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Reversed-Phase HPLC Determination of Alliin in Diverse Varieties of Fresh Garlic and Commercial Garlic Products.

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Reversed – Phase HPLC Determination of Alliin in Diverse Varieties of Fresh Garlic and Commercial Garlic Products

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by

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ABSTRACT

Reversed – Phase HPLC Determination of Alliin in Diverse Varieties of Fresh Garlic and Commercial Garlic Products

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Alliin is a predominant flavor precursor in garlic cloves. It interacts with the enzyme alliinase when garlic cloves are crushed, cut, or chewed to produce allicin, an unstable thiosulfinate that is the main biologically active component of fresh crushed garlic. Biological functions and health benefits of garlic include reduction of cancer risk in humans, improving immune system, and anti-microbial, anti-oxidant, and anti-hypertensive activities. The quality of fresh garlic and garlic products is usually related to its alliin content and allicin release potential. This research presents a simple, rapid, and precise HPLC method for alliin determination. It involves the use of 30:70% methanol: water and 0.05% sodium dodecylsulfate mobile phase composition, C_{18} 5 µm disc column of size 3.9 x 150 µm, and detector set at 210 nm. The method showed good reproducibility with 0.56%-4.11% relative standard deviations, a linear response of peak area to alliin concentration of 0.4 ng/mL–80 ng/mL, and average recovery of 93.5%-101%. Determination of alliin in eight garlic samples indicated the highest amount in garlic tablet that was expected. The method presented is economical and efficient and can be used in alliin determination. The method gave a satisfactory chromatograms with methanol–hydrochloric acid extract but not with hot water extract.

DEDICATION

This work is dedicated to the Apawu (Dad, mum, and siblings) and Ayaba (in-laws) families. Your support and your unrelenting confidence in me have given me no excuse to fail in this life.

May the good Lord prosper you in all your endeavors.

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Unto God my eternal father who has blessed me with the gift of life and success in all my pursuits be glory, honor, and dominion for evermore.

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To the Blevins and Nedderman community Bible study group at Grace Fellowship Church, I say God richly bless you for being unto me a family away from my home country.

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CHAPTER 1

INTRODUCTION

Garlic is grown all over the world and is used in various forms as food, spices, and medicine. Garlic is an indigenous herb of Western Asia and Mediterranean where it has been cultivated for centuries. The major garlic growing countries includes Korea, China, India, USA, Spain, Argentina, and Egypt, among which China is by far the largest producer (1).

Botany of Garlic

The garlic plant is made up of fleshy edible cloves that are encased in a white or pink, thin coat. It has leaves, stem, and flowers located on the head that are also edible. It is easy to grow and can be grown all year round. It is cultivated in temperate and tropical climates. Garlic plant grows well in well drained soil and requires a cool and moist period during growth and a relatively dry period as it matures. It is propagated using cloves obtained from the bulbs and is ready for harvest when the top turns yellowish or brownish. It is best stored in well ventilated room (2). Fresh garlic has a characteristic odor and is used for flavoring and also as a spice.

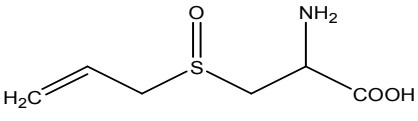
Taxonomy of Garlic

Recent taxonomy revisions place garlic in the family Alliaceae, which is made up of approximately 700 Species (3). It belongs to the genus *Allium*. A great number of species in this genus are perennial plants that have underground storage organs consisting of bulbs or rhizome. They have great economic value as well as enormous medicinal importance. The most common edible members include chives, (*A.Schoenoprasum L.*), leek (*A.porrum L.*), and

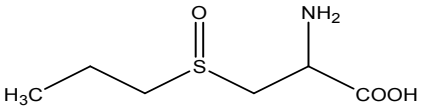
onion (*A.Cepa L*.) (4). *Allium* species are rich in sulfur containing compounds that have been identified to be responsible for their characteristic odor, flavor variation, and biological activities (3). Garlic, without exception, is one of the most extensively investigated *Allium* species both by chemists and biologists due to these compounds. It belongs to the species *Sativum* and has the scientific name *Allium Sativum L.*

The Chemistry of *Allium Sativum L.*

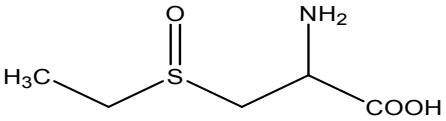
Garlic contains high levels of sulfur, zinc, phosphorus, and potassium; moderate levels of selenium, vitamin A, and vitamin C; and low levels of iron, manganese, calcium, magnesium, sodium, and B-complex vitamins. In addition to these, about 33 sulfur compounds and 17 amino acids that include alanine, arginine, aspartic acid, asparagine, histidine, leucine, methionine, phenylalanine, praline, serine, threonine, tryptophan, and valine have been identified and isolated (5). One unique constituent group of allium plants is S-Alk(en)yl–cysteine sulfoxides (ACSOs) that are responsible for their typical odor and flavors (6). These sulfoxides include S–Methyl–L–cysteine sulfoxide (Methiin), S–Allyl–L–cysteine sulfoxide (Alliin), S–propyl–L–cysteine sulfoxides (propiin), S–propenyl–L–cysteine sulfoxide(Isoalliin), S–Ethyl–L–cysteine sulfoxide (Ethiin) and S–n–Butyl–L–cysteine sulfoxide (Butiin). These compounds are shown in Figure 1.



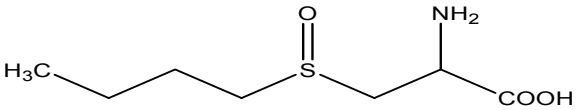
S-Allyl-L-cysteine sulfoxide
Alliin



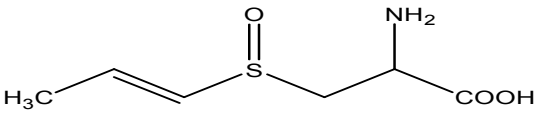
S-Propyl-L-cysteine sulfoxide
Propiin



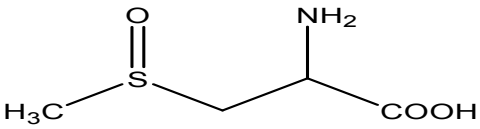
S-Ethyl-L-cysteine sulfoxide
Ethiin



S-n-Butyl-L-cysteine sulfoxide
Butiin



S-Propenyl-L-cysteine sulfoxide
Isoallin



S-Methyl-L-cysteine sulfoxide
Methiin

Figure 1. Examples of sulfoxides that are present in allium vegetables. The predominance of each compound varies from one vegetable to another.

In garlic the predominant flavor precursor is alliin, with lower concentration of isoalliin (source of lachrymatory factor in onion) and mithiin, and trace amount of propiin (7). Alliin was first identified in 1948 by Stroll and Seebrook (7). It is a stable non-protein amino acid that forms the parent sulfur compound that is responsible for the majority of the odorous volatiles produced from crushed or cut garlic (3). Alliin is located in the cytoplasm of the cell of a garlic bulb and is separated from allinase, an enzyme localized in the cell vacuole. When garlic is crushed, cut, or chewed, the enzyme from the vacuole is released to act on the alliin in a reaction that produces allicin (an odiferous alkyl – alkane thiosulfinate), with pyruvic acid and ammonia released as by products (8). The thiosulfinate formed is relatively unstable and storage over long period of time or steam distilled will cause it to undergo a number of

transformations depending on the temperature (3). A typical enzymatic reaction in garlic cell that produces allicin is presented in Figure 2.

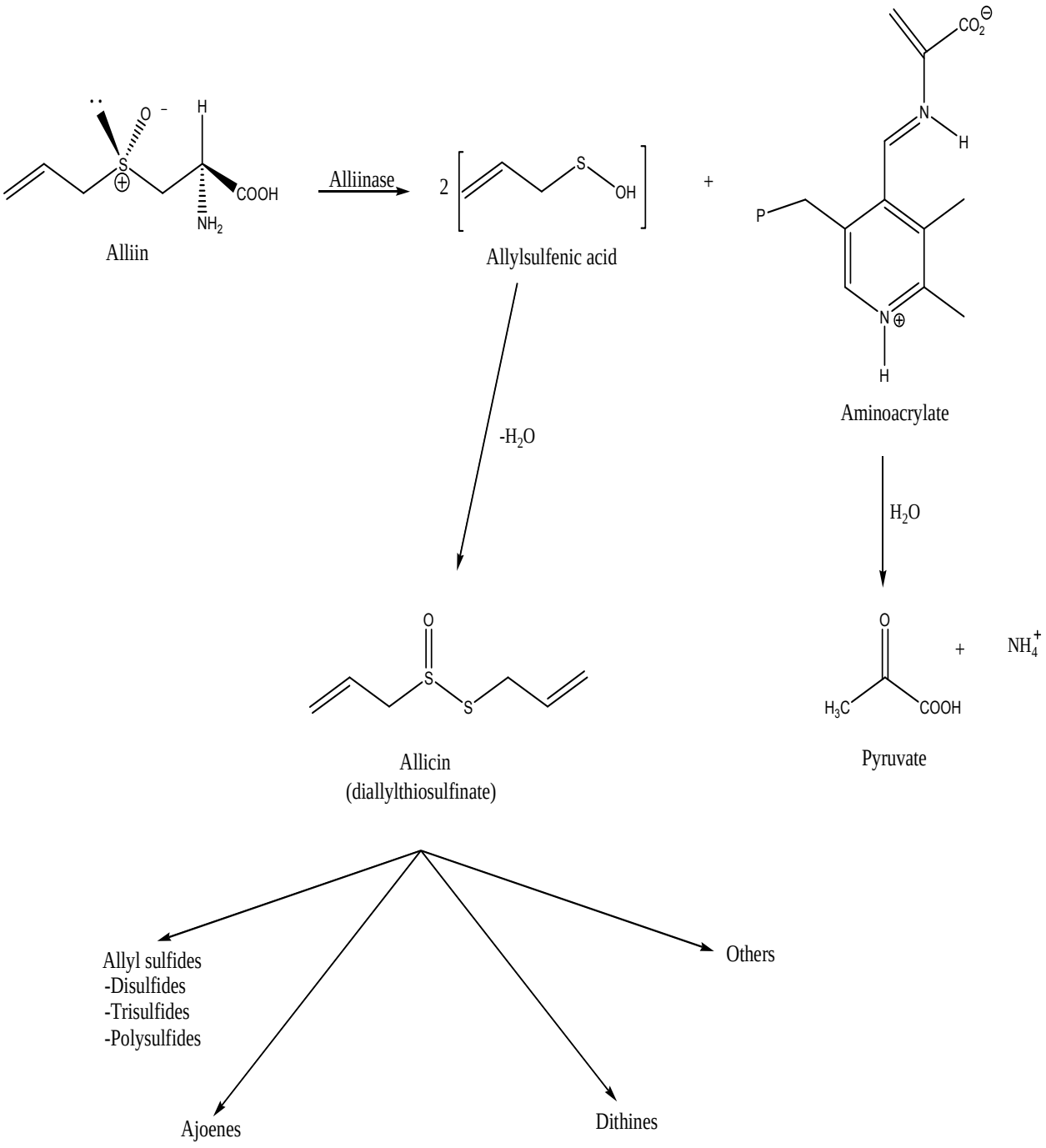


Figure 2. Ezymatic hydrolysis of alliin producing allicin as main product and pyruvic acid as a by product. Unstable allicin decomposes into several sulfur compounds.

Alliinase from garlic cloves has been isolated and well characterized. Linda et al. (8) in their paper published in 2006 described alliinase as a dimer with two equal sub-units, each having 448 amino acid residues with a total molecular mass of 103 kDa. It belongs to the family of glycoprotein and contains about 5.5%-6.0% mannose. The enzyme is known to form a stable complex with a mannose specific lectin, allium sativum agglutinin (ASA-1). The optimum pH for allinase activity is 6.5, and its isoelectric point was determined to be between 6.0 and 7.0 (8).

