isorhamnetin was obtained from Indofine Chemical Company, Hillsborough, NJ.

Onion Samples

All different types of commercial fresh onions were purchased from different stores in Johnson City, Tennessee. Natural wild fresh onion samples were obtained from a backyard garden.

Varieties of fresh onion used are given below:

1. Wild Onion - obtained from the backyard garden in Johnson City, TN

2. Yellow Onion – bought from the Walmart store in Johnson City, TN
3. Organic White Onion – bought from the Earthfare store in Johnson City, TN
4. Organic Red Onion – bought from the Earthfare store in Johnson City, TN

Procedures

Preparation of Solutions

The following standard solutions were prepared:

1. 6 M hydrochloric acid: 216 grams of HCl were diluted in a 1000 mL volumetric flask with distilled water.
2. Extraction solution mixture: 80 mL of methanol and 20 mL of 6 M hydrochloric acid was used as solvent mixture.

Preparation of Onion Stock Samples

The extraction solvent mixture was used for extracting the flavonoids from onions. The flavonoids compounds in onions are often polar, which dissolve better in methanol and HCl mixture. TBHQ is used to prevent oxidation. The extraction of onion flavonoids was carried out by the procedure given below:

- A few onion bulbs from each variety were taken and cleaned to remove all contaminants.
- Any damaged or ruined sections of the onion bulb were cut off.
- These samples were oven dried at 80^{o}C for 15 minutes to remove the moisture.
- About 2.0 g of each of the dried samples were taken and put it in the round bottom flask separately.
- In each round bottom flask 80 mL of methanol and 20 mL of 6 M HCl (extraction solution mixture) were added.

- To the extraction mixture 0.2 g teritary butyl hydroquinone (TBHQ) was added.
- These onion samples and the extraction mixture were carefully mixed and refluxed at 90^{o}C for 2 hours.
- After refluxing, the extract mixture was cooled and gravity filtered three times using a Whattman filter paper to insure the absence of particles in the solution.
- The solutions were made up to 100 mL with methanol and stored in the amber bottles and frozen until analyzed.

The flavonoids were also extracted from onions by using a sonication method.

- About 1g of fresh, cleaned slices of each onion were taken and placed in the ambered colored bottles separately.
- The samples were crushed in the bottles in helium gas atmosphere.
- 25 mL of methanol was added to the sample and mixed.
- The bottles were closed and were sonicated for about 98 minutes.
- After sonication the mixtures were gravity filtered three times to ensure absence of particles in the solutions.
- The mixtures were made up to 100 mL with methanol and stored in amber bottles and frozen until analyzed.

The sample stock solutions (wild onion, organic white onion, natural yellow onion) were diluted by pipeting 1:00 mL of stock solution into 10-mL of volumetric flasks and made up to volume with methanol. For the organic red onion stock solution, 1:00 mL and 5:00 mL were pipeted into two separate 10-mL volumetric flasks and diluted to the mark with methanol.

Preparation of Standard Stock Samples

Different flavonoids such as isorhamnetin, myricetin, quercetin-3-β-D glucoside, and quercetin dydrate stock solutions were prepared. The quantity of each compound used was different. The stock solutions are prepared as follows:

- Isorhamnetin stock solution: 10 mg of pure isorhamnetin was diluted using 25-mL of volumetric flask with methanol.
- Myricetin stock solution: 25 mg of pure myricetin was diluted by using 25-mL of volumetric flask with methanol.
- Quercetin- 3-β-D glucoside stock solution: pure quercetin- 3-β-D glucoside was diluted by using 25-mL of volumetric flask with methanol.
- Quercetin dydrate stock solution: 0.05 g of 99% quercetin dydrate was diluted by using 25-mL of volumetric flask with methanol.

Subsequently, all the standard stock solutions were diluted by pipeting 1 mL of stock solution into 10-mL volumetric flask and diluted to the mark with methanol.

HPLC Instrumentation

The analyses of flavonoids were conducted on Perkin Elmer (940 Winter Street, Waltham, Massachusetts, USA), Model 410 Series High Performance Liquid Chromatography. It is equipped with a model 235C diode array detector. A reversed phase column, an Alltech (2051 Waukegan Road, Deerfield, Illinois, USA) Econosil C_{18} 10 μ with the dimensions of 250 mm × 4.6 mm was used. The mobile phase was made up of 45% methanol and 55% de-ionized water. The standard solutions and their retention times were used to identify some of the compounds in the chromatographs. All the samples were

injected individually in triplicates via an auto sampler. All separations with HPLC were done with isocratic elution. The parameters for the HPLC procedure are stated in Table 2.

Table 2: HPLC parameters that are used for separation and analyzing the flavonoids

Loop size: 100 µL	Average pressure: 400 -3000 psi
Injection volume: 20 µL	Total run time: 25 minutes
Sampling rate: 2.44 pts/s	UV-Vis detector wavelength: 255 nm
Flow rate: 1 mL/min	Mobile phase: 45% methanol and
Column Length: 250 mm	55% water
Column internal diameter: 4.6 mm	Mode: Auto spectral mode
Column Temperature: 25^{o}C	

A schematic diagram of a typical HPLC system with its basic units is shown in Figure 5.

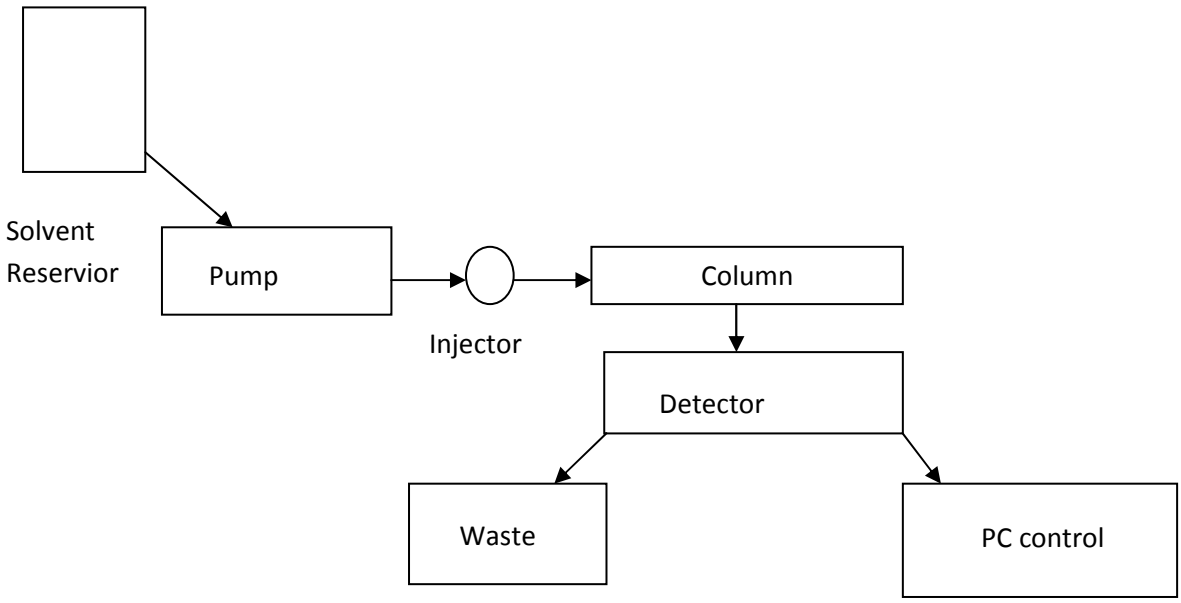


Figure 5: Schematic representation of high performance liquid chromatography

GCMS Instrumentation

The analyses of flavonoids in onions using GC-MS were conducted on a Hewlett Packard (3000 Hanover Street, Palo Alto, CA), Model 5890 Series II Gas Chromatography. It is equipped with a Series 5971A Mass Selective Detector. The column was a J & W Scientific (91 Blue Ravine Rd, Folsom, CA), with dimensions of 30.0 m × 0.53 mm, and 0.25 µm film thickness. The column temperature tolerance is up to 350^{o}C. The stationary phase is made up of 95% diphenyl polysiloxane and 5% phenyl. Most of the volatile compounds have very low boiling points and if the columns initial temperature starts with high temperatures, the compounds will be lost and the chromatogram will be cluttered. All the samples to be analyzed were injected in the GCMS individually. The amount of sample injected was 1 µL and run in triplicates. The parameters used were very important for obtaining a good chromatogram for the analysis of the flavonoids. The parameters of the GCMS are shown in Table 3.

