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AN INVESTIGATION OF CAPSAICINOIDS AND BIOACTIVE COMPOUNDS IN 'SCOTCH BONNET' AND SEVEN OTHER CULTIVARS OF PEPPER (*Capsicum annuum*)

By

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ABSTRACT

AN INVESTIGATION OF CAPSAICINOIDS AND BIOACTIVE COMPOUNDS IN 'SCOTCH BONNET' AND SEVEN OTHER CULTIVARS OF PEPPER (Capsicum annuum)

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The capsaicinoids and bioactive compounds in eight *Capsicum annuum* cultivars were investigated. Lyophilized peppers, grown in the greenhouse, were extracted with supercritical CO_2, hexane, ethyl acetate and methanol. Two major capsaicinoids, capsaicin and dihydrocapsaicin, were isolated and purified by column chromatography and preparative thin-layer chromatography. Capsaicin was quantified by reverse-phase high performance liquid chromatography and characterized by nuclear magnetic resonance, mass spectrometry and ultraviolet spectrophotometry. The biological activities of the crude extracts from eight cultivars of pepper (*C. annuum*), pure capsaicin and dihydrocapsaicin were studied on fungi, bacteria, nematodes and mosquito larvae. The hexane extract of 'Scotch Bonnet', at 250 ppm, killed all mosquito larvae (*Aedes aegypti*) in 30 min. Purification of this active extract afforded bonnetenol, characterized by ^{1}H, ^{13}C, ^{1}H - ^{1}H decoupling and DEPT NMR, and MS experiments. This compound gave 100% mortality against *Aedes aegypti* at 0.1 ppm in 24 h.

Dedication

This thesis is dedicated with love, to my wife, Cen.

ACKNOWLEDGMENT

I wish to express my sincere gratitude to my major professor, Dr. Muraleedharan Nair, for providing me with the opportunity to study in his laboratory, and for his patience and guidance in completing my research and this thesis. I would also to thank Dr. Jack Kelly, Dr. James Miller and Dr. Frank Dennis for participating as members of my guidance committee.

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LITERATURE REVIEW

General Introduction: Peppers have long been an important spice and flavoring in cuisines from one continent to another. The archaeological record at Tehuancan, Mexico, southeast of Mexico City, showed that wild peppers were eaten in Mesoamerica as early as 7000 B.C. and were domesticated probably by 2500 B.C. (Andrews, 1984). The fiery fruit of pepper is discomforting to eat and causes mouth burns, body perspiration and watery eyes. However, the flavor of pepper and its unique role in food preparation tempt many people to overlook the "heat" generated by the fruit.

Pepper, sometimes considered as the king of spices, is one of the earliest spices known to man. Peppers are an important vegetable commodity and are highly prized for their flavor, color, vitamins A and C, and pungency (Groviadaraian, 1979). The products made from peppers are sold in a variety of forms, ranging from whole fruits to ground powders. The fruit itself has been used in different forms, ranging from aid in child-birth to an instrument of torture. The Indians in Panama trail pepper pods behind their dugouts to repel sharks. Some city dwellers in the United States and Canada use sprays made from chili peppers to ward off muggers (Andrews, 1984).

In the international spice trade, peppers contribute

about 40% (77,000 MT) to the total world spice trade (Rathnawathie and Buckle 1984). The 1990 world production of peppers was 9.1 MT. Major areas of pepper production are Asia, Europe, and Africa, with about 44, 24, and 19 percent, respectively, of the total world production. Nations producing large amounts of peppers are China, Nigeria, Turkey, Mexico, the United States and Egypt (FAO, 1990). In the United States, with about 3 - 4 % of the total world production, the major producing states are California, Colorado, Florida, New Mexico and Washington (U.S.D.A. Agriculture statistics, 1990).

Horticultural Considerations of Pepper: Pepper (*Piper nigrum*) was the first oriental spice to reach Europe from Asia and remains today the most widely used spice throughout the world. In 1493, Columbus, while seeking a source of black pepper (*Piper nigrum*), discovered chili instead, and named chili as "pepper" (Andrews, 1984).

Pepper (chili) plants are shrubby perennials, but are usually grown as herbaceous annuals in tropical, subtropical and temperate regions (Andrews, 1984). Pepper has a perfect flower, and is primarily self-pollinated, and to a lesser extent cross-pollinated by insects. The plant is frost-sensitive and grows best at warm temperatures, preferably within a range of 25° C to 30° C. In fertile, fine-textured soils, such as sandy loams, the optimum pH range for high

productivity is between 6.0 and 6.5 (Purseglove, et al. 1981). Applying a water-soluble fertilizer high in phosphorus, and topdressing with high nitrogen fertilizer at bloom, will enhance yield (Cochran, 1932). Peppers are one of the few vegetables that germinate with very low soil moisture, therefore overwatering should be avoided. The water should be applied after emergence. Since growth of the pepper plants from seed requires considerable time, transplanting is commonly employed (Lafavore, 1983). Generally, a warm environment is required for rapid germination and to prevent damping off, and seeds will not germinate if the soil temperature is 15°C or lower. The optimum temperature range reported for germination is 25° to 30°C.(Andrews, 1984). Pepper plants are unable to withstand freezing (Kader, et al. 1982).