Allicin is the main thiosulfinate produced in garlic, representing 70% of the overall thiosulfinate present or formed by crushing garlic gloves (9). Allicin was discovered in 1944 by Cavalitto and Bailey (9). It is an unstable compound and cannot reach its target cell in the body via circulation (10). Allicin is a highly reactive molecule and readily degrades to form secondary sulfides. Allicin has a disullfur (-S(O)-S) bond that reacts with different thiol-containing molecules including SH-containing protein (9,11). Many recent studies have provided strong evidence that most of the biological functions and health benefits of garlic are attributed to allicin. This is made possible by its strong SH- modifying and antioxidant properties (12). In fact, no compound outside the thiosulfinates has been found that account for a significant portion of the pharmacological activities of crushed garlic at levels representing human consumption (2-5 g/day) (13)

Biological Functions and Health Benefits of Garlic

The medicinal value of garlic has been explored since antiquity (14,15). It has been used traditionally to treat several conditions such as cold, coughs, hypertension, high blood pressure, rheumatism, diarrhea, and snake bite. (14). Its traditional medicinal use varies from one

culture to another. For instance, in East Asia, hot water garlic extract has been used as an aphrodisiac and also to treat asthma. In England, hot water garlic extract is taken orally for diabetes. Garlic bulb is chewed together with other leaves in Ethiopia to treat stomach ache. In Guatemala, hot water extract of dried bulb is used externally for ring worm, fungal diseases of the skin, and skin diseases and irritations from leukorrhea, vaginitis, and infections of the skin mucosa. Water extract garlic is used in Japan to promote hair growth. In Nigeria, garlic is dried and soaked in juice of citrus aurantifolia and a pinch of copper sulfate and taken orally to treat convulsions in children (15).

Numerous pharmacological activities and clinical trials over the years have confirmed the biological functions and the health benefits of garlic. For instance, recent epidemiological studies had shown that consumption of large amount of garlic is associated with reduced cancer risk in humans, mostly stomach and colon cancer (16). Furthermore, studies have also shown the ability of garlic to reduce chemical carcinogens in different animals. Other well studied benefits of garlic include; antimicrobial, antithrombotic, antioxidant, improving immune- system, anticardiotoxic and anti–ischemic effects, antiaging, antibacterial, anticytotoxic, antifatigue, antifungal, antihypertensive, antihypotensive, antihypothermic, antimutagenic, antimycobacterial, antitoxic, and antiprotozoan activities. In addition, there have been studies on the inhibition of acetylcholinesterase, acid phosphatase, adenosine deaminase, adherence (bacteria to host cells), aflatoxin production, and alanine aminotransferose, as well as alamine aminotransferase and alkaline phosphatase stimulation (15).

Extensive investigation into garlic and its medicinal value has led to an improvement in the quality and yield of fresh garlic production. The countless benefits of garlic no doubt have given rise to the production of several garlic food supplements ranging from tablets to powder and are based on either allicin content or on the potential to produce allicin (12). This consequently has initiated a host of investigations to ascertain or debunk the claims that they have the same essential ingredients as raw garlic. One of such investigations has revealed that though garlic powder and granules can serve as important food supplement, if stored for a long time, the ingredients present in fresh garlic are often lost. In addition, because alliinase is irreversibly deactivated at the pH level in human stomach, if garlic powder is taken directly there would only be an insignificant amount of allicin that can be produced inside the human body. Based on this knowledge, garlic capsules coated with materials that can resist human stomach conditions in order to prolong the shelf life and protect alliinase activity through the stomach have been commercially produced. In this way, allicin can be released only in the intestine and consequently decrease the characteristic odor and after taste (17). Many other studies had been done to find out the pharmacological benefits of these food supplements, but not all of them can assumed equivalence in their composition and in their biological response (18).

CHAPTER 2

TECHNIQUES FOR ALLIIN DETERMINATION

In 1973, Freeman and McBreen developed the first method for analysis of volatiles of onions and garlic based on a rapid spectrophotometric determination (19). Twenty years later, many other methods were proposed (19). Currently, a number of analytical methods have been used to determine S-alk(en)ylcysteine sulfoxides in garlic. These include; capillary electrophoresis, spectrophotometric methods, gas chromatography, micellar electrokinetic capillary chromatography, thin layer chromatography, high performance ion-pair chromatography, and high performance liquid chromatography. They can be categorized into direct methods that allow determination of S-alk(en)ylcysteine sulfoxides content before their enzymatic hydrolysis to form allicin or indirect methods that are employed in the determination of diverse products arising from the enzymatic reaction (20). Some of the above named techniques involve a single step sample preparation, whereas others are associated with several steps during which the analyte is derivatized.

Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) combines aspects of both gel electrophoresis as well as high performance liquid chromatography (HPLC). The technique involves separation of species within the lumen of a small bore capillary filled with an electrolyte. The capillary is immersed in electrolyte filled reservoirs containing electrodes connected to a high voltage supply. When a sample is introduced at one end of the capillary, the components of the sample are separated

as they migrate through the capillary towards the other end (outlet) and are detected by a detector that sends electronic signal to a recorder (21). The separation depends on differential migration in an electric field. Capillary electrophoresis is similar to HPLC in many ways, in sample injection as well as data presentation and interpretation. Its advantages include high resolving power that sometimes may be higher than the conventional electrophoresis or HPLC. The use of narrow bore capillary with excellent heat dissipating properties allows the use of very high field strength that decreases analysis time and minimizes band diffusion. The major limitation of this technique has been reported in applying it to protein separation. The high surface volume ratio of the capillaries and the high surface activity of the fused silica capillary give rise to this limitation (21).

Measurement of sulfoxides by capillary electrophoresis is believed to have been first reported by Hories and Yamashita (6). In their work published in 2006, they explained that because capillary electrophoresis allows indirect detection of components with no specific ultra-violet absorption, it seemed likely that it could separate sulfoxides without difficult derivation. The advantages of this technique as reported in their article were simplicity and simultaneous detection of pyruvate and methiin as well as alliin peaks.

However, Kubec and Dadakora (22) mentioned that the reported method offers absolutely unsatisfactory results for analyzing real sample extracts. They made this assertion after they had repeated the previously reported procedure. They attributed this to the fact that, the capillary had to be rinsed thoroughly after every run and, in addition, the migration time variation and peak overlap rendered identification and quantification of individual peaks

extremely unreliable. Based on these aforementioned limitations, Kube et al. developed a completely new method. This was based on extraction of the sulfoxides with methanol, derivatizing them by fluorenylmethyl chloroformate, and subsequently separating them by miceller electrokinetic capillary chromatography. The derivatizing agent was known to readily form stable derivatives with compounds possessing primary and secondary amino groups in virtually quantitative yield. They reported that the reaction proceeded in aqueous solution within minutes with no time consuming clean–up procedure required. In addition, the derivatives produced a very high extinction coefficient, ensuring their specific and sensitivity detection (22).

Spectrophotometric Method

Spectrophotometric methods employ light to measure the chemical concentration of analytes. This absorption should be distinguishable from that due to other substances in the sample. The absorption by a sample is proportional to the total amount of material that absorbs the incident light that is referred to as chromophore. This is defined by the Beer – Lambert Law;

$$A = \varepsilon \text{bc} \qquad [1]$$

where ε, molar absorptivity is a constant that is a property of the material itself as

well as the wavelength of the measurement ; b refers to the length of the path through which the light travels in the sample; and c, the molar concentration of the material that absorbs the light (23). Alliin content in garlic extract can be indirectly determined using this method. This

determination is based on the principle that alliin in presence of allinase produces allicin that reacts rapidly with free thiol groups through a thiol–disulfide exchange reaction. Such exchange reaction can cause a shift in the optical absorption of a thiol-containing chromohpore, and therefore the molar concentration of alliin can be calculated from the difference in absorbance according to the equations:

[alliin] = [absorbance without alliin] – [absorbance with alliin] x dilution x [ε]-1 [2]

where ε =molar absorptivity (12).

Miron and his co-workers (12) used this method in their assay for allicin, alliin, and alliinase. In their recent work, they used 4-mercaptopyridine (4-MP) rather than the previously used 2-nitro-5-thiobenzoate (NTB) because the former is a commercially available chromogenric thiol. Their determinations were based on the reaction of 4-MP (which has maximum absorption at 324 nm) with the activated disulfide bond of thiosulnates -S(O)-S- to form 4-allylmercaptothiopyridine, which has no absorbance in this region. The structure of the 4-allylmercaptothiopyridine was confirmed by mass-spectrometry. The difference in absorbance obtained was thus used to calculate the content of the analyte. This method though indirect, was reported to be sensitive, fast, and non-costly and gives highly efficient throughput assay of alliin, allicin, and alliinase in garlic extracts.

Gas Chromatography

Gas chromatography (GC) involves the separation of components of vaporized sample as a result of partitioning between a gaseous mobile phase (called carrier gas) and a liquid or

solid stationary phase. The carrier gases commonly used are helium, nitrogen, or hydrogen gas. The choice of carrier gas depends on the detector and the desired separation efficiency and speed (24).

The liquid stationary phase is a non-volatile liquid bonded to the inside of the column or to a fine solid support. This is used in gas-liquid partition chromatography. The solid stationary phase is used in gas-solid adsorption chromatography in which the analyte is adsorbed directly on solid particles of stationary phase. The essential elements of gas chromatography include a regulated supply of carrier gas, a device for vaporizing the sample (injector), a thermostatted oven in which the column is housed, a detector, and a data processor (25). When a volatile liquid or gaseous sample is injected through a septum into a heated port, it is rapidly evaporated and the vapor is carried through a hot column by carrier gas causing separation of components of sample to take place. The column is kept hot enough to provide sufficient vapor pressure for analyte to be eluted in reasonable time. The separated components flow through a detector and their responses are recorded by a recording device and processed (24). The columns used are generally open tubular columns and packed columns. The open tubular columns are used in wide range of analyses. It is made up of fused silica (SiO_2) and coated with polyimide for support and protection from atmospheric moisture (24). An open tubular column has the advantage of better separation efficiencies and greatly improved sample detectability for a given analysis time than any packed columns (25). It also gives greater sensitivity and shorter analysis time (24). Open tubular columns however have disadvantages that include

more demanding of instrument performance, less forgiving of poor operator technique, and possess a lower sample capacity than the packed columns (25).

Packed columns are made up of fine particles of solid support coated with nonvolatile liquid stationary phase, or the solid itself may be the stationary phase. It offers a greater sample capacity but gives broader peaks, longer retention times, and lower resolution. However it is useful for preparative separations or separation of weakly retained solutes such as gases and low molecular weight hydrocarbon. Different detectors are used in gas chromatography. These include flame ionization detector, thermal conductivity detector, and electron capture detector. Others are flame photometric detector, sulfur chemiluminescence detector, and photoionization detector (24, 25).

Gas chromatography gives excellent resolution and has mass identification capabilities (26). For this reason gas chromatography and gas chromatography-mass spectrometry (GC-MS) have been widely used in the characterization of allium volatiles. Gas chromatography determination (GC-FPD) of alliin in garlic and garlic products was first elaborated by Saito et al. in 1988 (20). Their method involved derivatizing alliin with trifluoroacetic acid anhydride (TFAA) followed by GC analysis using a short packed column. Saito and his co-worker found out that the trifluoroacetic derivative was unstable and consequently decomposed after it was exposed to sunlight for 15 minutes. This was thus, a major limitation of their method. Another limitation that was also reported was poor column resolution that renders the method unsuitable for routine work.

In their work published in 1992, Block et al. (26) emphasized that gas chromatography as typically performed with high injector and column temperature present an erroneous picture of the composition of room temperature extracts of Alliin species and that HPLC provides a reliable quantitative and measure of what is actually present in the species.

Auger and Ferary and their team also reiterated that gas chromatograph–mass spectrometer (GC-MS) does not seem to be a suitable method for the analysis of true garlic flavors as it gave artifacts. They proposed high performance liquid chromatography as a preferable alternative (27).