Table 3: GCMS parameters are used for separation and analysis of flavonoids

Column Length: 30.0 m Column internal diameter: 0.530 mm Column Film Thickness: 0.25 µm Flow rate: 1.0 mL/min Pressure: 21 kPa Carrier gas: Helium Solvent Delay: 3 minutes	Initial Temperature: 50^{o}C hold upto 2 minutes Rate of Temperature: 10^{o}C/min Final Temperature: 230^{o}C Total Run Time: 21 minutes Inlet Temperature: 250^{o}C Injection volume: 1 µL

A schematic representation of gas chromatography is shown in Figure 6.

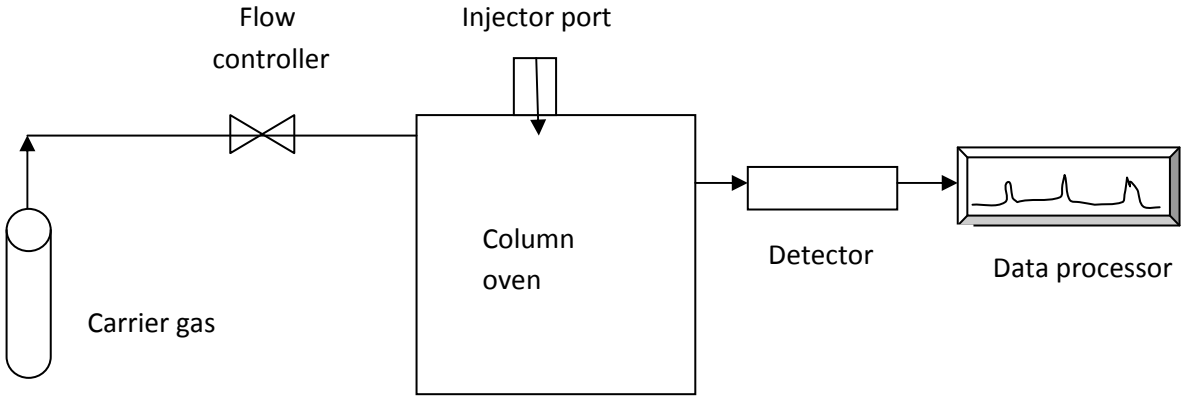


Figure 6: Schematic representation of gas chromatography

Data Analysis

All natural onion samples and standard flavonoids samples were chromatographed on both the HPLC and GCMS. Each of the samples was injected and chromatographed in triplicates. The retention times that were obtained from the chromatograms of each sample were used for statistical results by using Microsoft Office Excel 2007 (Microsoft Corporation One Microsoft Way, Redmond, WA, USA). The data were input into the Excel spread sheet. By using Excel's statistical pack software, the average, the standard deviation, and relative standard deviation of each sample were calculated. The correlation coefficients were also calculated by comparing the retention times and peak areas of all samples to each other. By highlighting the target samples peak areas, then clicking on FORMULAS, MORE FUNCTIONS, STATISTICAL and then CORREL, the correlation coefficients were calculated for all experimental data obtained from GCMS and HPLC.

The data stored on the Excel spread sheet were also used to plot the scatter plots. The average retention times of the natural compounds were compared to the averaged retention

times of the commercial compounds. The scatter plots were plotted by clicking INSERT, SCATTER and SCATTER WITH ONLY MARKERS.

The statistical studies of data were also done by analysis of variance (ANOVA) using Excel. The retention times of the commercial compounds were compared to the natural compounds retention times. This shows if differences seen between the samples were significant or not.

CHAPTER 5

RESULTS AND DISCUSSION

All the extracted samples were injected into HPLC and GCMS to obtain the chromatographic profiles. The retention times versus peak areas were subjected to different statistical analysis. From these results, we will attempt to determine if we have attained the research goals. Figure 7 shows the HPLC chromatograms of the available four standard compounds purchased.

Optimization of Mobile Phase with Surfactants in HPLC

Surfactants have been used to reduce the band widths because they increase both diffusion coefficient and eluent strength (69). Sodium dodecyl sulfate, decyltrimethylammonium bromide and 1-octane sulfonic acid were mixed in the mobile phase to reduce the surface tension and solvent viscosity. Into the mobile phase, 0.05 g of each surfactant was added separately to obtain the chromatograms of samples. But, there was no improvement seen in reduction of the band width. Therefore, adding surfactant to the mobile phase did not help in separation and was thus not pursued in this study.

Chromatograms of Onion Samples

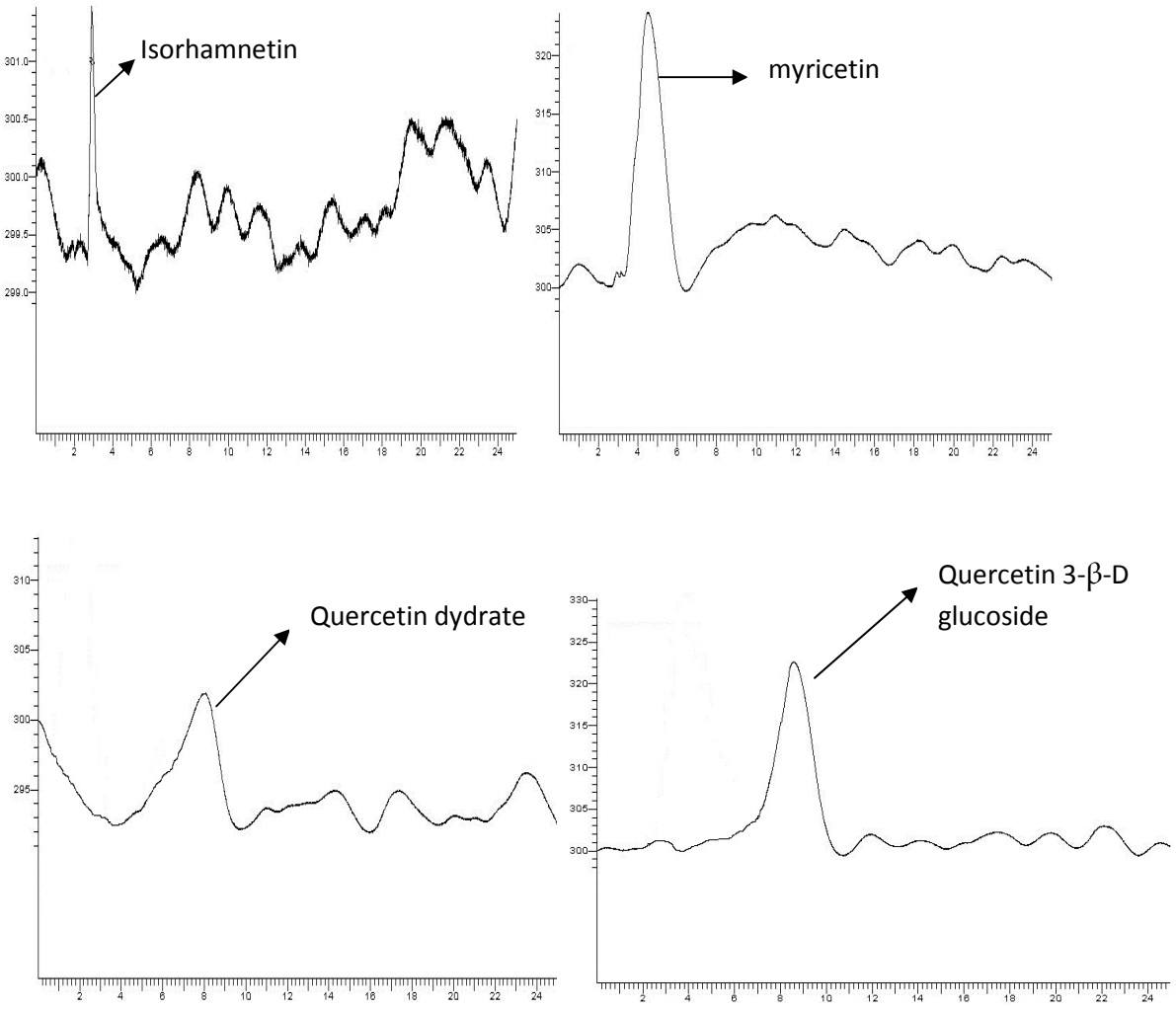


Figure 7: HPLC chromatograms of, isorhamnetin, myricetin, quercetin dydrate and quercetin-3-β-D glucoside samples using 45:55 methanol:deionized water mobile phase. The retention times were identified for the standard compounds from the chromatograms.