Peppers are affected by many of the same diseases and insects as tomatoes, including bacterial spot, Fusarium wilt, aphids and pepper weevil. These problems can be reduced by planting resistant cultivars or use of fungicides and insecticides rotations (Boswell, 1964).

Peppers can be harvested at any stage, but quality is generally best if left to maturity. For the pungent types, the flavor reaches its peak only when fruits are mature. The red mature fruit is reported to be higher in vitamins A and C (Kitagawa, 1973). In order to maintain product quality and avoid mold growth, peppers should be stored at near 15°C at

low humidity.

There are a number of varieties of "peppers" , and not all varieties or types are called pepper. Black pepper, for example, is obtained from the corn or seed of the climbing vine of *Piper nigrum* which is native to India (Govindorajan, 1977). Chili pepper, from plants of the genus *Capsicum*, has many varieties which differ in pungency, color and flavor (Andrews, 1984; Erwin, 1932). There are also other "peppers" which belong to different genera. For example, Jamaican pepper (pimento or allspice) belongs to *Pimenta dioica*; Japanese pepper, the black fruit from the tree *Zanthoxylum piperatum DC.*, belongs to the family Rutaceae, and the African or Negro pepper, a pod-like fruit of a shrubby tree, *Xylopiasethiopica Dunn*, belongs to the family Anonaceae. (Groviadarajan, 1977).

Nearly 200 varieties of domesticated peppers (*Capsicum annuum*) are grown in North, Central and South America. The name capsicum is derived either from the Greek " Kapso " meaning to bite or the Latin " Capsa " referring to the fruit pod or capsule (Domenici, 1983). Capsicums are members of the Solanaceae or nightshade family, which includes tomatoes, eggplants and potatoes, and probably evolved from an ancestral form in the area of Bolivia or Peru (Heiser, 1976). Capsicums are perennial in suitable climatic conditions but are often cultivated as an annual. Geographical and climatic conditions also cause variations in *Capsicum* species throughout the world

(Andrews, 1984). The genus *Capsicum* includes 20 to 30 species native to the New World tropics and subtropics. Within this genus there are five domesticated species - *Capsicum annuum*, *Capsicum frutescens*, *Capsicum pubescens*, *Capsicum chinense*, and *Capsicum baccatum var pendulum*. *C. annuum* is the most widely cultivated species and includes almost all of the varieties grown in the U.S. and Europe. This plant, an annual in temperate climates, can be distinguished by its white flowers. The fruits vary in length and color when mature. *C. frutescens*, of which the tabasco variety is a member, is perennial usually and has smaller fruits. They are cultivated mainly in warm regions. Fruits of wild forms of *C. chinense* are spherical in shape, but the cultivated forms produce variable-shaped fruits. Plants have large leaves and are grown mainly in tropical South America. *C. baccatum* is widely distributed in South America and cultivated to a very limited extent in the U.S. The climatic requirements for *C. baccatum* are similar to those of *C. annuum*. It can be identified by the yellow or brown spots on its flowers, which form conical pungent fruits. *C. pubescens* is found only at high elevations in the tropics or subtropics. The plant is characteristically hirsute and requires a long and cool growing season. *C. annuum*, *C. frutescens* and *C. chinense* can be hybridized. However, no hybrids are known for *C. baccatum* and *C. pubescens*. Therefore, relatively few cultivars of *C. pubescens* and *C. baccatum* are commercially

significant (Smith, 1987; Andrews, 1984).

Chemistry of Pungent Principles In Peppers: The two different classes of peppers, black pepper and chili pepper, contain very different compounds responsible for their pungency and flavor. Black pepper is one of the most important spices, used in many dishes, meat products and sauces. Its pungency is due to many non-volatile compounds such as piperine alkaloid and its isomers, piperlonguminine, piperylin, piperanine, piperine, piperettyline and piperttine (Figure 1) (Kenneth and Michael, 1988). The most abundant and major pungent principle in black pepper is piperine (Genest, et al. 1963). The geometric isomers of piperine (I), i.e., chavicine (cis-cis)(II), isopiperine (cis-trans)(III) and iso-chavicine (trans-cis)(IV) (Figure 2) also occur in black pepper, but in very small amounts (Kulka, 1967).