However, Kubec and his colleagues (20) reported a new method allowing a highly sensitive and reproducible determination of S-alk(en)ylcysteine sulfoxides (including minor derivative) using gas chromatography. The method was based on isolation of amino acid fraction by ion-exchange chromatography followed by derivatization with ethyl chlorofomate at ambient temperature and reduction of derivatized S-alk(en)ylcysteine sulfoxides by sodium iodide. They reported that all preliminary attempts to analyze S-alk(en)ylcysteine sulfoxides by GC immediately after derivatization failed. They used two different capillary columns, various temperature programs, and injector temperatures and realized that under all the conditions there was a substantial decomposition of S-alk(en)ylcysteine sulfoxides similar to what had been described in connection with GC determination of glucosinolates having a sulfoxide moiety in the side chain. They attributed this to the presence of the highly polarized and extremely labile sulfoxide group and suggested that the best way to analyze S-alk(en)ylcysteine sulfoxide by GC is removing this group prior to the injection, hence the use of sodium iodide in

their method. Even though this method offered outstanding sensitivity, excellent resolution capacity, accuracy, and reliability, time requirements are a serious drawback for use in routine analysis. In addition, the method is unable to resolve between S-alk(en)ylcysteine and their sulfoxides (20).

Thin Layer Chromatography

Thin layer chromatography (TLC) involves movement of a mobile phase through a thin layer of sorbent (coated on an inert, rigid background such as aluminium, plastic, or glass) by capillary action. The separation of sample is a result of the differences in migration of sample components in the direction the mobile phase travelled. It is measured in terms of retention factor or retention index, RF given as;

$$Rf = \frac{\text{distance travelled by spot from origin}}{\text{distance travelled by solvent from origin}} \quad [3]$$

TLC, unlike column chromatography, allows simultaneous analysis of a number of samples and standards. It also allows samples that are difficult to resolve to be developed in two different solvents run in perpendicular directions. In addition, because TLC plate is used only once, harsh separation conditions that can degrade and rapidly destroy an analytical column can be used for TLC (23).

The conventional TLC technique has seen improvement in the quality of adsorbent layer as well as its methods of sample application. This new technique called high performance thin layer chromatography (HPTLC) is more rapid, efficient, and sensitive than conventional TLC (25). Comparing HPTLC with HPLC, the former is an open bed while the latter is a closed system (25).

Poole et al. (25) in their book noted that the two techniques complement each other and hence selection should be based on the type of problem to be solved. Even though HPLC techniques offer a greater separating power than HPTLC considering individual samples, the enormous advantages of HPTLC over the HPLC cannot be over emphasized. In HPTLC the selection of mobile phase does not limit the choice of detector. This is because the solvent is completely evaporated between development and measurement so it does not influence the detection process. This is not so in HPLC, because, for example, UV–absorbing solvents cannot be used with UV–detectors (25). The detection process in HPTLC is more flexible and variable than that of HPLC because the former is dependent on distance rather than time even though detection limits under optimum conditions are approximately the same for both techniques.

HPTLC techniques allow simultaneous sample analysis with the possibility of substantially reducing the time required for the analysis of a large group of samples. This is not so in the case of HPLC because analysis in HPLC is by necessity performed in a sequential manner due to the nature of the method development (25).

Thin layer chromatographic method of analysis of garlic constituents has been developed involving the use of ninhydrin detection reagent to optimize the differentiation between cysteine sulfoxides and other amino acid derivatives (19). The shortcoming of this method has been reported to be enzymatic degradation of alliin due to preparation of sample extract by homogenizing garlic. This allows the interaction between alliin and allinase that otherwise are present in separation components in the intact cells (28).

In 2005, Niranjan and his group (28) published a proposed HPTLC method for the analysis of garlic and its formulation for its alliin content. This method involves densitometric evaluation of alliin after resolving it by HPTLC on silica gel plates with n–butanol: acetic acid: water (6:2:2 v/v) as the mobile phase. After derivatizing the resolved bands with ninhydrin reagent, the peak areas were recorded at 540 nm in densitometric evaluation. They found the relation between the concentration of alliin and the corresponding peak area to be linear within the range of 250 to 1500 ng/ spot. They recommended this method for use in routine quality control of garlic and its formulation due to its good precision, specificity, sensitivity, and accuracy (28).

High Performance Liquid Chromatography

High performance liquid chromatography has been used widely in analysis of diverse varieties of samples since most compounds are not sufficiently volatile for gas chromatography. Its advantages include; high speed resolution, sensitivity (femtograms–nanograms), good reproducibility, recovery, accuracy, precision, and ease of automation. It has proven to be a reliable technique for quantitative and qualitative determination of sulfoxides in allium extracts (26). For this reason a number of HPLC techniques have been developed and have been used in the determinations of organosulfur compounds in allium species

In 2003, Arnault and his coworkers (20) published a report on a rapid HPLC method suitable for routine analysis of sulfoxides. This method involves the use of 3 µm particle Hypurity Elite C_{18} column of dimension 150 x 3mm, an ultra-violet detector operated at 208 nm, and a gradient elution involving the use of mobile phase consisting of (a) 20 mM sodium

dihydrogenphosphate, 10 mM heptanesulfonic acid, 85 % orthophosphoric acid and (b) acetonitrile, 20 mM sodium dihydrogenphosphate, 10 mM heptanesulfonic acid. They used eluents containing an ion–pairing reagent to ensure a sufficient separation between alliin and the more retained dipeptide at very low pH. Arnault et al. reported that their method that was without derivatization allowed simultaneous quantification of alliin, allicin, as well as dipeptides and requires no particular sample preparation. The method also yielded good linearity for each compound and gave a run time of 30 minutes. However, the sensitivity of the method was weaker compared to a pre-column dervatization.

Ichikawa and his group have also reported another method for simultaneous determination of sulfoxides (30). The method involved one step sample preparation procedure followed by normal phase and reversed phase HPLC techniques to determine the sulfoxides. Alliin , isoalliin, methiin, cycloalliin, and γ-L-glutamyl-S-methyl-L-cysteine were determined by normal phase HPLC using an aminopropyl-bonded column, whereas γ-L-glutamyl-S-(2-propenyl)-L-cysteine and γ-L-glutamyl-S-(trans-1-propenyl)-L-cysteine were separated on an octadecylsilane column. They reported overall recoveries of 97.1%-102.3% and relative standard deviation values of intra- and interday precision lower than 2.6% and 4.6% respectively. The advantages of their method include specificity, speed, and ease of use. They confirmed that the method was useful for chemical and biological studies of garlic and its preparations.

In 2007, Diego et al. developed and validated a reversed phase HPLC assay for quantitative determination of allicin in garlic powder and tablets (18). Their chromatographic

separation was performed on an RP-18_e column of dimensions 124 mm x 4mm. They used a mobile phase made up 50:50 methanol: water, a flow rate of 0.5 mL/min, and ultra-violet detection at 220 nm. The method also involved the use of ethylparaben as internal standard. Diego and his colleagues reported that the method was linear for allicin concentrations of 5.0-60.0 µL/mL and gave relative standard deviation for precision to be less than 6.14% with accuracy above 89.11%.

Although these publications demonstrate great success in the development of high performance liquid chromatographic techniques for thiosulfinate and sulfoxide determinations, new chromatographic techniques continue to evolve from already existing ones with the aim of using environmentally friendly reagents, improving efficiency, and simplicity of method as well as cutting down the cost for analysis.

CHAPTER 3

METHOD USED IN THIS WORK

This chapter discusses the methodology that was used in the project. It focuses on the instrumentation, the chromatographic process, some advantages it has in sulfoxide determination, and the research objective.

High Performance Liquid Chromatography (HPLC) Process

HPLC employs high pressure to force solvent through a closed column containing chemical groups bounded to very fine particles producing high resolution separation (25). The technique involves the use of a solvent delivery system, injection system, a column, a detector, controller, and recorder. A good chromatographic separation requires unlimited supply of solvent, a high resolution stationary phase, with porous pack materials, high pressure pump, and a desirable flow rate of solvent. Furthermore, the solvent should be volatile and have a low viscosity (31). The use of pure HPLC grade solvents prevents degradation of column by impurities and decreases detector background signals from contaminants. Solvents are degassed with helium to eliminate gas bubbles that otherwise degrade pump and detector performance (24, 25). However, it is essential to prevent degassing in the detector where solvent pressure may be reduced to atmospheric pressure, in order to prevent baseline drift or continuous spikes. Samples must be dissolved in a relatively large volume of solvent and injected with valve injector rather than syringe injection (31). When a sample is injected into the column, it is carried through the stationary bed by the mobile phase resulting in separation.

The sample components flow through the detector connected to the end of the column that monitors the separation and then the resulting chromatogram is recorded (31). Figure 3 shows a schematic diagram of a typical HPLC system with the basic units described above.

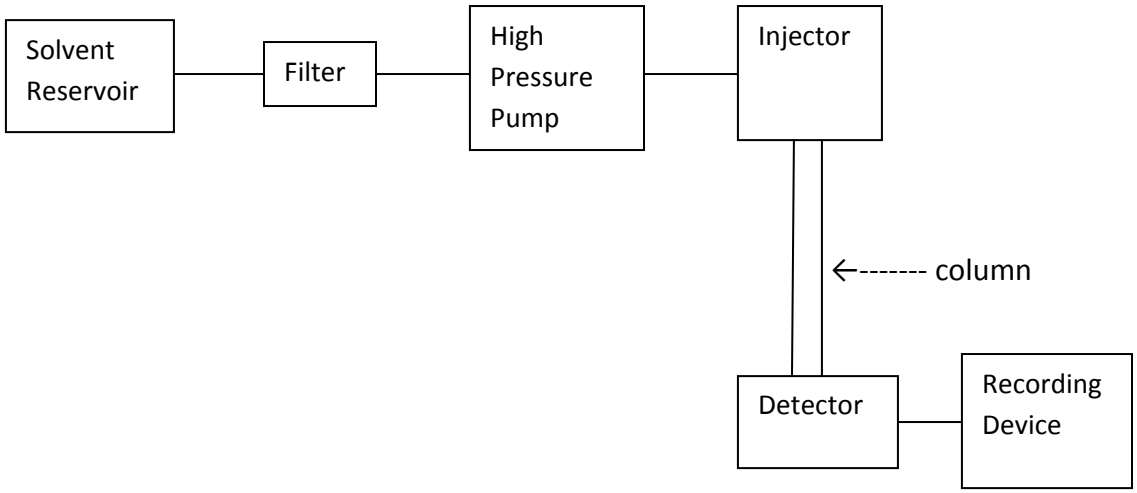


Figure 3. Schematic Diagram of a typical high performance liquid chromatography System.

Normal Versus Reversed Phase HPLC

Normal phase HPLC (NP-HPLC) involves the use of non-polar mobile phase and a polar stationary phase. Reversed phase HPLC (RP-HPLC), as the name implies, refers to the reversal of the normal phase chromatography. It employs the use of a polar mobile phase and a non-polar stationary phase in the chromatographic separation. Due to its simplicity and versatility, RP-HPLC can separate a broad spectrum of non-ionic, ionizable, and ionic compounds. Because the stationary phases are chemically bonded, columns are stable and separations are reproducible. In addition, due to the weak surface energies of bonded phases, analyses are rapid and re-equilibration time is short. It can be used to determine physiochemical properties such as hydrophobicity, dissociation constants, and complexation constants. The limitations of

the reversed phase technique include the fact that silica–based column packings limits the usuable pH range to 2–7.5. The unreacted silanol groups present on the silica surface can cause adsorption of the solute to the silica surface giving rise to poor peak shapes. The retention mechanism is more complex than in other forms of chromatography and a better understanding is needed to control it (25).

Isocratic Versus Gradient Elution

In isocratic elution, the mobile phase composition is kept constant throughout the separation. This method of elution is simple and convenient to use. It also gives good reproducibility of chromatographic data. There are also a lower assay costs and increased reliability in using isocratic method. In addition, when samples are separated using the isocratic method, the column is in equilibrium with the same mobile phase composition throughout the run, so the next sample can be injected as soon as the last band from the previous sample has eluted. These, among many others, account for the common use of isocratic assay for routine work (32). However, in separating samples containing components of widely different polarities, the isocratic method is ineffective and produces poor resolution. Gradient elution is thus preferred under such conditions. A typical gradient elution uses a weak solvent and a strong solvent as mobile phase. Elution is done by varying the strength of the mobile phase during separation by continuously changing the mobile phase composition (21, 24). Problems associated with gradient elution include drifting baselines, solvent demixing, and artifactual peaks that are a result of separating any UV-absorbing impurities in the mobile phase causing

appearance of peaks that do not correspond to sample bands. Also, in using gradient method re-equilibration of the column require a longer time (32).