The retention times observed on these chromatograms of the standard compounds are used to identify their presence in the chromatograms obtained from the natural and commercial onions. In the Figure 7, the first chromatogram shows the presence of isorhamnetin (retention time of 2.75 min) and from the second, third, and fourth chromatograms the standard compounds myricetin, quercetin dydrate, and quercetin-3-β-D

glucoside were eluted from the column with retention times of, 4.50 min, 7.55 min, and 8.51 min respectively. The chromatograms were somewhat noisy because the sample sizes were small because only small quantities of the pure compounds were purchased as they were costly. However, the chromatograms were of sufficient quality to a certain the retention times.

Figure 8, 9, 10, and 11 are the HPLC chromatograms of different types of onions samples used in this study.

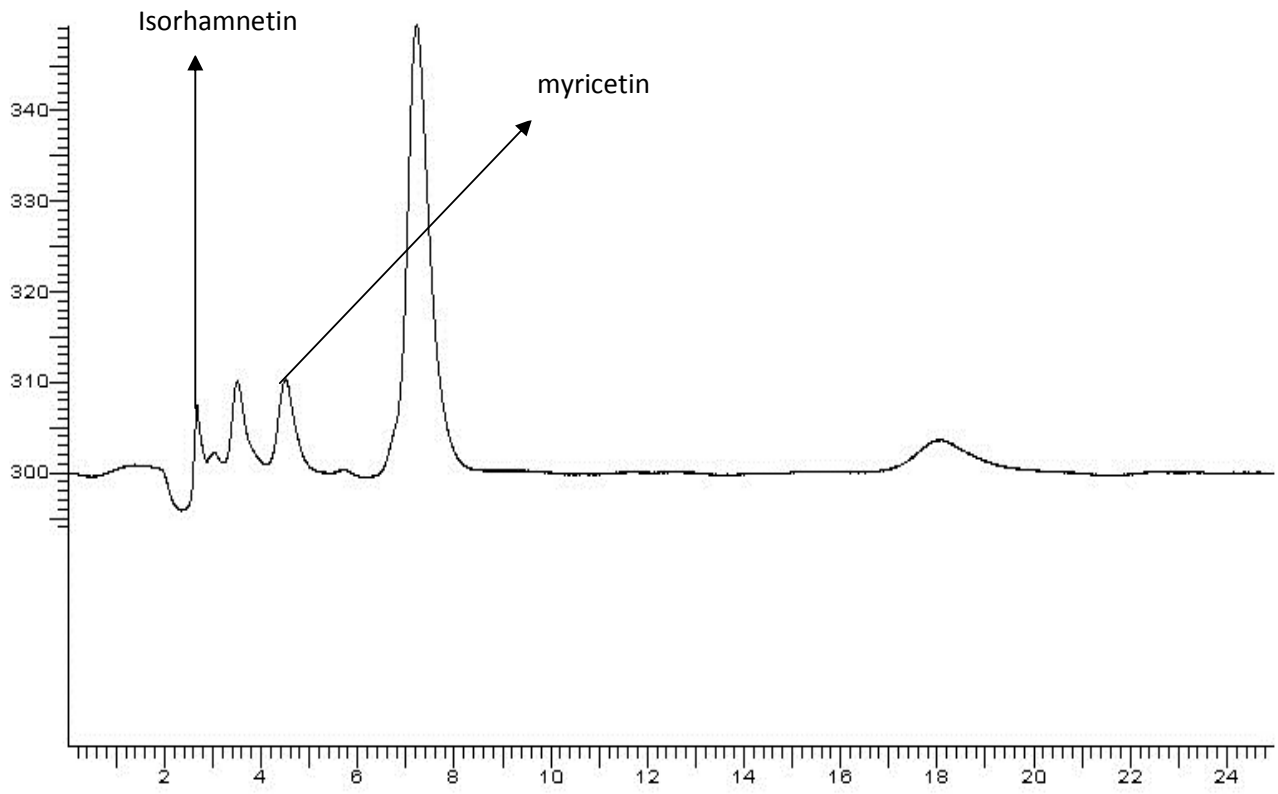


Figure 8: HPLC chromatogram of commercial yellow onion sample using 45:55 methanol: deionized water mobile phase. Based on retention times, the standard compounds that were found in the sample were myricetin and isorhamnetin.

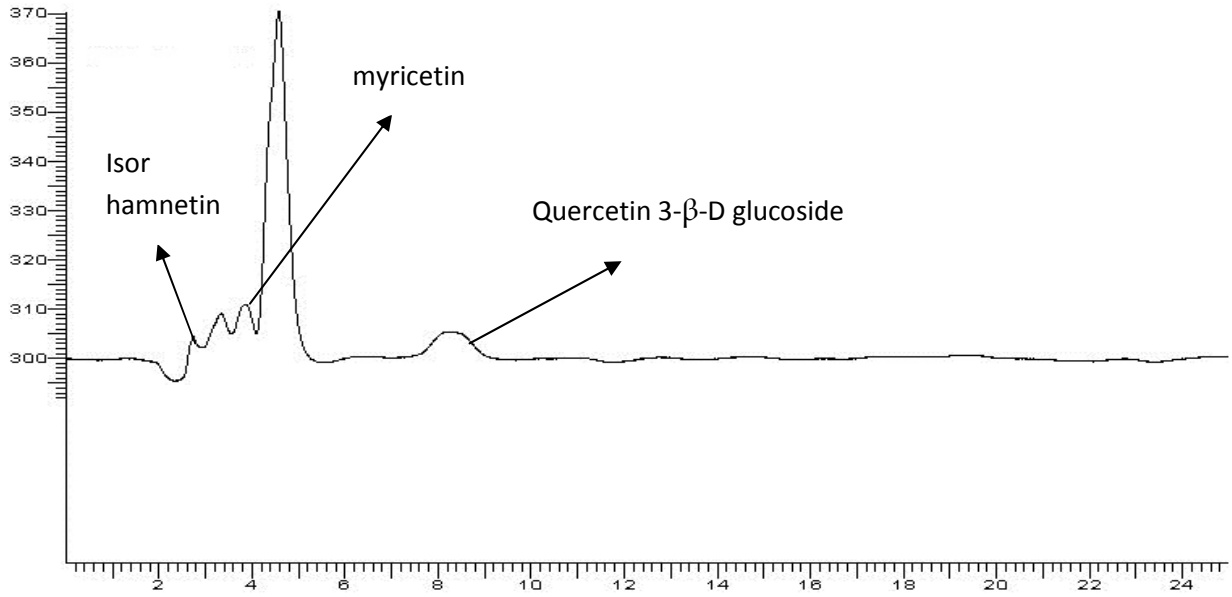


Figure 9: HPLC chromatogram of commercial organic white onion sample using 45:55 methanol: deionized water mobile phase. Quercitin 3-β-D glucoside, isorhamnetin and myricetin were the compounds that were identified based on the retention times of the standard compounds.