The primary compounds responsible for the pungency of capsicum fruits are capsaicinoids (N - vanillyl nonamides), and the most pungent compound among them is capsaicin (Figure 3), which is 100 times more pungency than piperine (Groviadaraian, 1979). Capsaicinoids are amino acids of vanillylamine and C_9 - C_{11} branched-chain fatty acids. These fatty acids are secreted from the cells of both pungent and nonpungent cultivars of peppers (Fujiwake, et al. 1979). The vanillyl amides with varying side-chain lengths responsible for the flavor and hotness of pepper, are capsaicin,

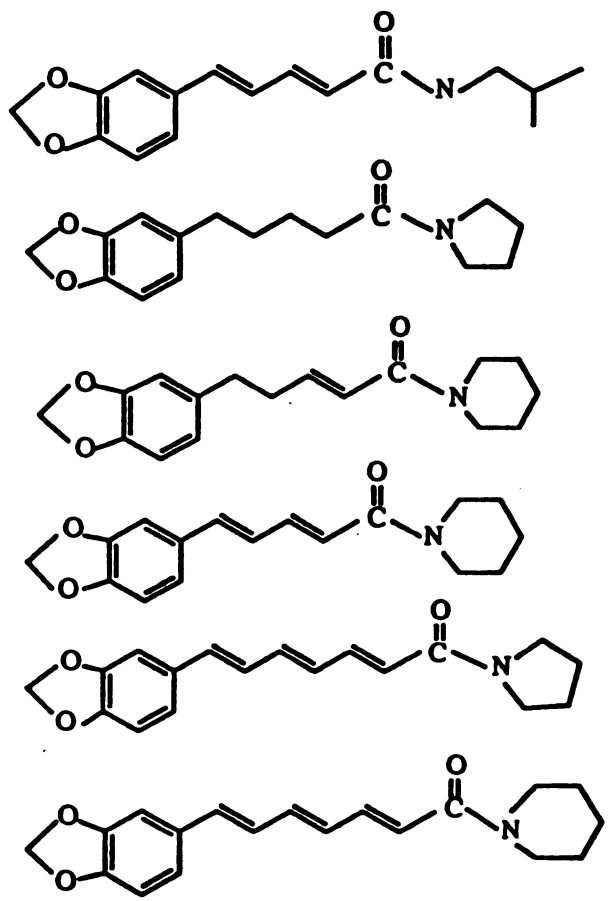
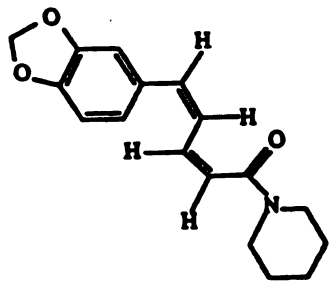


Figure 1: Structures of piperine alkaloids



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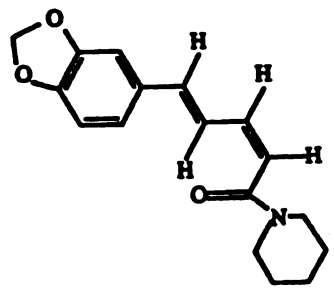

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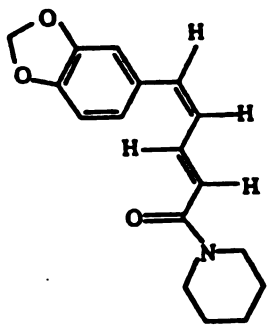

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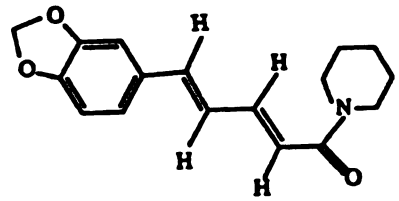