Columns Used in HPLC

Column is an integral component of HPLC. It is sensitive and easily degraded by dust or particles in the sample or solvent and by irreversible adsorption of impurities from sample or solvent. Narrow column are usually used because they require less sample and produce less waste. They are also useful because heat generated by friction of solvent flow inside the column is more easily dissipated maintaining isothermal condition. The column is packed with small, rigid particles with narrow particle size distribution (25). Silica is by far the most commonly used adsorbent in HPLC (24). It is however ineffective at high pH because it dissolves in base (25). Column efficiency is expressed in terms of theoretical plate. It is a measure of the broadening of a peak during passage through the chromatographic column (21). The efficiency of a packed column increases with decreased particle size. Smaller particles size leads to higher plate number, high pressure, shorter optimum run time, and low detection limit. However, small particle size can cause resistance in solvent flow leading to high pressure. Typical particle size in use today in HPLC columns is 3-5 μm (24).

Controlling column temperature is critical in HPLC. Heating the column usually decreases the viscosity of solvent and allows faster flow. It also decreases retention time and improves resolution by hastening mass transport of solutes. However, increased temperature can degrade the stationary phase and decrease column life time (25). Example of analytical

column used in reversed phase HPLC include C_{18} (ODS), C_8, phyenyl, trimetylsilyl (TMS), and cyano (33).

Detector Used in HPLC

A detector is a useful component of the HPLC system. It produces a signal that is dependent on the concentration of sample and relays it on a data processor for display and storage. Its performance includes the ability to detect a component over the background of event and matrix signal, including noise. This property is referred to as selectivity (21). Another important parameter of the detector is the detection limit, which is the minimum concentration of analyte that can be detected with a given confidence limit. Detector noise may result from instrument electronics, line voltage surges temperature fluctuations, flow changes, and pulse from the pump causing a change in the output signal of the detector that is not directly attributed to the analyte. Other parameters are detector drift, which is a steady movement of the baseline either up or down scale, and absolute sensitivity of the detector (34).

Detectors used can be classified into two categories; the bulk property or general detectors and solute property or selective detectors. The former measures the difference in some physical property of the solute in the mobile phase compared to the mobile phase alone. Examples of this kind of detector include refractive index, dielectric constant, and conductivity detectors. The solute property or selective detectors respond to a physical or chemical property of the solute that, ideally, is independent of the mobile phase. Examples of these detectors are spetrophotometric, electrochemical, and fluorescence detectors (24,34).

Differential refractometer detectors measure the changes in refractive index of eluent as a result of the presence of solutes as they come out from the column. It is rugged and is able to detect concentrations of about 10^{-5} to 10^{-6} g/mL. However, it cannot be use effectively with gradient elution (due to a change in base line) nor when the solvent has a refractive index close to that of the solute. It is also highly sensitive to temperature changes.

UV detectors have good sensitivity to 10^{-8} g/mL and is not temperature sensitive. It is also relatively inexpensive and can be used with gradient elution. In addition, it is sensitive to a large number of organic compounds. However, it cannot be used with solvent that have significant absorption in the UV region or with sample component that do not absorb in the UV region.

In diode array detector, the focus radiation source passes through the detector flow cell and is dispersed by grating to a photodiode array for detection. The detector can record absorption spectra instantaneously and provides an additional resolving power.

UV-Vis detectors are selective detectors. They are the most commonly used detectors for HPLC and are based on ultraviolet and visible spectrophotometers. The underlying principle of the operation of this detector is the Beer Lambert law that relates absorbance to concentration. Because the path length and absorptivity for a particular compound in a given detector are constant, absorbance only depends on the concentration. Examples of lamps used in UV detection include, cadmium, mercury, and zinc discharge lamps. The advantages of the UV-Vis absorbance detector includes high selectivity, high sensitivity (10^{-10} – 10^{-11}g), and easy operation. The detector is nearly universal and has low background with many HPLC solvent,

allowing gradient elution without excessive background drift. However, in using the UV-Vis detector, analyte must have absorbance in the ultra violet or visible region. Another limitation is that it cannot operate at wavelength below the UV cut off of the solvent. Also, at a given wavelength, response varies between molecules based on their absorptivity (21).

Other detectors that are also used in HPLC are fluorescence and amperometric detectors. Fluorescence detectors can provide selectivity over the UV absorption detectors and they have good sensitivity. Amperometric detectors are useful in detecting electroactive substances and have wide biological application (35).

Resolution of Bands

The extent to which two bands are disengaged from each other determines the quality of a chromatographic separation. This is referred to as resolution. It is defined quantitatively as;

$$R_s = 2(t_2 - t_1)/ (W_2 - W_1) \qquad [4]$$

where t_1 and t_2 are retention times of adjacent bands, W_1 and W_2 are the peaks width measured at their respective bases in the same time units as the retention time. Bands that overlap have small values of R_s, whereas those that are totally separated from each other have big R_s values. Resolution is dependent on retention, selectivity, and efficiency of separation. While retention refers to the ability of a molecule to interact with the stationary phase or the affinity of the molecules for the matrix, selectivity is the discriminating power of the matrix that

produces differential interaction for at least two compounds. Efficiency refers to the ease of movement of the solute through the column. These factors are related as

$$R_s = N/4\ (\alpha - 1)(k_1/1+k_1) \qquad [5]$$

where $(\alpha - 1)$ is the selectivity term that becomes $(\alpha - 1)/\alpha$ if the plates and capacity factors are associated with second peak. N is efficiency and k_1 refers to the retention term (21).

Method Development

This section focuses on establishing the best analytical conditions for sample determination. New methods may be necessary because of poor existing ones or the need to analyze a new analyte. Most often, new methods are derived from existing ones or from similar methods used in the literature (21). Method development may be by trial and error. This approach is inefficient and time consuming. In order to develop a good method, the chromatographer's indepth knowledge of the chemistry of the analyte and its matrix is paramount. Models based on properties of analyte have been developed in order to narrow down on the optimum conditions suitable for a particular analyte determination (25). This minimizes the trouble chromatographers go through during method development. Developing an HPLC method may be done by first varying the mobile phase composition with the aim of obtaining adequate resolution, a reasonable run time, and easily detected narrow bands. Other steps include varying the mobile phase pH, addition of surfactants, or changing the analytical column (33). The success of the new method is evaluated by validating its parameters such as robustness, linearity, accuracy, precision, and limits of detection (21).

The Use of Surfactants in HPLC

Some proteins and peptides are analyzed effectively in the presence of surfactants. These surfactants enhance their solubility and prevent aggregation (21). Surfactants are made up of a non-polar end that is usually a hydrocarbon responsible for their hydrophobic properties and a polar head that accounts for their hydrophilic behavior. Surfactants are grouped into anionic, cationic, amphoteric, and non-ionic compounds considering the charge on the hydrophilic group (36). Surfactants have the ability to adsorb on to aqueous solution at interfaces. When they adsorb onto hydrophobic surface, they normally orient their hydrophobic end towards the surface and expose the hydrophilic group towards the water making the surface hydrophilic. The surface tension between the surfaces is thus reduced (37). Surfactants are added to the mobile phase during HPLC determinations to reduce viscosity and surface tension. They increase the eluent strength and diffusion coefficient causing reduction band width (21). Tang and Deming studied the effect of surfactants in reversed-phase chromatography and reported that the addition of surfactant to eluent can reduce the interfacial tension and thus decrease the retention time of the injected sample (38). Although, surfactants in chromatography are useful, they should be used with caution as they can bind strongly on the column walls and consequently damage it (21). Examples of surfactants commonly used in high performance liquid chromatography are shown in Figure 4a and 4b.

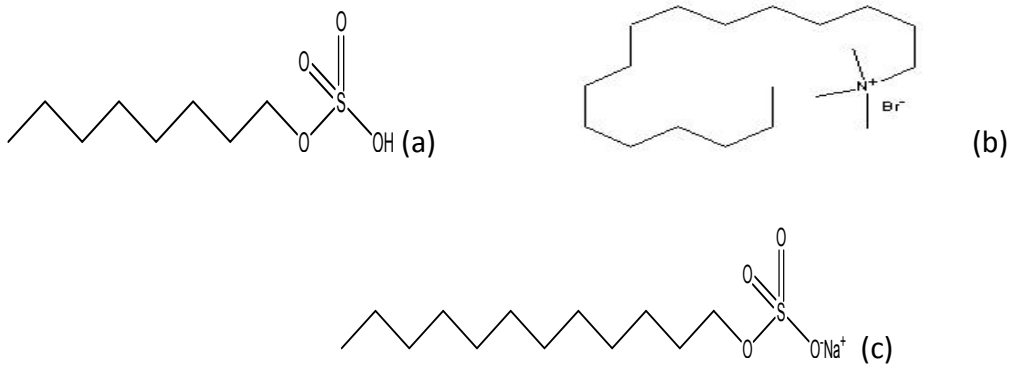


Figure 4: Example of surfactants commonly used in HPLC determinations, (a) 1-Octanesulfuric acid (OSA), (b) Cetyltrimethyl ammonium bromide (CTAB), (c) Sodium dodecyl sulfate (SDS)

Research Objective

From the literature and discussion above, the use of HPLC as a powerful analytical technique cannot be over-emphasized. Its role in the analysis of sulfoxides has thus been well examined and has been noted to provide a reliable quantitative and qualitative measurement (25).

This work seeks to develop a simple but efficient HPLC method for alliin determination. The objectives of this method are as listed below:

1. T o use a simple but effective extraction procedure to extract alliin in fresh garlic and garlic products.

2. To establish an optimum reversed phase HPLC conditions for alliin determination using :

    a) Suitable HPLC instrumentation.

    b) A mobile phase composition that is economical and environmentally friendly.

3. To establish the figures of merits of the proposed RP-HPLC method by verifying its linear dynamic range, reproducibility, and recovery.

4. To verify the applicability of the method in the determination of alliin in fresh garlic and commercial garlic products.

CHAPTER 4

EXPERIMENTAL PROCEDURE RESULTS AND DISCUSSION

Reagents

1. Optimum grade methanol and Hydrochloric acid used were ACS Certified and products of Fisher Scientific (Fair Lawn, NJ).
2. Surfactants; Sodium dodecyl Sulfate (SDS), Cetyltrimethylammonium bromide, (CTAB) and 1- Octane Sulforic acid were obtained from Sigma Chemical Company (St.Lousi, MO).
3. Alliin standard used was a product of Sigma Aldrich (Steinheim, Germany).

Plant Materials

Fresh garlic and garlic products were purchased from different stores in Johnson City Tennessee and Toledo Ohio. One fresh garlic sample was obtained from a backyard garden. Varieties of fresh garlic and garlic products used are shown below:

White Garlic – from Oriental Market in Johnson City

Garlic Tablets 1 – from Wal-Mart in Johnson City

Elephant garlic – from Toledo, Ohio

Organic garlic – from Toledo, Ohio

Pure white garlic – from Toledo, Ohio

Garlic Tablets 2 – form Kroger, Johnson City

Hybrid garlic – from a backyard garden

California garlic – from Food Lion, Johnson City

Experimental Procedures

Preparation of Reagents, Standard, And Stock Solutions

The extraction solvent used was 90:10 methanol: hydrochloric acid mixture. This was prepared and used as extraction solvent to inhibit enzymatic activity on alliin. In preparing the extraction mixture, 0.5 mL of concentrated hydrochloric acid was added to 450 mL methanol in a 500-mL volumetric flack and diluted to the mark.

The alliin standard solution was prepared by dissolving 10 mg of pure alliin in 100 mL of the methanol–acid mixture and saved in a bottle. A stock Solution of garlic was prepared by homogenizing about 10 g of garlic cloves in 25mL of the extraction solvent using a laboratory blender. This was carried out for 5 minutes and the homogenate was quantitatively transferred into a 250-mL beaker using 40 mL of the methanol – acid mixture. It was then sonicated for another 5 minute to mix well. The homogenate was filtered by gravity into a 100 mL volumetric flask and the residue washed three times, each with 10 mL of the solvent. The filtrate was made up to the 100 mL mark to obtain the stock solution of each sample.