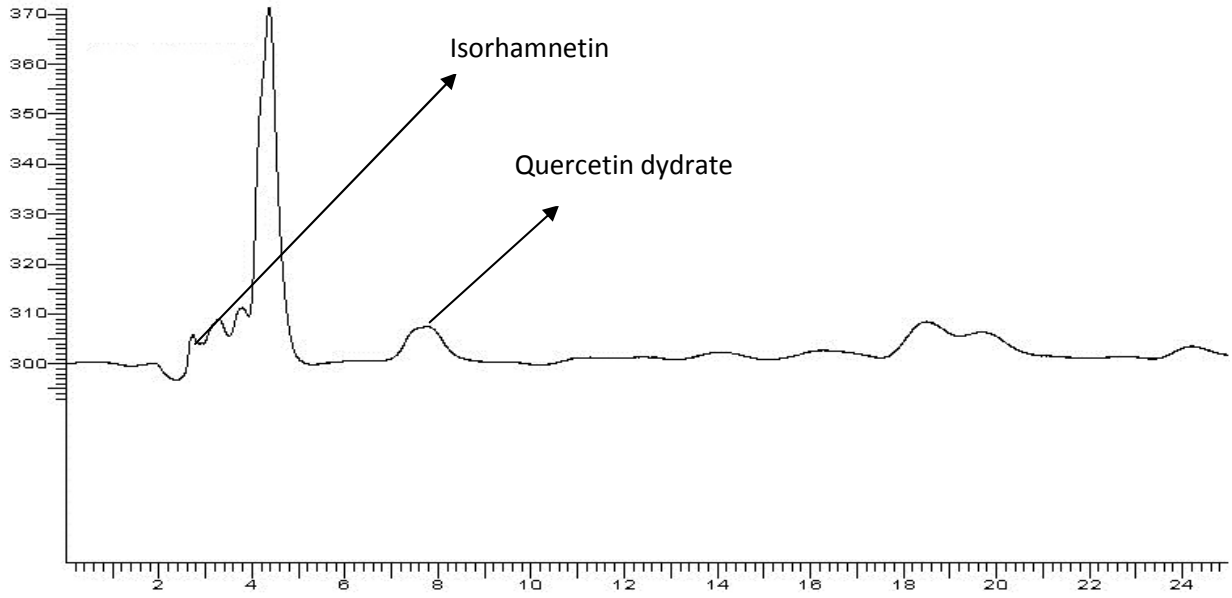


Figure 10: HPLC chromatogram of commercial organic red onion sample using 45:55 methanol: deionized water mobile phase. Quercitin dydrate and Isorhamnetin were the compounds that were observed in this sample when compared with the retention times of the standard compounds.

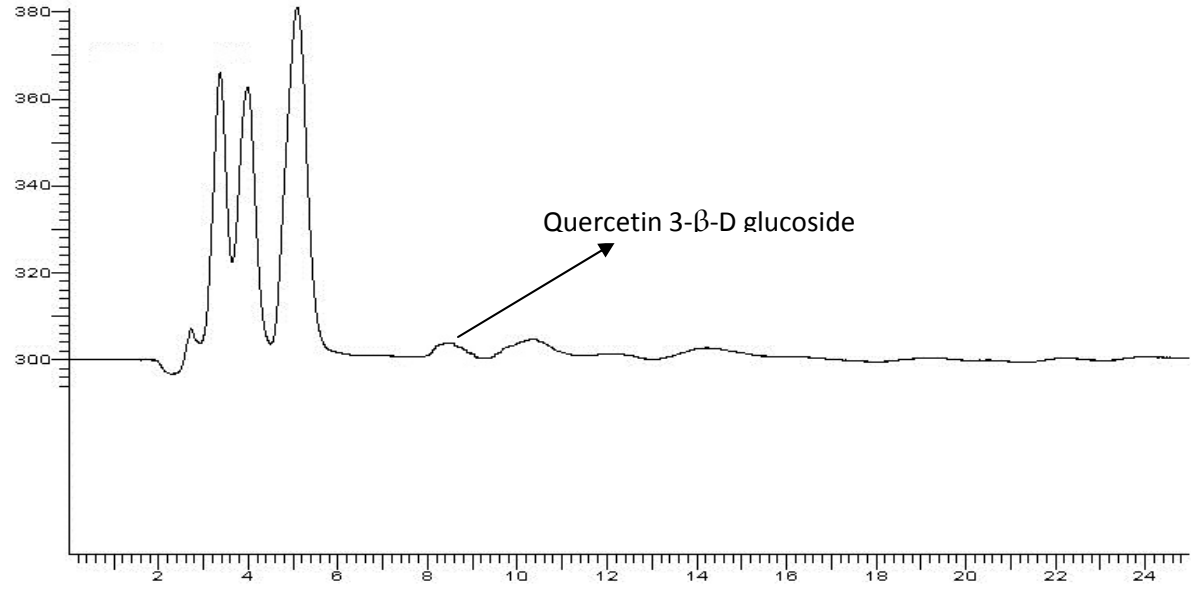


Figure 11: HPLC chromatogram of natural wild onion sample using 45:55 methanol: deionized water mobile phase. Quercetin 3-β-D glucoside was the only compound that was identified in this sample, based on the retention time of the standard compounds.

Using this mobile phase, all the chromatograms obtained were neat and have peaks eluting between 2.0 to 19.0 minutes.

In Figure 8, we can observe that in commercial yellow onion, there are other unidentified compounds present beside the standard compounds available commercially to us. Based on the retention times of the standard samples, we can only find the presence of myricetin and isorhamnetin (retention times of 4.50 min and 2.75 min, respectively) in this sample. The other peaks were not identified. The detector allows one to identify any of the compounds by their retention times only.

Figure 9 is the chromatogram of the commercial organic white onion sample. It has several compounds present also. Three peaks were identified by comparing their retention times to these of the standard compounds. Quercitin 3-β-D glucoside, isorhamnetin, and myricetin, are the compounds that were identified at retention times, 8.51 min, 2.75 min, 4.50

min, respectively. However, the amounts seem to be slightly less and the chromatogram was not as well resolved as that of yellow onion.

In the chromatogram of Figure 10, one can see that the commercial organic red onion sample have more peaks eluted at beyond 14 minutes. In the sample, quercetin dydrate, and isorhamnetin, are the compounds that were identified at retention times, 7.75 min and 2.77 min, respectively. The elution profile between time of injection and 10 minutes was quite similar to that of the organic white onion.

Figure 11 is the chromatogram of the natural wild onion sample. We observe that the natural wild onions have some of the compounds found in the commercial onions. When compared with the standard compounds quercetin 3-β-D glucoside was identified at retention time 8.52 min from the chromatogram. The peaks were, however, more prominent and matched with the retention times of the standard compounds. Also there were some other unidentified compounds present in quite large amounts as indicated by the size of the peaks. It seems that the wild onion have larger quantities of the flavonoids present.

Figures 12, 13, 14, and 15 are the GCMS chromatograms of the different onion samples used in this study.