IV

Figure 2: Structures of isomers of piperine

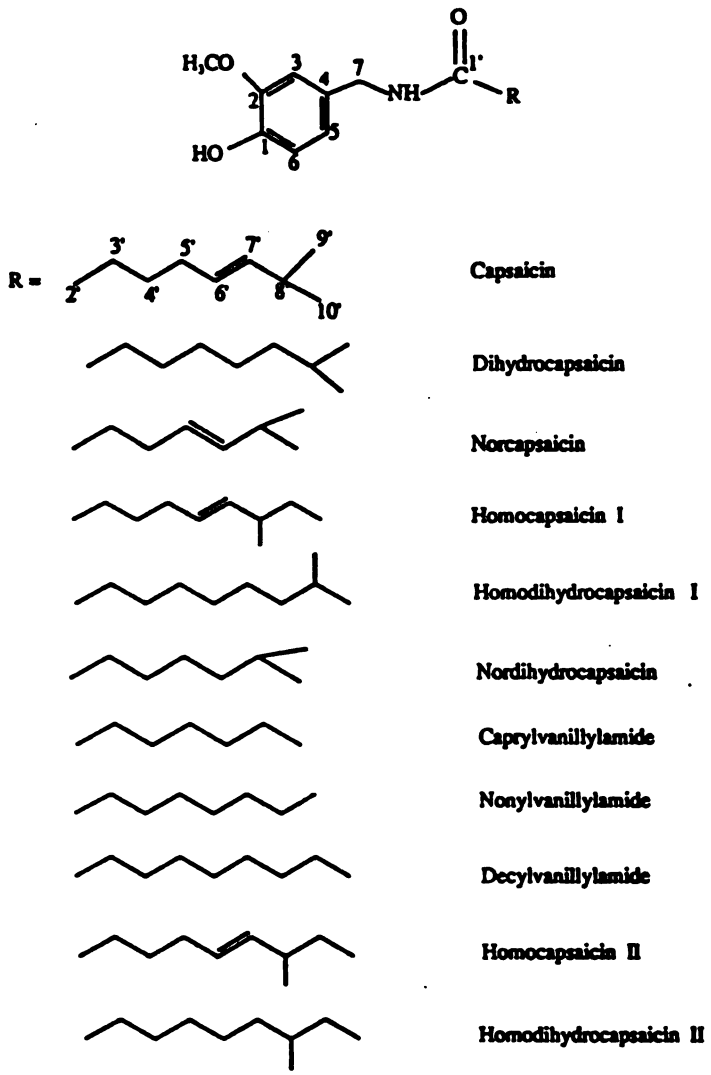

Figure 3: Structures of capsaicinoids

dihydrocapsaicin, norcapsaicin, nordihydrocapsaicin, homocapsaicin I, homocapsaicin II, homodihydrocapsaicin I, homodihydrocapsaicin II, caprylvanillylamide, decylvanillylamide and nonylvanillylamide (Figure 3)(Patric, et al. 1983). The most abundant capsaicinoids are capsaicin and dihydrocapsaicin. Capsaicin is the most pungent compound of all the capsaicinoids and is present mainly in the placental tissues of the inner walls of the fruit. The hull and seeds of chili pepper contain little or no capsaicin (Hoffman, et al. 1978; Newman, 1953).

Capsaicin has a melting point of 65°C and boils at 210 - 220°C. It has a strong absorption at 280 nm in the ultraviolet spectrum. Capsaicin is practically insoluble in cold water but freely soluble in ethanol, methanol and chloroform, and slightly soluble in carbon disulfide (Merck Index, 10th edition, 1989).

Chemical synthesis of dihydrocapsaicin was achieved first by Nelson and Dawson (1923). The synthesis involved the treatment of 4-methyl pentanoic acid with sodium ethoxide at room temperature for 4 h. The resulting 4-methyl-pentyl alcohol was refluxed with hydroiodic acid for 3 h to obtain 4-methyl-pentyl iodide; this was condensed further with aceto-acetic ester to produce 4-methyl-pentyl aceto-acetic ester. The ester was saponified by sodium ethoxide for 4 h, and the resulting product, ethyl-6-methyl-heptylate, was converted into 6-methyl-heptyl alcohol by reacting with sodium ethoxide

in ethanol at room temperature. The alcohol thus obtained was reacted with hydroiodic acid to form 6-methyl-heptyl iodide followed by condensation with aceto-acetic ester. The 8-Methyl-nonanoic acid, obtained by saponification of ethyl-8-nonanoate, was further converted into its acid chloride by thionyl chloride. The acid chloride finally was condensed with vanillyl amine to form dihydrocapsaicin (Figure 4).

Newman (1953) reported the first synthesis of capsaicin using isobutyl zinc iodide as the starting material. 8-Methyl-nonan(6)on-(1)ic acid was prepared by reacting isobutyl zinc iodide and monoethyladipylchloride in acetic acid-toluene solution at room temperature and then reduced with sodium powder in ethanol to form 8-methyl-nonan(6)on-(1)ic acid(6)-ol. This was converted into 8-methyl-nonan(6)-Br-(1)ic acid(6)-ol by heating with HBr at 100-105^{0}C in a closed ampule. 8-Methyl-non(6)en-(1)oic acid was produced by the distillation of 8-methyl-nonan(6)-Br-(1)ic acid (6)-ol with HBr. The capsaicin was obtained from the reaction of 8-methyl-non(6)en-(1)oic acid with vanillyl amine at room temperature (Figure 5).