The same steps were followed using boiled water as extraction solvent and the stock solutions prepared as well as that of the methanol-acid extracts were saved in labeled bottles and kept in a laboratory refrigerator until analysis.

Preliminary Gas Chromatography-Mass Spectrometry (GC-MS) Studies

Preliminary determinations of alliin in the garlic extracts prepared were performed using Hewlett Packard Model 5890 Series II Gas chromatograph equipped with Series 5971 mass selective detector. These were done using HP5MS column of dimensions 30 m x 0.25 mm and 0.25 µm film thickness with polar stationary phase consisting of 5% phenyl and 95% dimethyl polysiloxane. The instrument was operated at 170 ^{0}C injection temperature, 180 ^{0}C interfaces temperature and ion source temperature of 200 ^{0}C. The temperature program on the instrument was varied in an attempt to get the optimum conditions for the alliin determination. Figures 5 and 6 presents samples of chromatograms and mass spectra obtained in the preliminary studies.

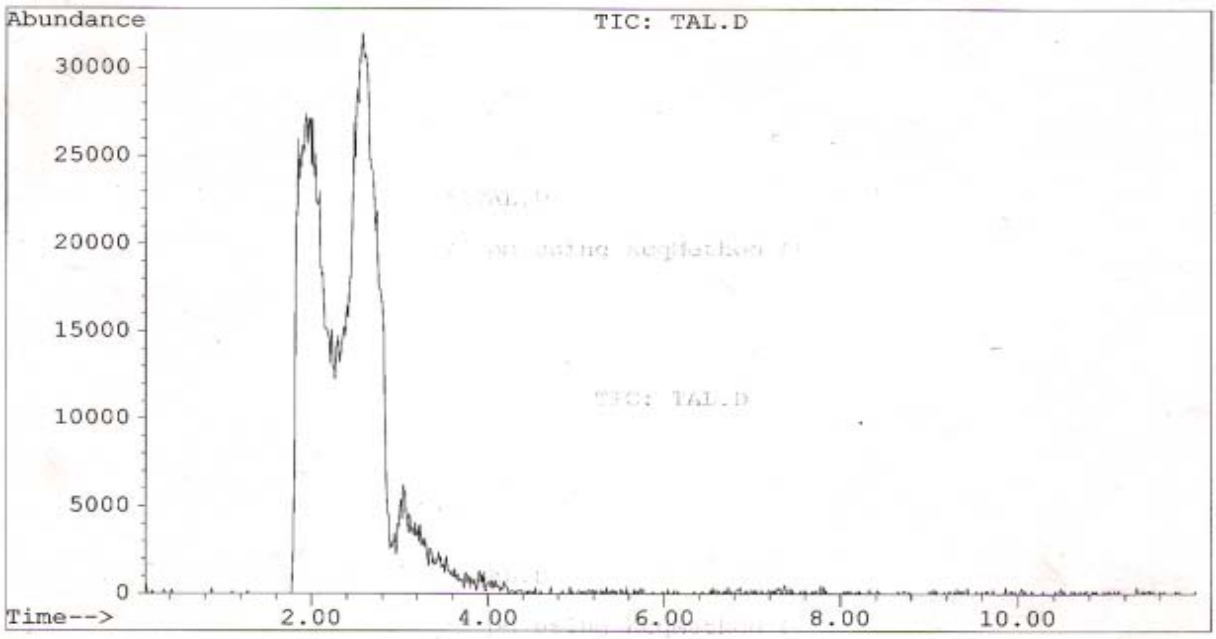


Figure 5. GC-MS chromatogram of methanol extract of garlic.

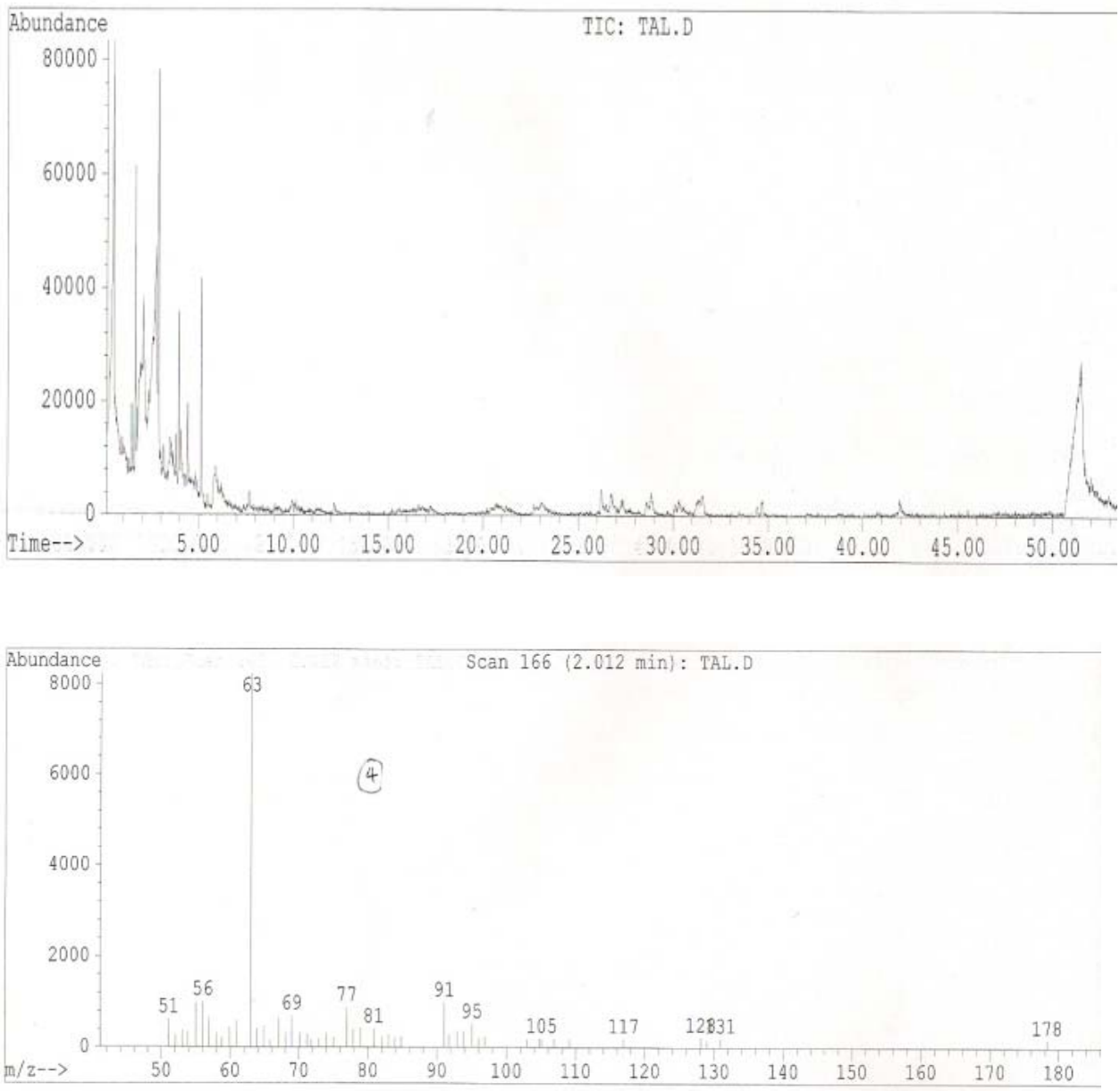


Figure 6. GC-MS Chromatogram of methanol extract of garlic. Temperature program; linear increase from 170 ^{0}C–300 ^{0}C at 3 ^{0}C/min; then 300^{o}C–350 ^{0}C at 10 ^{o}C/min and kept constant for 10 minutes.

The GC-MS procedure used in the preliminary studies proved to be futile as the chromatograms obtained did not give good representation of sulfoxides in the extract. The procedure yielded inconsistent results and poor resolution of bands. This may be due to the

assertion that sulfoxides and thiosufinates are thermally unstable and may decompose into several sulfur compounds at high temperature (19, 25, 36). Thus the GC-MS appeared not to be a technique of choice for alliin determinations in the garlic extracts prepared.

HPLC Instrumentation

Shimadzu LC–IOAS system with SPD–1OA UV – VIS detector, a product of Shimadzu Scientific Instrument Incorporated, 7102 Riverwood drive Columbia, MD 21046 was used. It has a scan range of 190–300 nu and SCL–1OAVP system controller. Reversed phase HPLC conditions including C_{18} 5 µm Disc column of size 3.9 X 150 µm with column temperature set at 25 ^{o}C. The UV–VIS detector was set at 210 nm after several trials. All HPLC separations were done with isocratic elution at a flow rate of 1.0 mL/min and an average pressure of 400–1600 psi.

Data Analysis

The experimental data obtained were analyzed mainly with Microsoft Office Excel 2007 software. The software was used to calculate averages of the instrumental response for triplicates runs as well as relative standard deviations. It was used in making calibration plots of alliin standard solutions showing error bar within 5% value. The software was also used to calculate the alliin concentrations in samples with the aid of the calibration curve.

Statistical studies of data were done by analysis of variance (one way ANOVA) using Minitab and SPSS softwares.

Results and Discussion

Following the results obtained in the preliminary studies and the recommendations published in literature that HPLC presents a suitable approach in determining sulfoxide in garlic extracts (26, 29), an HPLC method was developed and used in alliin determination. This section discusses the results obtained starting from the method development through to the application of the method in alliin determination.

Method Development and Validation

Developing the best analytical conditions for a reversed phase HPLC determination was an integral portion of the entire work. In doing so, much emphasis was on simplicity of method without compromise on its efficiency. In addition, the use of reagents that are environmentally friendly and economical was vital at this stage. Preliminary experiments were done to develop a method for alliin determination. These include wavelength optimization and mobile phase optimization. After these experiments, figures of merits such as reproducibility, linearity, and recovery studies were done to evaluate the proposed method.

Optimization of Wavelength

Ultra violet-visible (UV-Vis) detection of alliin has been reported at the wavelength range of 205 nm -280 nm. (17, 29, 29). Detection at these wavelengths was explored using alliin standard solutions and the optimum wavelength that gave a high absorption peak was 210 nm. This was used in the method development and subsequent determinations.

Optimization of Mobile Phase

A suitable solvent for HPLC analysis must be readily available in a pure form and have low viscosity. It must also be compatible with detection system and have low flammability and toxicity (25). In this study, the mobile phase composition for the determination of alliin in garlic samples was explored with the aim of finding the optimum mobile phase that will give the best separation. It is known that a change in percent organic composition often leads to significant changes in separation factor, α, for reversed phase HPLC and this provides the easiest way of optimizing band spacing (33). Based on this fact, mobile phase with different solvent strengths beginning from 80:20 , 70:30 , 60:40 , 55:45 , 30:70 , to 20:80 methanol: water compositions were used. Methanol was used because it has low viscosity and UV transparency. It is also totally miscible with water which is readily available (33). Each mobile phase prepared was degassed by passing helium gas through it for 15 minutes and then purged before it was used in the optimization studies. Alliin standard solutions and aliquots of garlic extracts were run with these mobile phase preparations on the reversed phase HPLC instrumental conditions mentioned previously using an injection volume of 20 µL and their chromatograms were compared.

Figure 7 shows the chromatograms of garlic extract obtained by running samples at a flow rate of 1 mL/min at 210 nm wavelength using 80:20, 70:30 ,and 60:40 methanol:water mobile phase compositions.