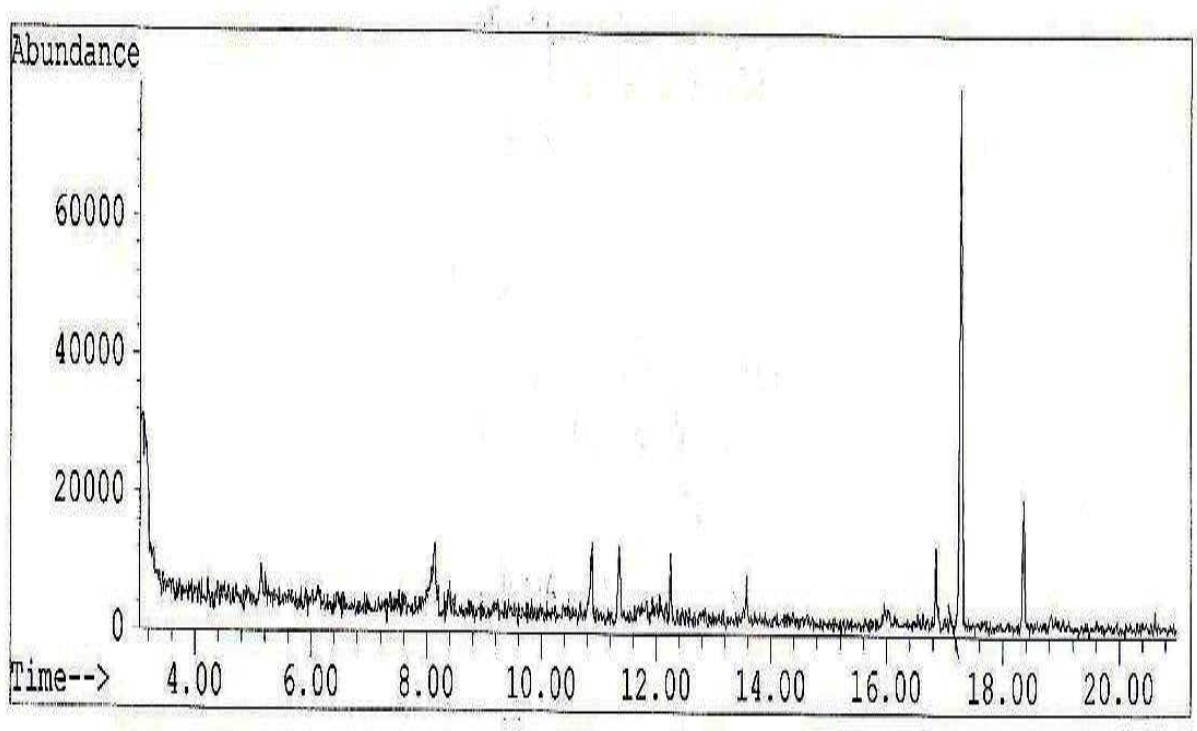


Figure 12: GCMS chromatogram of commercial yellow onion sample

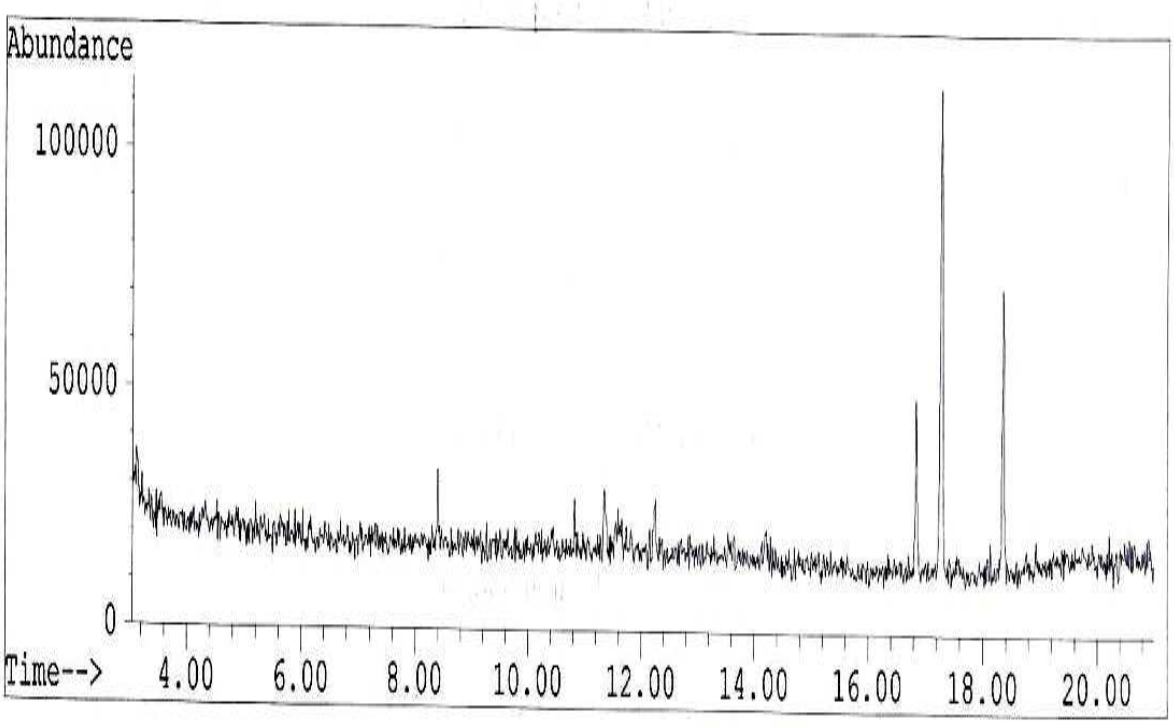


Figure 13: GCMS chromatogram of commercial white onion sample

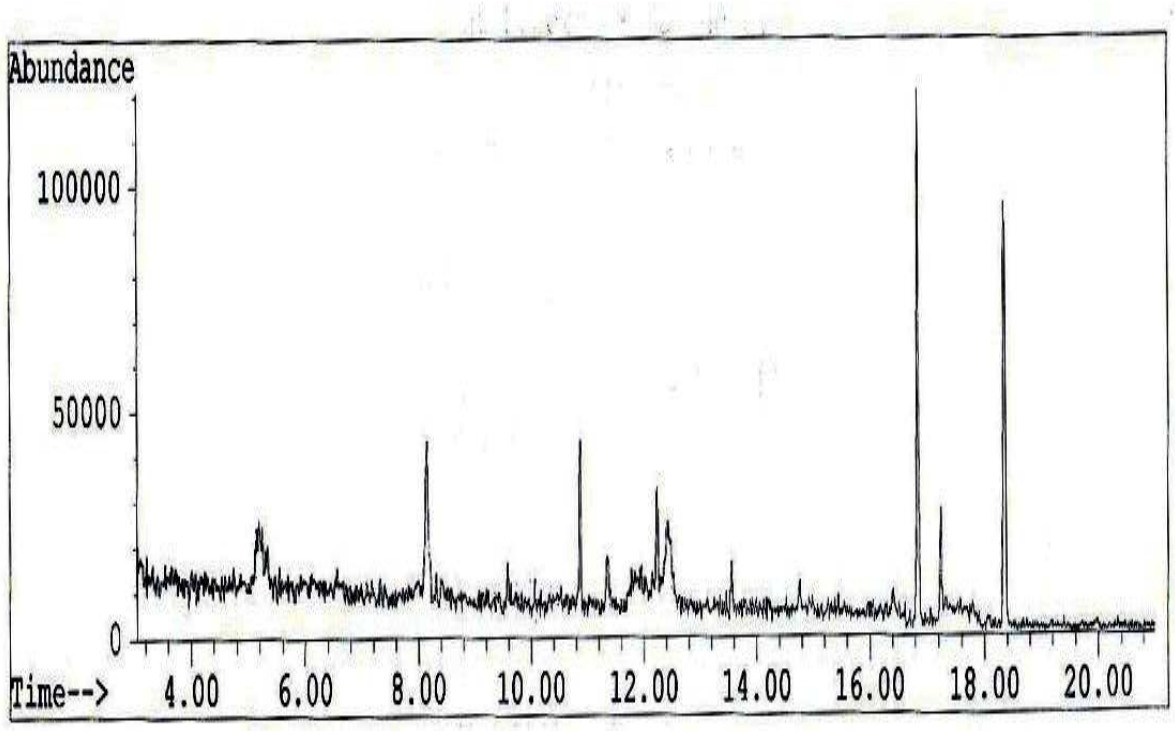


Figure 14: GCMS chromatogram of commercial red onion sample

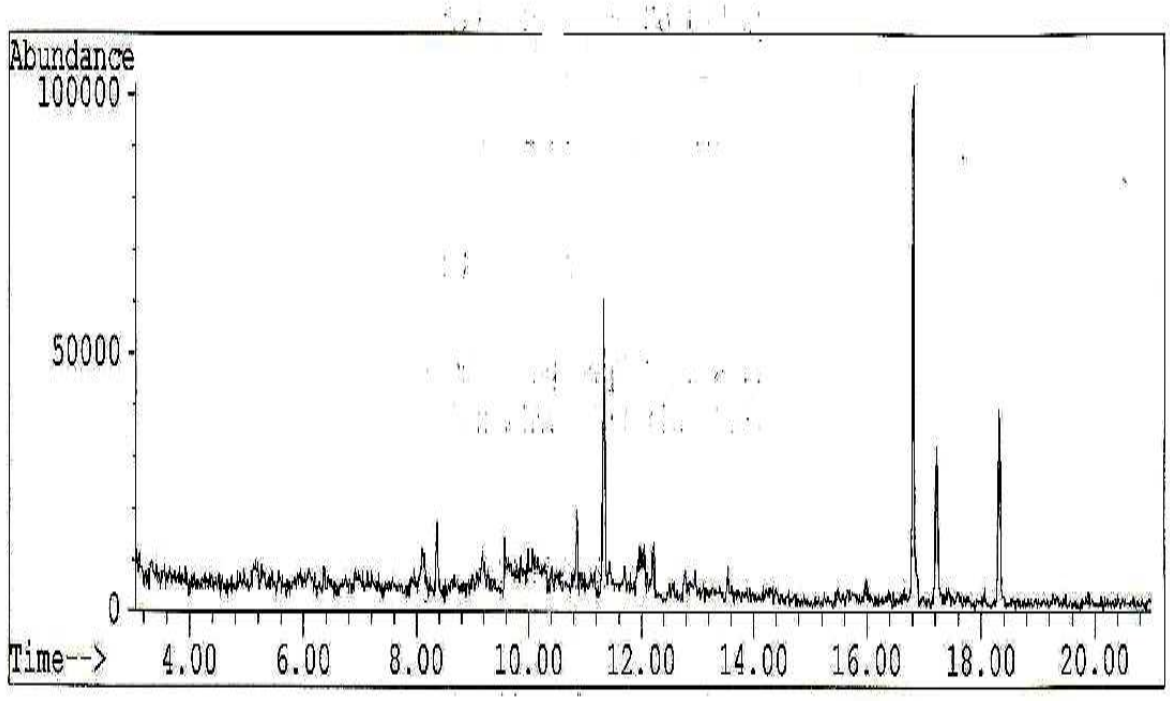


Figure 15: GCMS chromatogram of natural wild onion sample

Figure 13, 14, and 15 are the gas chromatograms of the commercial onion samples. As one can see from these chromatograms, each sample contains many flavonoid compounds. From the visual observations it is found that red onions contains a larger number of flavonoid compounds than the other two commercial onion samples. The range of elution is between 4.0 to 19.0 minutes in these chromatograms.

Figure 15 presents the chromatogram of natural wild onion sample. Visual inspection of this chromatogram seems to show that it is quite similar to that of the commercial organic red onion in terms of more peaks than white and yellow onion samples similar to the case in the HPLC chromatograms. The commercial samples most likely have only the most prominent compounds of natural onions present but not the other natural flavonoids. None of these