Crombie et al. (1955) reported that Newman's synthesis of capsaicin resulted in a mixture of isomers. Therefore, Crombie et al. synthesized capsaicin using an unambiguous stereo-specific method as follows: 2,3-dichlorotetrahydropyran was slowly added, with stirring, to isopropylmagnesium bromid in ether at 0^{0}C, and the resulting 3-chlorotetrahydro-2-

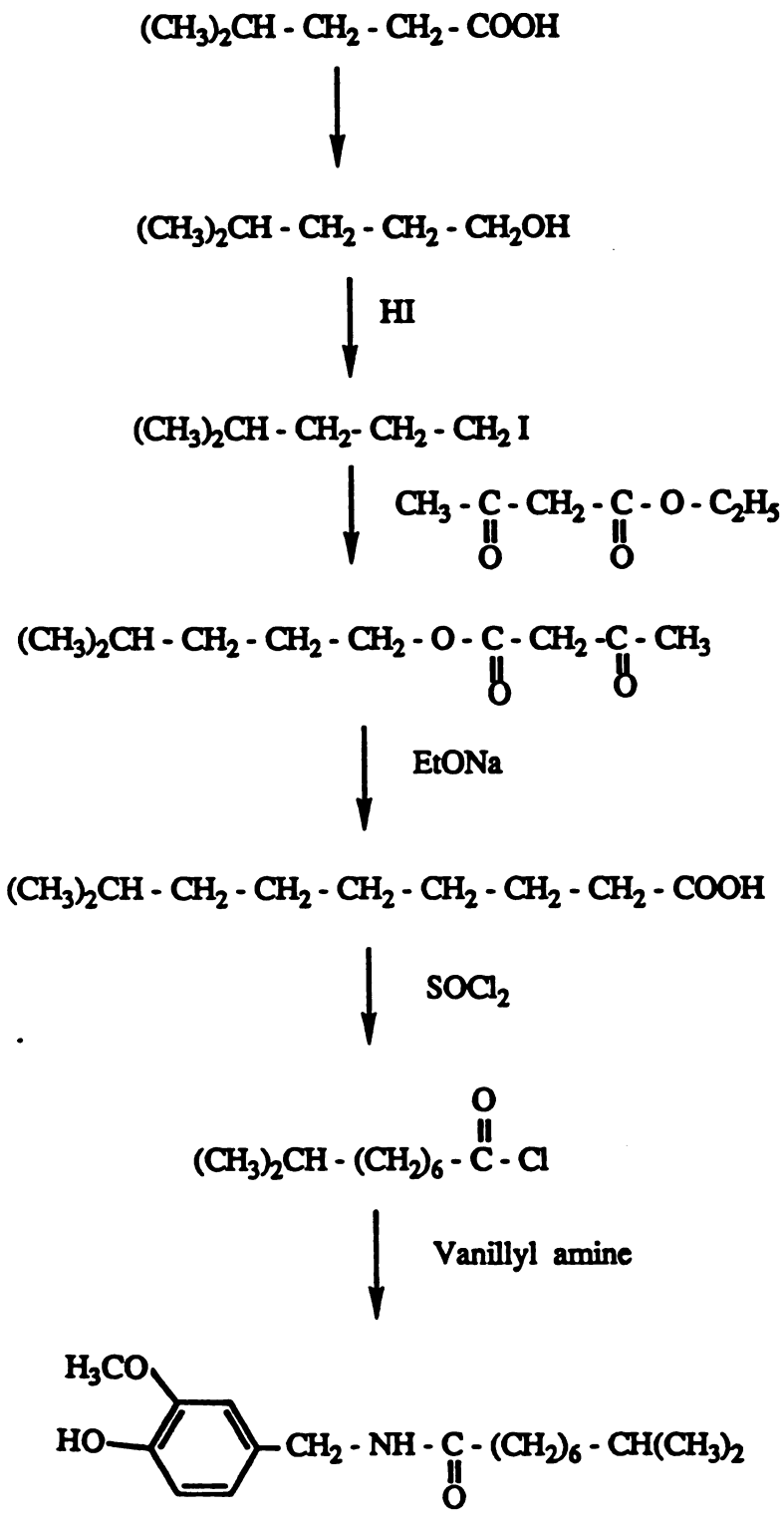

Figure 4: Synthetic scheme for dihydrocapsaicin

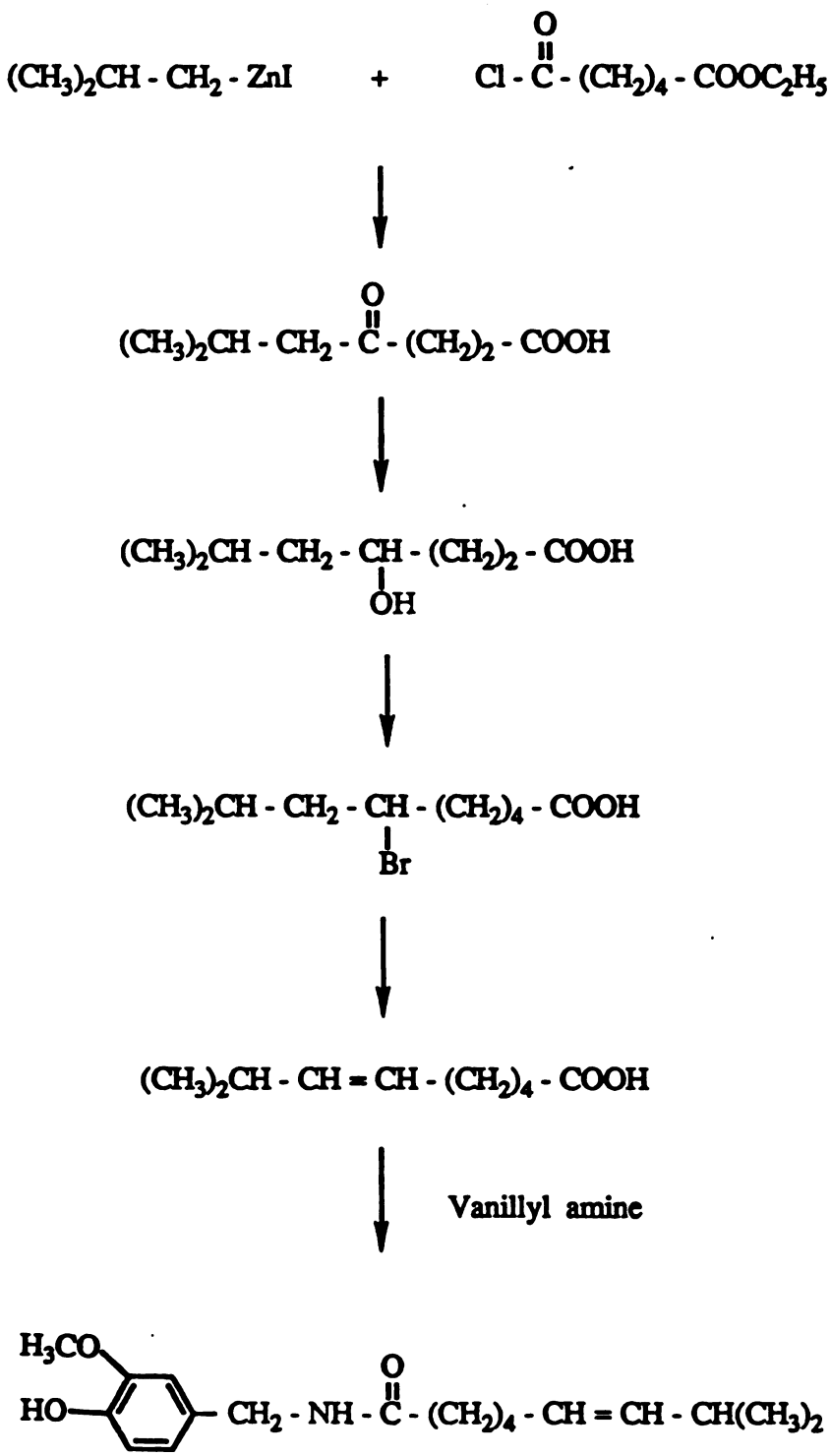

Figure 5: Synthetic scheme for capsaicin

isopropylpyran was converted to 6-methylhept-trans-4-en-1-ol by adding 3-chlorotetrahydro-2-isopropylpyran slowly into powdered sodium in anhydrous ether. 6-Methylhept-trans-4-en-1-ol was reacted with phosphorus tribromide in pyridine at 0^0C for 30 min., and refluxed with ethyl sodiomalonate for 2 h to form 8-methylnona-trans-6-enoic acid. This compound was hydrogenated in dioxan with the catalyst and the resulting product was treated with thionyl chloride for 18 h to produce 8-methylnona-trans-6-enoic acid chloride. Finally, the trans-capsaicin was prepared by adding 8-methylnona-trans-6-enoic acid chloride to vanillyl amine suspended in ether and shaking for 3 days (Figure 6).