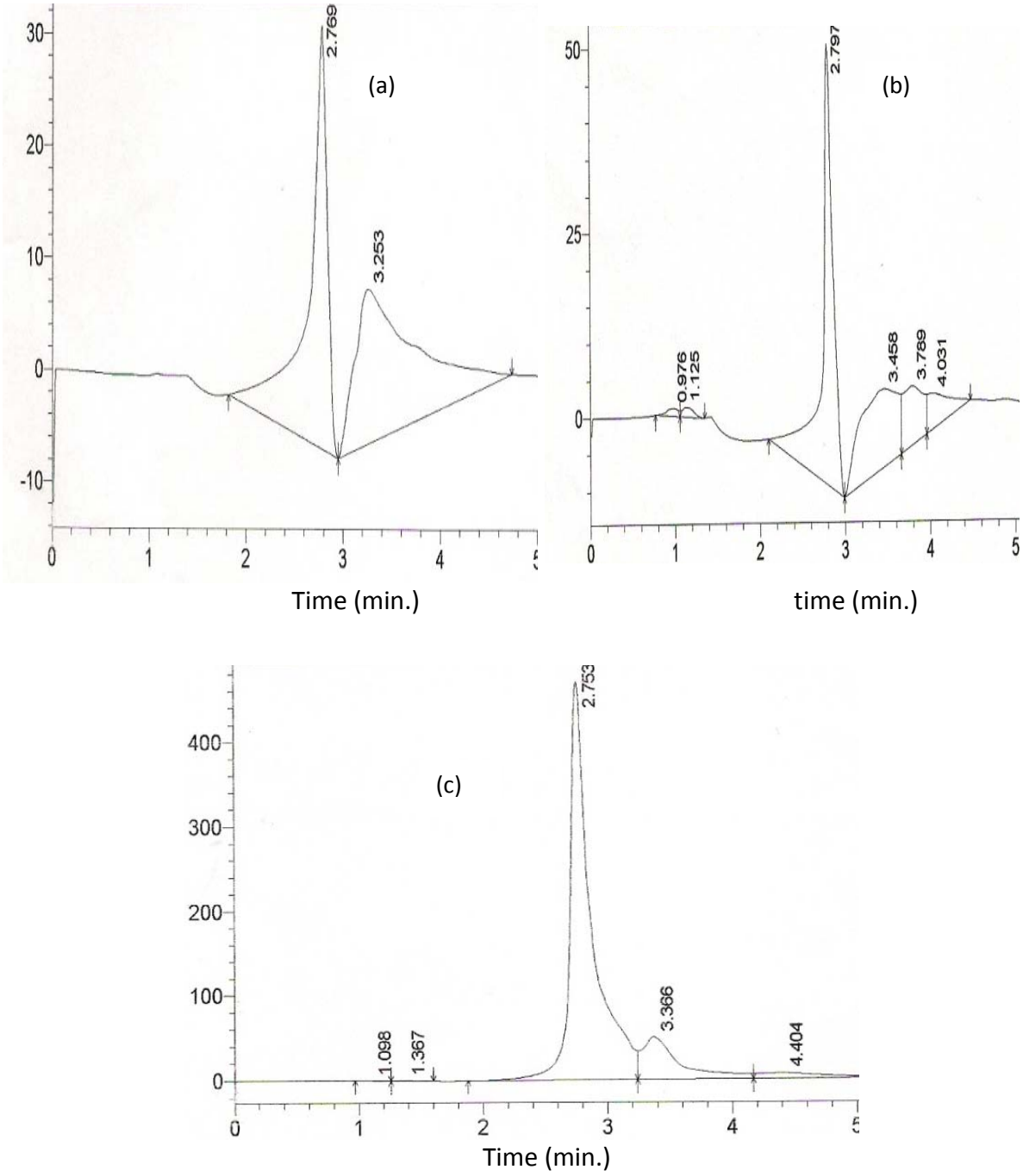


Figure 7. HPLC chromatograms of methanol-acid extract of garlic using (a) 80% methanol: 20% water, (b) 70% methanol:30% water mobile phase compositions. (c) 60:40 methanol:water mobile phase composition. Samples were run at 1mL/min at 210nm wavelength

Using these mobile phases, all the chromatograms obtained had very good run times of about 5 minutes with the alliin peak eluting between 2.5-3.0 minutes. However, the major problem with these mobile phases was poor resolution of bands. In the chromatogram (a) of Figure 7 in which elution was done with the highest methanol concentration, only two noticeable bands were observed. As the concentration of the methanol in the mobile phase was decreased other peaks showed up in the chromatograms but these peaks were wide and overlapped with each other. Also very prominent was the occurrence of negative peaks appearing as an extension of the suspected alliin peak in (a) and (b). Varying the solvent composition from more methanol to less methanol, representing a decrease in solvent strength, improved the band resolution and eliminated the negative peak extension. These comparisons showed that decreasing the solvent strength could improve baseline separation and give better chromatograms. This observation is in line with known fact that mobile phase more than any other variable has a major effect on band resolution (33).

Optimization of mobile phase pH

The pH of various mobile phases was adjusted using glacial acetic acid buffer. Starting from a mobile phase composition of 80:20 through to 20:80 methanol:water ratios, the acid buffer was added to study the effect of pH on the separation. It was observed that addition of the buffer showed no significant improvement in the resolution of the peaks. Instead, at low pH (less than 3), a sharp negative peak was noticed adjacent to the alliin peak and this interfered with the alliin peak area.

Optimization of mobile phase with surfactants

Surfactants are known to reduce band widths because they increase both eluent strength and diffusion coefficients (21). Sodium dodecyl sulfate is an anionic surfactant. It aggregates in dilute aqueous solution to form micelles at a characteristic concentration called critical micelle concentration of 8.6 at 40 ^{o}C temperature. In presence of buffer the critical micelle concentration reduces to 4.6 at 30 ^{o}C (39). Cetyltrimethyl ammonium bromide (CTAB) is a cationic surfactant and appears as white crystal powder. The critical micelle concentration of CTAB increases with increased concentration of methanol under room temperature (32, 40, 41).

Sodium dodecyl sulfate (SDS), Cetyltrimethyl ammonium bromide (CTAB), and 1–Octane sulfonic acid (OSA) were added to the mobile phase to reduce solvent viscosity and surface tension. The best elution of garlic components was observed when samples were run using 30% methanol:70% water, and 0.05% sodium dodecyl sulfate. This gave the best resolution of bands compared with all the other trials. The run time was very short and there were no negative peaks. Standard solutions of alliin were also run and the alliin peak was deduced to elute at a retention time of 2-3 min. Figure 8 shows the chromatograms of garlic sample run using the various methanol:water: surfactant mobile phase mixture.

The addition of cetyltrimethyl ammonium bromide and 1– Octane sulfonic acid gave broad peaks and poor base line separation. Thus,the optimum mobile phase composition for alliin determination was chosen to be 30% methanol:70% water, and 0.05% sodium dodecyl sulfate. The pH of the mobile phase was determined to be 3.5.

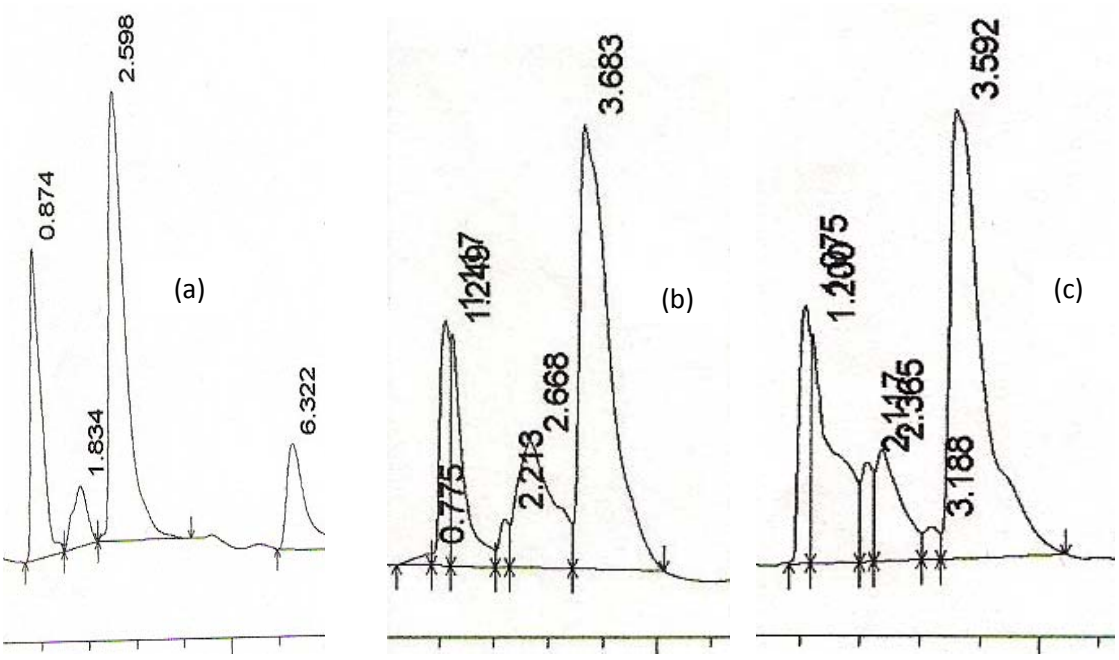


Figure 8. HPLC chromatograms of methanol-acid extracts of garlic using (a) 30 % methanol:70% water and 0.05% SDS; (b) 30% methanol:70% water and 0.05% CTAB; (c) 30% methanol:70% water and 0.05% OSA phase compositions. Samples were run at 1 mL/min at 210 nm wavelength

Reproducibity Studies

Repetition of a method of measurement several times reveals how reproducible the method is. This figure of merit is precision. The precision of an analytical method is vital in measurement, especially in routine work. In this study, the precision for the quantitative method was demonstrated with two sets of solutions, one at high and another at low concentrations of analyte. For the sets of solutions of high analyte concentration, eight aliquots of working solutions were prepared by pipeting 600 µL of stock garlic solution into eight 5-mL volumetric flasks and diluted with distilled water to the mark. Each aliquot of this solution was run on the reversed phase HPLC in triplicate and the alliin retention times as well as the peak

areas were recorded. The procedure was repeated using 10 µL of the stock garlic solution instead of 600 µL and the peak areas were compared. The averages and relative standard deviation for each triplicate run were calculated for all eight samples. These eight averages were then averaged again and the relative the relative standard deviation calculated. These results for both the high and the low analyte concentrations are tabulated in Table 1.

Table1: A summary of results obtained from reproducibility studies of the method for the measurement of alliin content in garlic samples. Trial 1 is the result for the higher analyte concentration while Trial 2 is for lower analyte concentration.

Overall	Trial 1	Trial 2
Mean	1.73 E+07	2.32 E+06
Relative standard Deviation	4.11%	0.56%

The results showed that the method gave excellent precision. The relative standard deviations were 4.11% and 0.56%, for the sample with the higher and lower analyte concentrations respectively. It was quite unexpected that the set of samples with lower concentration gave better precision. Overall, the results indicated the proposed procedure was feasible for the determination of alliin in a sample, even at very low concentration of analyte.

Linearity Studies

Linearity measures how linear the response of analyte is in relation to its concentration (25). The linear dynamic range of the method was studied using different concentrations of alliin standard solution. Into different 5-mL volumetric flasks, 10 µL, 500 µL, 1000 µL, and 2000

µL of alliin standard solutions were delivered and the flasks filled up to the mark with distilled water. Three aliquots of each solution were prepared and run on the reversed phase HPLC in triplicate and their peak areas were recorded. Linear relationship between concentrations and corresponding peak areas was obtained as shown in Table 2.

Table 2: Results of linearity studies showing linear relationship between concentration and instrumental response. Note: Three aliquot of each concentration were prepared and run in triplicates. The average peak areas are shown.

Standard (std.)ID	Volume of standard in 5 mL solution (µL)	Concentration (ng/mL)	average peak area
1	10	0.4	1.69E+04
2	500	20.0	8.85E+05
3	1000	40.0	1.68E+06
4	2000	80.0	3.40E+06

From the data tabulated, linearity of the measurements was demonstrated graphically in a calibration plot as presented in Figure 9. Regression equation and correlation coefficient were calculated from the calibration curve using Microsoft Office Excel 2007.

A common measure of linearity is the correlation coefficient, R^2 for the equation of the line. For a calibration curve to be considered linear R^2 must be close to one and the intercept of the calibration curve should be close to zero (25). Based on these criteria the method yielded linear response with varying concentrations, showing a good correlation coefficient

of 0.9998 and equation of the line y = 42272x +10833.