compounds were identified in these GCMS chromatograms. Identifying the standard compounds that are available also proved to be a difficult task as the retention times do not match. Also we do not have a library of mass spectral library on this instrument, so none of the compounds could be identified. When GCMS chromatograms were compared, it was found that a smaller number of peaks was observed in commercial white onions as were observed in commercial red onions.

Scatter Plot Study

A scatter plot is used for visual comparison of samples with each other and to find out how significantly different they are to each other. Figures 16 and 17 are the scatter plots of the averaged retention times of the flavonoids compounds found in the commercial onion samples against those of the natural wild onion samples, run through HPLC and GCMS, respectively. A scatter plot chart of commercial onion sample against wild onion samples from HPLC chromatograms and GC-MS chromatograms is shown in Figure 16 and Figure 17, respectively.

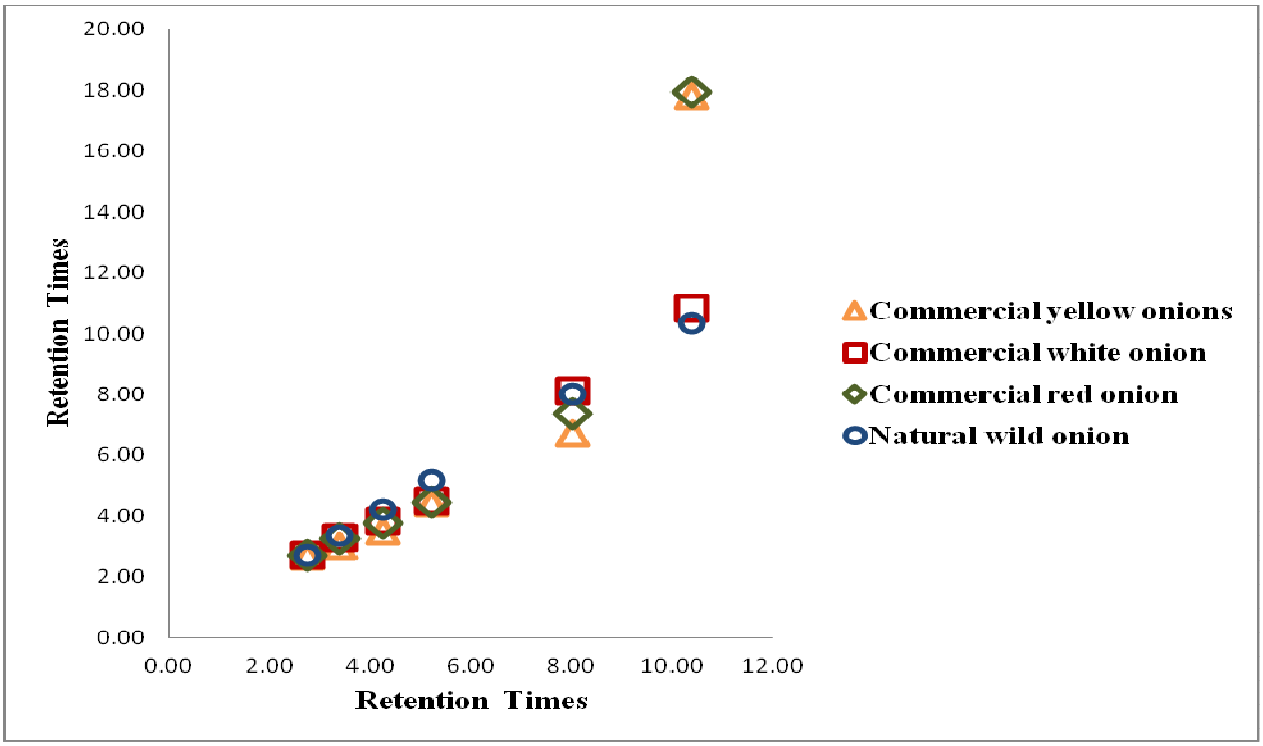


Figure 16: Scatter plot of natural onion samples averaged retention times compared to those of the averaged retention times of commercial onion samples run through HPLC were taken and plotted.