Bennet and Kirby (1968) concluded that both phenylalanine and tyrosine are the precursors for the biosynthesis of the aromatic portion of capsaicin. The conversion of phenylalanine into capsaicin proceeded via hydroxylated cinnamic acids. Bennet and Kirby (1968) also reported that vanillylamine might be the most direct precursor; whether the amino group of vanillylamine was retained during the biosynthesis of capsaicin in the plant was not established. In most cases, natural and synthetic capsaicin differ in the cis-trans arrangement at the double bond in the fatty acid side-chain moiety; the trans or cis arrangement may not be retained during chemical synthesis. However, natural and synthetic capsaicin can be distinguished by their infrared absorption spectra (Datta and Susi, 1961)

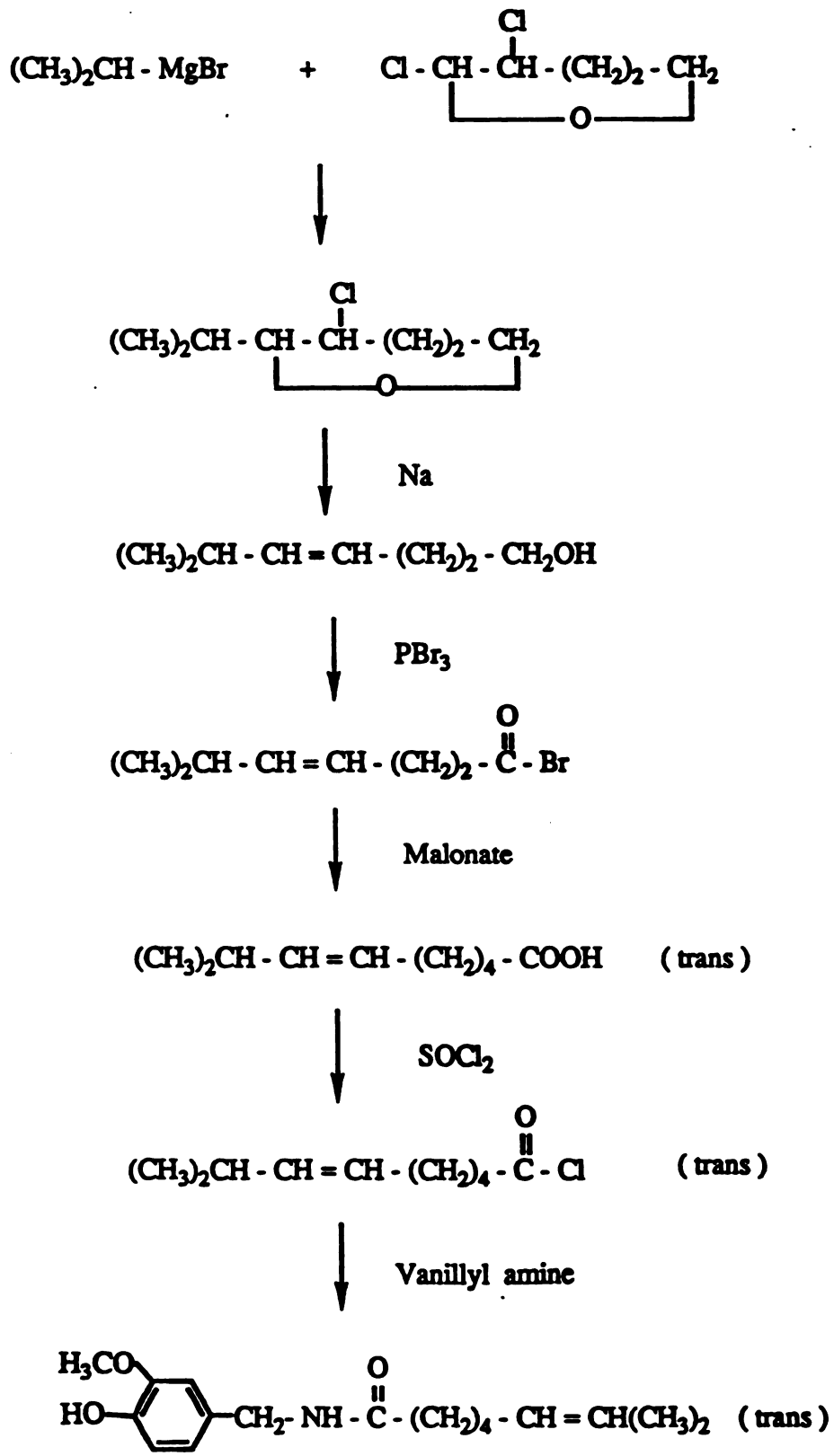

Figure 6: Stereospecific synthetic scheme for capsaicin

and also by evaluating the fatty acids associated with the amide portion of capsaicin (Todd and Perum, 1961). The latter method was not accurate because of interference by various fatty acids from the lipid fraction resulting from other biosynthetic pathways (Masada, et al. 1971).

The capsaicinoids can be extracted from the fruits of peppers by several methods. Roshchina et al.(1986) used petroleum ether, methanol and hot water, respectively, as solvents under different conditions. They found that methanol extraction gave the highest yield of capsaicin, even though hot water and petroleum ether could yield a purer extract of capsaicin. Many published reports describe various solvent systems under specific conditions for extracting capsaicinoids from chili peppers (Mary, 1984; Hoffman, 1978 and Roshchina, et al. 1986).