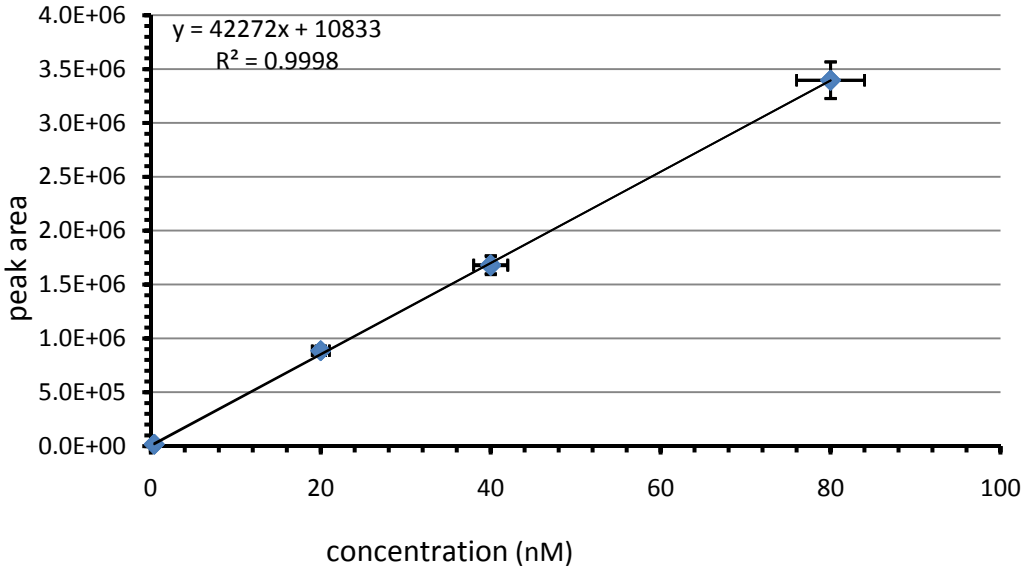


Figure 9. A plot of calibration curve of Alliin standard solution showing linearity of method. The plot displays error bar with 5% value

The calibration curve presented in Figure 9 also shows error bars that give a general indication of how accurate measurements are. These have been displayed within 5% value using the excel software. By inspection, the error bars show that the measurements made in the linearity studies were satisfactory and are within experimental error.

Recovery Studies

In the recovery studies, the accuracy of the developed method was accessed and verified. Accuracy describes the closeness of the value or result determined to the expected value. It is measured as the percentage of analyte recovered using spiked samples (21).

Two different samples were used in the recovery experiment, the white garlic and garlic tablet. For the white garlic sample, nine aliquots of the working solutions were prepared by pipeting 10 µL of stock solution into 5-mL volumetric flasks and diluted to the mark with distilled water. To three of these aliquots, no standard solution was added. To another three, 50 µL each of standard solution was added. To the last set of three, 100 µL each of standard solution was added. The same procedure was repeated with the garlic tablet. However, this time 0 µL, 20 µL and 40 µL standard solutions were respectively added to the working solution. A standard calibration curve was also obtained by running different concentrations of alliin standard solutions. Table 3 shows data obtained for triplicate runs of alliin standard solutions and the resulting calibration curve is presented in Figure 10.

Table 3. Standard calibration data used in the recovery studies. Note: Three aliquot of each concentration were prepared and run in triplicates.

Standard solution ID (std.)	Volume of Std. in 5 mL solution(µL)	concentration (ng/mL)	alliin peak area
Std1 run1	500	20.0	1671880
Std1 run2	500	20.0	1684239
Std1 run3	500	20.0	1672978
Std2 run1	1000	40.0	3449196
Std2 run2	1000	40.0	3489779
Std2 run3	1000	40.0	3469160
Std3 run1	2000	80.0	5280206
Std3 run2	2000	80.0	6233435
Std3 run3	2000	80.0	6241045

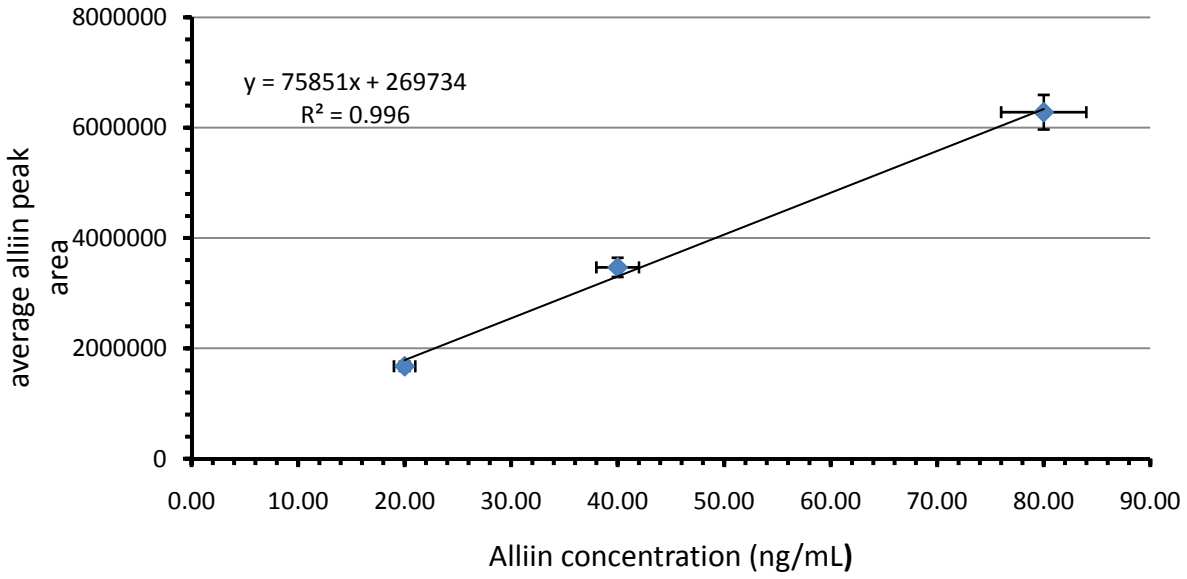


Figure 10. Standard calibration curve used in recovery studies displaying error bar 5 % value.

Using the calibration curve the data obtained for the recovery studies for both types of garlic samples were analyzed. The amount of alliin each aliquot of the garlic sample to which no alliin standard solution had been added were calculated. Similarly, the total amount of alliin all the spiked samples were calculated. From these results the amount of alliin added to the original aliquots of the garlic samples were calculated. The calculated amount of alliin added over the actual amount added multiply by 100% give the percent of recovery. The result of these calculated recoveries are tabulated in Table 4 for the white garlic sample and Table 5 for the garlic tablet sample.

Table 4. Results for recovery studies carried out with high concentration of standard solution. The sample used in these studies was white garlic and experiments were done in triplicate.

Sample Id	Amount added (ng/mL)	Amount recovered (ng/mL)	% recovery	Relative standard deviation (%)
Sample + 50 µL standard solution	1.98	2.01	102 %	1.23
Sample + 100 µL Standard solution	3.92	3.94	101 %	0.36

Table 5. Results for recovery studies carried out with low concentration of standard solution. The sample used in these studies was garlic tablet 1 and experiments were done in triplicate.

Sample Id	Amount added (ng/mL)	Amount recovered (ng/mL)	% recovery	Relative standard deviation (%)
Sample + 20 µL standard solution	0.80	0.73	91.3 %	5.76
Sample + 40 µL standard solution	1.59	1.52	95.6 %	2.99

As shown by the results obtained in the recovery studies tabulated in Table 4 and 5, the proposed procedure yielded satisfactory recoveries. That is, the accuracy of the proposed method was good.

The recovery for white garlic samples was very good with very small experimental error of 0.36%-1.23%. The results from the experiments performed on the garlic tablet were not as good but even still acceptable and within the experimental errors. The reason for the

difference may be in the smaller amount in the alliin standard added to the aliquots of the garlic tablet sample. Thus, the error was larger and recovery not as good as the one performed on the white garlic sample.

In all, the recovery studies presented an interesting result in that when higher concentrations of the standard solutions were added to both samples, the amount recovered improved. This was, in fact, evident with the same sample and between the two samples. Thus the accuracy of the method was found to be better at comparatively high concentrations of the analyte.

Comparing Methanol-Hydrochloric acid (MeOH-HCl) Extract With Hot Water Extract

From the chemistry of garlic, we learned that alliin is converted to allicin by the enzyme alliinase when the garlic cell is crushed. Allicin then decomposes rapidly into other sulfur compounds (3, 8). This means that in order to identify and quantify alliin in garlic samples, there is the need to irreversibly inhibit the enzyme activity on alliin. Inhibition of alliinase activity in garlic extract has been done by either extracting samples with methanol-hydrochloric acid mixture or the use of hot water as extracting solvent (30). In the methanol-hydrochloric extraction, the hydrochloric acid provides an acidic medium unfavorable enough to hinder the activity of the enzyme. A number of studies have revealed examples in which sulfoxides had been extracted with methanol-acidic solution. The acid provides an acidic medium in order to irreversibly inhibit alliinase activity at a pH value below 3.6, whereas high methanol concentration in the extracting solvent protects the analytical column due to decrease in solubility of carbohydrates and proteins in garlic (20, 30).

Ichikawa and his colleagues (30) explored and evaluated the effect of hydrochloric acid and methanol concentrations on extraction efficiency of organosulfur compounds and reported that increasing methanol concentration resulted in a decrease in extraction efficiency of γ-glutamyl peptides in the presence of 0.001 M HCl. They attributed this to solidification of prepared samples that decreases the solubility of polar compounds. They also realized that at high HCl concentrations (i.e. 0.01 and 0.1 M HCl) samples extracted with 80% or 90% methanol showed higher contents of γ-glutamyl peptides than those with 95% methanol. They explained that this might be due to enhancement of solubility in all concentrations of methanol by suppression of ionization of γ-glutamyl peptides at high HCl concentrations. According to them, the sample extracted with 90% methanol and 0.01 M HCl resulted in the highest contents of sulfoxides compared with that of other extraction solvent compositions.

Hot water extraction of alliin in garlic seeks to elevate the temperature of the garlic extract above the optimum temperature of 35 ^{o}C–37 ^{o}C to irreversibly inhibit the enzyme's activity. The denaturing of enzyme begins at 42 ^{o}C and temperatures above 60 ^{o}C inactivate it (43). In light of the research objective of going green and cutting down on cost without compromising the efficiency of the method of alliin determination, the two extraction procedures were compared. To do this, 10 µL of MeOH–HCl extracts of white garlic sample were pipetted into a 5 mL volumetric flask and diluted to the mark. This was run on reversed phase HPLC using 30:70 MeOH: H_2O and 0.05 % SDS mobile phase. Water was heated to boiling and was used to extract alliin in garlic using the same procedure described for methanol

–acid extraction. Aliquots of hot water extract of the same sample also were prepared and run the same way and their chromatograms were compared.

Methanol–hydrochloric acid extract yielded satisfactory results from the method developed. However, the hot water extract did not. This is evident in the chromatograms presented in Figure 11.

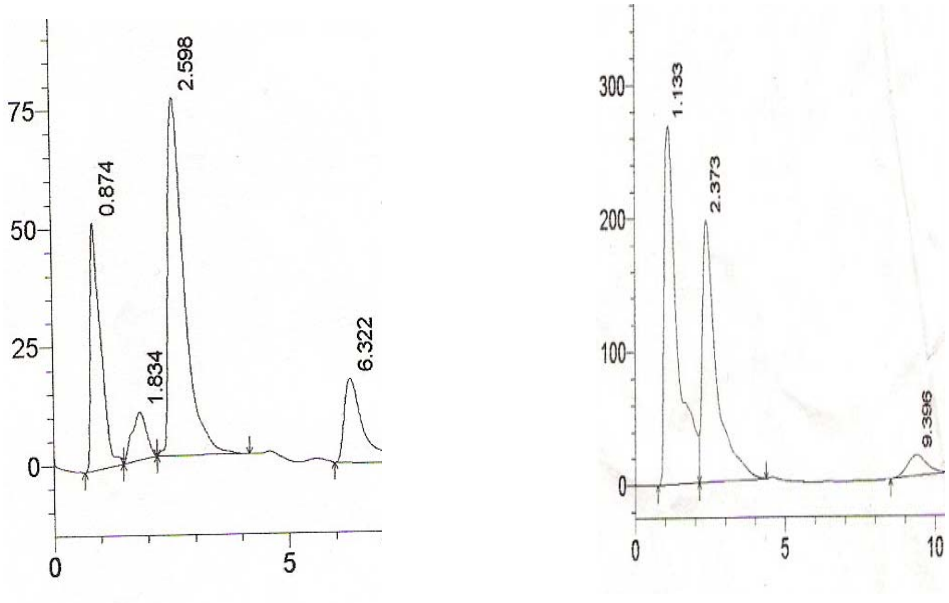


Figure 11: Chromatogram of garlic sample extracted with (a) 90% Methanol-0.01M Hydrochloric acid compared with (b) chromatogram of hot water extract.