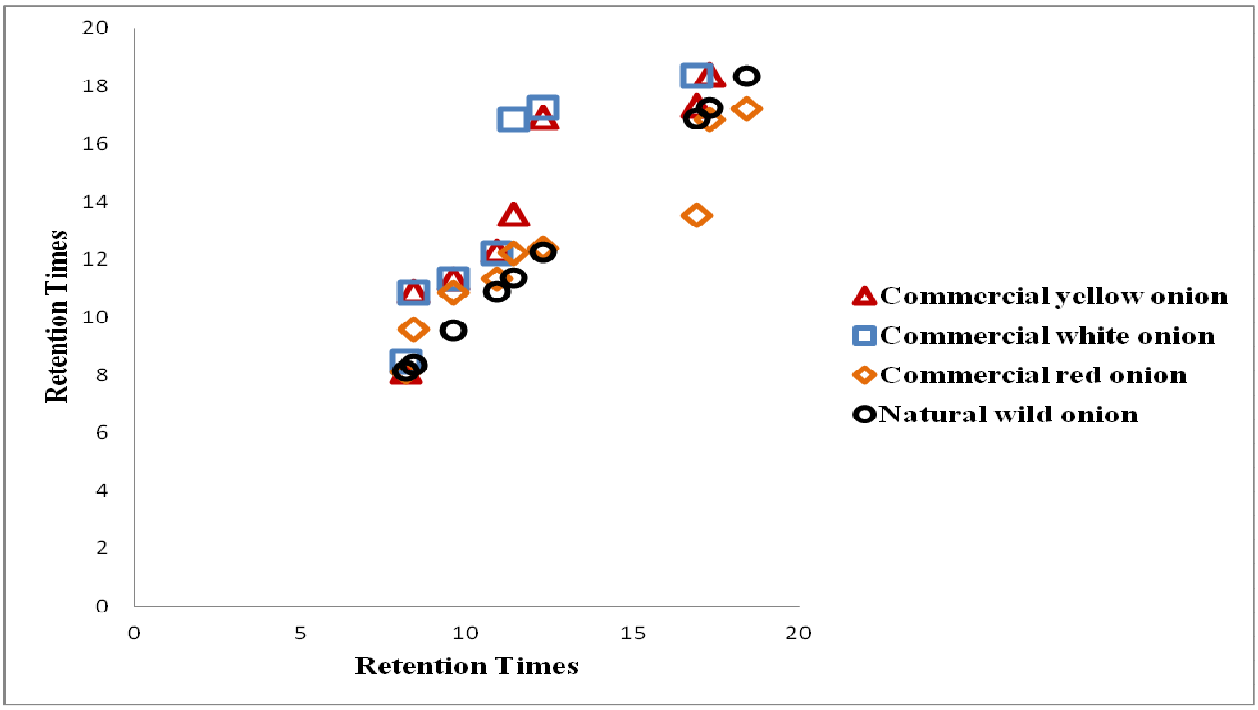


Figure 17: Scatter plot of natural onion samples averaged retention times compared to those of the averaged retention times of commercial onion samples run through GCMS were taken and plotted.

Figure 16 is the combined scatter plot showing the commercial organic white onion sample, commercial organic red onion sample, and commercial yellow onion sample against the natural wild onion sample from HPLC chromatograms. Figure 16 shows that all the commercial onion samples patterns are almost similar to that of the natural wild onions for the first few peaks of the chromatograms. The scatter plot shows that almost all the average retention times fall on top of one another. At longer retention times their chromatograms deviate from the chromatogram of the natural wild onion. From the HPLC chromatograms one can observe that more peaks were found in commercial red onions than were observed in yellow and white onions. The concentration of flavonoids seems to be high in wild onions. The organic red onions seem to be have the closest resemblance to the wild onions. From this scatter plot, one can conclude the commercial onions that were obtained from the market are similar to one another but differ from the natural wild onions.

Figure 17 is the scatter plot showing the averaged retention times of the compounds found in the GC-MS chromatograms from the commericial onions samples against that from the natural wild onion. From the scatter plot of Figure 17, the compounds in the commercial yellow onion sample track that of the compounds in the commercial white onion sample somewhat closely. The compounds from the natural wild onions were different from the compounds in commercial organic red onions, but they seem to be quite similar in terms of more peaks. The commercial white onion when compared with the red onion samples seem to have fewer compounds similar in their flavonoid distribution. The commercial red and yellow onions seem to have more similar flavonoids when compared with white and red onion samples. There were fewer peaks observed in white onion sample chromatogram when compared with the other three onions and a grerat number of peaks were observed in the commercial red onion sample.

Tables 4 and 5 are the averaged retention time values and the relative standard deviation of the triplicates injection of 9 samples of each onion, from the chromatograms obtained using HPLC and GCMS, respectively.

Table 4: Averaged retention times and their relative standard deviations from the HPLC chromatograms of the onion samples

Peak Number	Averaged retention time (r) (minutes) and RSD (%)	Commercial yellow onion	Commercial white onion	Commercial red onion	Natural wild onion
1	r	2.71	2.74	2.74	2.73
	RSD	1.28	0.22	0.29	0.31
2	r	3.04	3.32	3.28	3.39
	RSD	0.84	0.38	0.99	0.77
3	r	3.52	3.85	3.80	4.23
	RSD	0.53	0.33	0.05	4.85
4	r	4.45	4.52	4.47	5.19
	RSD	4.03	2.57	2.36	5.91
5	r	6.74	8.14	7.40	8.02
	RSD	10.30	4.68	3.72	3.49
6	r	17.81	10.84	17.95	10.36
	RSD	11.01	7.65	17.59	10.48

Table 5: Averaged retention times and their relative standard deviations from the GCMS chromatograms of the onion samples

Peak Number	Averaged retention time (r) (minutes) and RSD (%)	Commercial yellow onion	Commercial white onion	Commercial red onion	Natural wild onion
1	r	8.14	8.47	8.13	8.16
	RSD	0.06	0.69	0.35	0.49
2	r	10.89	10.88	9.59	8.39
	RSD	0.27	8.15	0.78	0.87
3	r	11.36	11.36	10.88	9.58
	RSD	0.23	3.47	4.46	0.22
4	r	12.30	12.25	11.34	10.91
	RSD	1.44	0.31	0.36	0.42
5	r	13.59	16.85	12.24	11.38
	RSD	0.05	0.006	0.47	0.43
6	r	16.91	17.28	12.42	12.27
	RSD	0.27	0.09	3.05	0.47
7	r	17.33	18.36	13.55	16.88
	RSD	0.33	0.13	0.12	0.35
8	r	18.41	0	16.84	17.28
	RSD	2.35	0	0.06	0.33
9	r	0	0	17.25	18.37
	RSD	0	0	0.40	0.33
10	r	0	0	18.35	0
	RSD	0	0	0.20	0

These averaged retention times were used to plot the scatter plot graph, by comparing the natural wild onion sample versus commercial onion samples. Correlation coefficient data were determined by using the retention times between each other. To find out the significance between the different onions, ANOVA was performed on their retention data. The recorded retention times used for ANOVA were between 2.0 to 18.0 minutes from HPLC chromatograms, and between 8.0 to 19.0 minutes from the GCMS chromatograms.

Statistical Analysis

Tables 6 and 7 shows the correlation coefficients of the retention times of the compounds in the natural wild onions, and those of the commercial onion samples, obtained from HPLC and GCMS, respectively. The retention times obtained from the chromatograms of natural and commercial onions were taken and compared against each other. The closer the correlation coefficient is to 1.00, the more similar the two kinds of onions.

Table 6 is the correlation coefficients of the different samples compared to each other by using the retention times of the samples run through HPLC. From the results, one can observe that the correlation coefficient between commercial yellow onion and the natural wild onion is the smallest, about 0.91, of all the values obtained. The correlation coefficient between the commercial yellow onion and commercial red onion, and also between commercial organic white onion and natural wild onion are both 0.99. This means that the red and yellow onions are very similar, so do the red and the natural wild onion. The correlation coefficients between the yellow onions and the red onion and the white onion, are respectively, 0.93 and 0.94. Thus there are small differences between these three onions. From the data of HPLC analysis, due to the fewer number of peaks one can obtain from the method, the onions are shown to be basically similar with minor differences.

Table 6: Correlation coefficients of the samples obtained from the HPLC chromatograms

Sample names	Commercial yellow onion	Commercial organic white onion	Commercial organic red onion	Natural wild onion
Commercial yellow onion	1	0.93	0.99	0.91
Commercial organic white onion		1	0.94	0.99
Commercial organic red onion			1	0.92
Natural wild onion				1

Table 7 lists the correlation coefficients of the different samples compared to each other by using the retention times of the samples run through GCMS.

Table 7: Correlation coefficients of the samples obtained from the GCMS chromatograms

Sample names	Commercial yellow onion	Commercial white onion	Commercial red onion	Natural wild onion
Commercial yellow onion	1	0.71	0.77	0.61
Commercial white onion		1	0.48	0.88
Commercial red onion			1	0.42
Natural wild onion				1

The GC-MS results were more interesting as we now have a technique that has a higher resolution and sensitivity. Thus, there are more peaks seem in the chromatograms indicating more compounds are detectable. So, now we would expect differences to be discussed easier.