As can be seen from the chromatogram in Figure 11b, the hot water extract gave poor peaks separation and resolution. Thus, hot water extract, though cheaper and simple, is not the best extraction procedure for the proposed method. Hence, all the extractions for alliin determination in fresh garlic and garlic products were done using methanol–hydrochloric acid mixture

Application of Method

The method for alliin determination involves the use of extraction solvent made up of 90% methanol and 0.01 M HCl as well as a mobile phase composition of 30% methanol:70% water:0.05% sodium dodecyl sulfate. Separation and quantification of alliin were done on 5 µm C_{18} column in Shimadzu LC system connected to a UV–Vis detector with optimum wavelength selected to be 210 nm and a flow rate of 1 mL/min.

Eight fresh garlic samples and garlic products were analyzed. Each garlic sample was extracted and prepared and triplicate aliquots were injected into the HPLC. The resulting chromatographic data were used with the help of the calibration curve to calculate the amount of alliin in 1 g of each garlic sample. Figure 12 shows chromatograms of white garlic and elephant garlic cloves as well as that of alliin standard.

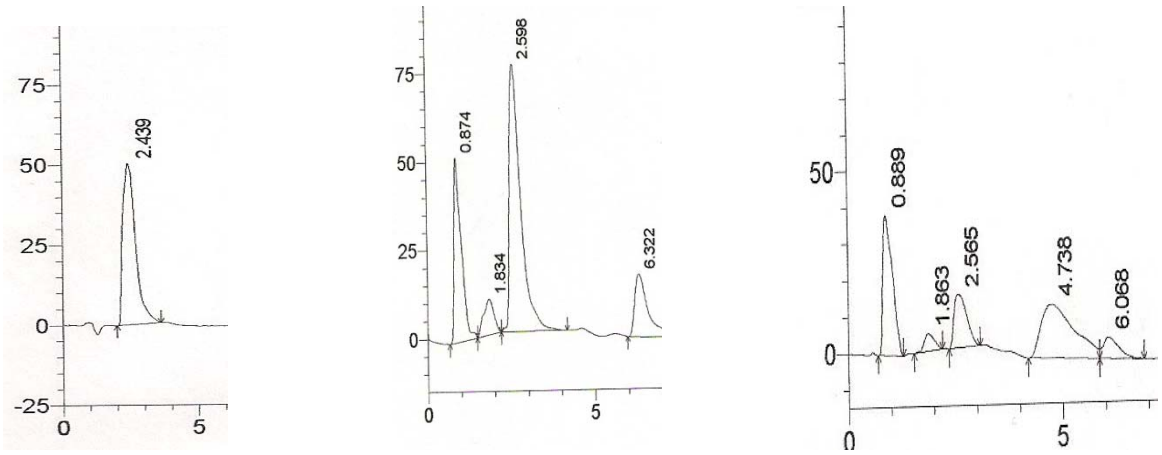


Figure 12: Chromatograms of (a) alliin standard in (b) white garlic and (c) elephant garlic obtained using 30 % Methanol:70% water and 0.05% Sodum dodecyl sulfate. Alliin elute at 2.4-2.6 minutes.

The retention time of the alliin peak was recorded between 2.4–2.6 minutes. From the chromatographic data the concentrations of alliin in the various fresh garlic and garlic products were calculated with the help of a calibration curve. These results have been tabulated and are presented in Table 6.

Table 6: Concentration of alliin in diverse varieties of fresh garlic and garlic product.

Varieties of garlic	Mass of garlic	Concentration (ng/mL)	mg of alliin/g of garlic clove
Pure white garlic	10.4030	43.2	0.207
White garlic	10.2237	57.3	0.281
Elephant	10.1753	7.5	0.037
Organic	10.3695	5.2	0.025
California	10.9683	0.6	0.003
Hybrid	10.6473	30.1	0.141
Tablet 1	10.5531	76.2	0.361
Tablet 2	10.1147	9.9	0.049

The data were subjected to statistical analysis using analysis of variance (one-way ANOVA) and the results showed significant differences among alliin content in the varieties of garlic at 95% confident limit. Tablet 1 recorded the highest alliin content indicating a concentration of 0.361 mg/g of the garlic sample. However Tablet 2 recorded a lower alliin concentration of 0.049 mg/g of the garlic sample. This was so because the two are different samples with different brand names and were manufactured under different conditions and

specifications. White garlic recorded relatively higher amount of alliin than the pure white garlic. These are however the same garlic variety but purchased from different stores in different cities (Johnson City, TN and Toledo, OH respectively) at different times. The expression 'pure" was used to distinguish between them. The possible reason for this difference might be the effect of environmental factors on the two samples. These samples, though the same variety, were grown under different environmental conditions (temperature, humidity, soil composition) as well as different farming practices (use of sulfur containing fertilizer), and were also kept under different storage conditions before purchase. The difference in the period of time that these samples have been on the shelf might also have affected the alliin content. The hybrid type of garlic that was locally grown in a back yard garden yielded 0.141 mg of alliin per a gram of the garlic clove, an amount greater than that of Elephant garlic that gave an alliin content of 0.037 mg/g of fresh garlic clove. Alliin content of the hybrid was also more than that of the organic variety and garlic tablet 2. It was even so much more than that of California garlic that recorded the least concentration of alliin (0.003 mg/g of fresh garlic). This observation encourages small scale cultivation of garlic where expensive and sophisticated storage facilities are not required to preserve the vegetable in order to retain essential chemical constituents such as alliin. Alltogether, the differences between the alliin levels can be attributed to genetic and environmental factors. A graphical distribution of alliin levels in the various samples is presented in Figure 13.

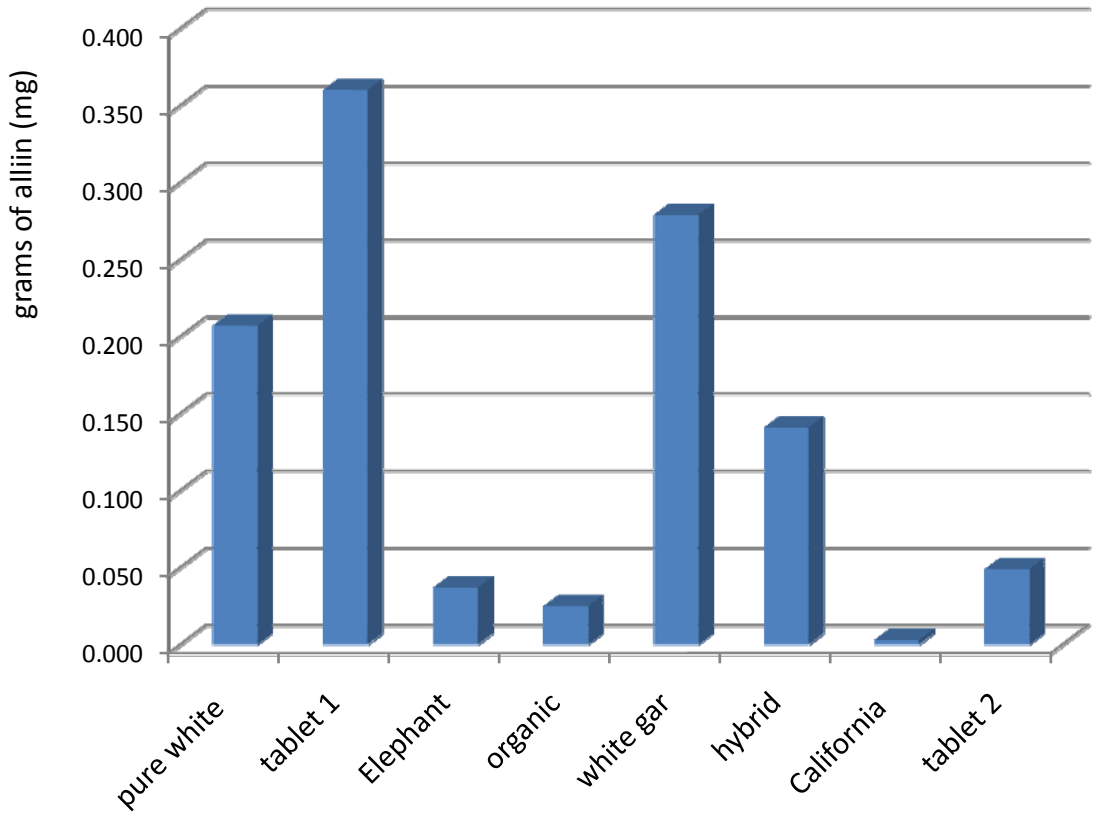


Figure 13. Histogram showing the distribution of alliin in diverse varieties of garlic and garlic products

Figure 12 gives a pictorial comparison of alliin content among varieties of samples. For example, the alliin content in the hybrid variety represent less than one half of the amount in tablet 1, with tablet 2 forming about a quarter of the content in pure white garlic. Alliin concentration in the organic variety comes next after that of the California cultivar that has the least amount of the analyte.

In all the method gave a good quantification of the analyte in the various varieties and can be useful in routine routine work.

CHAPTER 5

CONCLUSION

Developing an HPLC method that is economical, efficient, and green to be used in alliin determination is useful due to the enormous medicinal value of the compound. In this study, a simple reversed phase high performance liquid chromatographic method was developed. The proposed method works best with methanol–hydrochloric acid extract. This approach of extracting alliin in garlic cloves is simple and can be accomplished in one step. It involves homogenizing garlic sample with the extraction mixture and filtering it to obtain garlic extract containing alliin rather than allicin that is unstable. The use of hydrochloric acid in the extraction solvent provides an acid medium to inhibit alliinase activity, whereas the methanol protects the analytical column (30). Hot water extract of garlic does not work well with the method developed. This yielded broad peaks with poor resolution.

The HPLC method developed utilizes the mobile phase composition of 30% methanol:70% water with 0.05% sodium dodecyl sulfate. The mobile phase is economical and more green because water is readily available and the use of large volumes of it reduces the toxicity of methanol drastically. It is efficient because methanol has low viscosity and UV transparency and is totally miscible in water (33). The addition of the surfactants increased eluent strength and diffusion coefficient and consequently reduced bandwith (21). However addition of pH buffer showed no significant effect or improvement on the separation.

The alliin peak was noted at a retention time of 2.0–3.0 minutes and was identified in comparison with alliin standard. Reproducibility of method was good ranging between relative standard deviation of 0.56%–4.11%. The method exhibited good linearity with correlation coefficient of 0.9997. Accuracy of method was satisfactory showing average recovery of 93%–101%. The use of the method in alliin determination in fresh garlic and commercial garlic products was successful. Garlic tablet 1 recorded the highest level of alliin giving a concentration of 7.19 ng/mL in 1 g of the tablet. California garlic had 0.003 mg of alliin per 1 g of the garlic clove. The differences in alliin levels found could be attributed to genetic and environmental factors such as temperature. Different agricultural practices such as the use of sulfur containing fertilizers as well as the period of storage of fresh garlic have also been proven to affect their alliin concentrations. Significant variations were found between the alliin content of the commercially produced garlic products because they were prepared under different manufacturing conditions and with different chemical specifications. The method developed is cheap, accurate, precise, efficient, and thus can be used in routine determination of alliin in fresh garlic and commercial garlic products.

For further work, it would be helpful to compare this method with currently accepted ones by determination of alliin levels in garlic samples that are cultivated and stored under the same conditions. Also, the effects of the period of storage of garlic on their alliin content should be studied.

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