The natural wild onions sample had the highest correlation coefficient of 0.88 with the commercial organic white onion sample. Based on the correlation coefficients data, the organic white onion sample is most similar to the natural wild onion sample and their chromatograms have the highest number of peaks in common. The correlation coefficient is 0. 77, when the retention times are compared between the commercial organic red onion sample and the commercial yellow onion sample. The retention times compared between the commercial white onion sample and commercial yellow onion, and the retention times compared between the commercial yellow onions and natural wild onions ends up with a correlation coefficient of, 0.71 and 0.61, respectively. The correlation coefficients of the organic red onion with the commercial white onion sample and the natural wild onion sample, were 0.48 and 0.42, respectively. The values show that the biggest difference in the flavonoids were found in the white onion and red onion and there between the wild onion and red onion. The yellow onion seems to have flavonoids somewhat similar to all kinds of onions studied.

Using visual and the statistical comparisons, the HPLC and GCMS results did not completely match each other. From the HPLC results, one can observe that all the samples were mostly similar to each other (correlation coefficients were all close to 1.0). From the GCMS results, one observe that only the natural wild onion sample have flavonoids distribution similar to that of commercial white onions (correlation coefficient is 0.88). All others were not as strongly correlated to each other while that between red onion and the

white and wild are both very low. The major difference is most likely that separation of flavonoids using HPLC allows fewer of them to be detected when compared with GCMS. When samples were run on GCMS, more peaks were observed. The reason is in HPLC only a particular wavelength is used to identify the compounds, and therefore some of the flavonoids separated were not detected at that particular wavelength. But, in GCMS once the sample is volatized, all the compounds above the detection limit are detected. Comparing between fewer number of peaks is easier and high correlation results are obtained if there were than comparing a greater number of peaks with greater probability that differences can occur. Due to this the correlation coefficient data obtained from HPLC would be higher when compared with the GCMS results.

The averaged retention times of all the samples were also compared to each other using analysis of variance (ANOVA). It was used to determine whether two independent samples have the same distribution. The null hypothesis is determined. The ANOVA test is used to decide whether to reject or confirm the null hypothesis. For this project, the null hypothesis is that the commercial onion samples are similar to the natural wild onion samples. A significance level of 0.05 (α) was considered. If the 'significance value' or 'α' or 'p-value' is less than 0.05, the null hypothesis is rejected. If α value is greater than 0.05, it fails to reject the null hypothesis. This confirms that the samples are similar at least at some point. The HPLC data were used to find the values of significance between the samples. All the significance values observed were more than 0.05, which determined that the samples had similar distribution when compared with each other. The highest significance value (0.96) is for the wild onions versus the white onions where the null hypothesis fail to reject. The second significance was for the red onion sample against

yellow onion sample is 0.95 and the null hypothesis was confirmed. The significance in the order of most similar is in agreement with the correlation coefficient results.

Tables 8 and 9 lists the ANOVA p-values of the different samples compared to each other by using the retention times of the samples run through HPLC and GC-MS , respectively.

Table 8: ANOVA p-values of the samples obtained from the HPLC chromatograms

Sample names	Commercial yellow onion	Commercial white onion	Commercial red onion	Natural wild onion
Commercial yellow onion	1	0.77	0.95	0.79
Commercial white onion		1	0.73	0.96
Commercial red onion			1	0.71
Natural wild onion				1

Table 9: ANOVA p-values of the samples obtained from the GC-MS chromatograms

Sample names	Commercial yellow onion	Commercial white onion	Commercial red onion	Natural wild onion
Commercial yellow onion	1	0.46	0.69	0.59
Commercial white onion		1	0.25	0.89
Commercial red onion			1	0.52
Natural wild onion				1

The GCMS samples data were also used to find the significance between the independent samples. The significance values obtained were above 0.05, where the null hypothesis was failed to reject or confirmed. The significance between commercial white onions shows high similar distribution against the natural wild onions with a p-value 0.89 when compared with the other samples. All other samples follow the same pattern. This leads one to have a greater confidence in the conclusions to be made for the project. ANOVA test is an excellent statistical method to find if the independent samples are similar or dissimilar.

CHAPTER 6

CONCLUSIONS

This chapter presents a summary of the project. It includes extraction of flavonoids compounds from the natural and commercial onion and the procedure to separate the flavonoids by using HPLC and GCMS. This chapter also summarizes the results obtained by visual and statistical methods used for comparing the distribution of compounds in the samples.

This research is focused on the distribution of compounds (especially flavonoids) in natural onions compared to the distribution of compounds in commercial onions. Onions are often obtained wild as well as from commercial sources. Onion is from the family Alliaceae and the genus Allium. They are often considered for their health benefits and as a food spice.

One of the goals of this research was to evaluate a simple, economical, and practical extraction method. In this study, a simple reflux extraction method was used for extracting the flavonoids from the onion. The extraction works better with methanol, hydrochloric acid, and TBHQ. This approach of extracting flavonoids works better and is simple and also can be done in one single step. After extraction the extracts were filtered by passing through the whatmann filter paper and then injected into HPLC and GCMS for separation. The procedure was satisfactory as shown from the data obtained. The other extraction method using sonication was also tried. Although it was simpler, the chromatograms obtained were not useful.

A simple reversed phase high performance liquid chromatographic method was developed to separate and identify the compounds. Another method with simple

parameters was developed on GCMS to separate the flavonoids compounds. The HPLC method uses the mobile phase composition of 45% methanol and 55% deionized water. The mobile phase is more economical because it is readily available and use of more water than methanol reduces chemical waste and lesser methanol's harmful effect to the environment. It is more efficient because methanol is totally miscible in water and has low viscosity. The chromatograms from both techniques showed that the commercial organic white onions are very similar to those of the natural wild onions when compared with the other varieties of onions. But the correlation coefficients obtained via HPLC is different from that of GCMS. This conclusion is obtained from various ways of analyzing the data from the experiments. The methods include scatter plots, correlation coefficients, and Anova tests.

The scatter plot study is a visual aid to compare the commercial onion samples against the natural onion samples. These are plotted by using the averaged retention times. From HPLC data, these pattern plots show that the commercial samples were totally superimposable to the pattern of natural onion samples. The results also show that the flavonoids eluted earlier from all onion samples were quite similar to those from the wild onion samples. From the GCMS results, however, it is observed that the patterns track are more scattered. This shows that fewer flavonoids are similar among the onion samples studied.

The correlation coefficient for the commercial white onion against natural wild onions was 0.99 from HPLC results and 0.88 from the GCMS results. The correlation coefficients are both the highest among all correlations between onion types compared. From this calculated correlation coefficient, the commercial white onion is concluded to have the greatest similarities to that of the natural wild onion albeit the numerical value being smaller from GC-MS data. The comparison between commercial yellow and red

onions seems to show that they have similar flavonoids compounds between them with a correlation coefficient of 0.99 from HPLC results and 0.77 from GCMS results. The correlation between white and red onions, red and wild onions shows the greatest difference. The yellow and white onions and yellow and wild onions show less similarity even though the yellow onion and the red onion are somewhat similar similar in flavonoids distribution.

The ANOVA tests support the results obtained from the correlation coefficient and the scatter plot study. The null hypothesis of the commercial onions were similar to the natural onion samples was confirmed by ANOVA. The significance value obtained between the commercial white onions and wild onions was the greatest which shows that they have similar flavonoids distribution to each other. The overall results of ANOVA tests track those of the pattern plots and correlation coefficient values well.

From all the different methods of evaluating the HPLC and GCMS data of the different samples, natural and the commercial onions, this project shows that HPLC and GC-MS can be used to compare the distribution of flavonoids in different onions. With the aid of different statistical procedures one can reach the same conclusions in different ways.

It would be interesting in the future to expand the studies by including more varieties of onions in the study. Also, one can obtain HPLC chromatograms using several different wavelengths of detection to expand the data sets for statistical analysis.

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