Sensory Properties Of Capsaicin: The traditional method for assessing pepper pungency is the Scoville procedure, in which a sample of capsaicin is diluted until it becomes undetectable by taste to a panel of judges (Nagy, 1982). An aliquot of an ethanol extract of the capsicum product is diluted serially with a 5% sucrose solution. The concentration at which 3 out of 5 panelists perceive a slight burning sensation in the throat upon swallowing 5 ml of the solution establishes the numerical Scoville heat value or dilution factor (ASTA, 1968). This method has been criticized severely for

irreproducibility and the time required for the need for extensive training of the panelists (Meilgaard, et al. 1987).

Lou (1984) suggested that HPLC is the best analytical method for quantifying the capsaicin content and evaluating the heat value in pepper and pepper products. Spectrophotometric methods employing the reactions of capsaicinoids with several reagents to produce a colored complex were reported by Rymal, et al. (1984). Reverse-phase is preferred over normal-phase chromatography for the purification of capsaicin in dehydrated *Capsicum annuum var.* Separation of the capsaicinoids on normal-phase silica gel is difficult because the only difference in the structures of capsaicinoids is their fatty acid moiety (Chiang, 1986; Weaver and Awde, 1986).

The savor for peppers consist mainly in their pungency or 'heat' value. Ironically, the enjoyment of chili pepper is actually due to the irritation to nerve endings by capsaicin. The trigeminal nerves, which are distinct from the taste receptors, are chemically irritated by capsaicin (Anonymous, 1986). These nerves also are responsible for sensing touch, heat, cold, and pain in the mouth (Nagy, et al. 1982). The contribution of trigeminal nerves to the flavor sensation of foods often is overlooked. In the nose, the trigeminal nerves respond to irritants in foods and beverages such as carbon dioxide and to a variety of aromatic flavors that normally are considered "just smells". Therefore, the enormous number of

nerves making up the trigeminal system is very important for the flavor sensation (Nagy, et al. 1982).

One can taste all the other food flavors through the pepper burn. However, research in this area has produced mixed results. To some degree, pungency seems to mask bitter and sour tastes (Cowart, 1987). Overall, trigeminal sensations interact very little with other flavors under the influence of " hot " pepper.

Pepper also has a number of pharmacological functions such as anaesthetic properties (Jancso, et al. 1977; Anonymous, 1986). Endorphin, an opiate-like substance, reportedly is secreted by the brain when capsaicin irritates the trigeminal nervous system (Jessell, et al. 1977). Some studies indicate that peppers, especially hot peppers, are cancer-producers and are not recommended for good health (Virus, 1979).

The aromatic ring in capsaicinoids plays an important role in the pungency of chili pepper in addition to the alkyl side chain. The optimal chain length of alkyl group is 8 - 10 carbon atoms for the highest pungency; increasing or decreasing the carbon chain length would reduces the 'heat' value of capsaicinoids. The degree of unsaturation of the alkyl side chain does not have much influence or at least is not essential for the pungency produced by these compounds (Nelson 1919). However, the acylamide part exhibits the relationship between chemical structure and pungency.

Replacing the nitrogen atom in capsaicin with oxygen has very little effect on pungency. However, when nitrogen was replaced by elements or groups such as ethylene, pungency of capsaicinoids was reduced significantly (Szolcsanyi, 1975).

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CHAPTER TWO

INVESTIGATION OF CAPSAICINOIDS IN *CAPSICUM ANNUUM* CULTIVARS

ABSTRACT

Capsaicin content in various *Capsicum annuum* cultivars were investigated. Supercritical carbon dioxide was used to extract 'Scotch Bonnet' pepper at 50°C and 450 atm, 50°C and 600 atm for 30 min and 1 h, respectively. The SFE results were compared with those obtained by solvent extraction using hexane, ethyl acetate and methanol, respectively. Preparative thin-layer and column chromatographic methods were employed for the separation of capsaicin and dihdrocapsaicin. A reverse-phase high performance liquid chromatographic method was used to quantify capsaicin. The capsaicin and dihydrocapsaicin were characterized by nuclear magnetic resonance, mass spectrometric and ultraviolet spectroscopic methods. The SFE extract of 'Scotch Bonnet' with the heat value of 300,000 Scoville units, afforded 3.2% capsaicin /g dry weight compared to the 0.5% capsaicin /g dry weight from the total solvent extracts. Chili with heat value of 8,000, Jalapeno with heat value of 5,000, Cayenne with heat value of 3,000 Scoville units and bell pepper with no heat value were found to contain 0.09%, 0.07%, 0.03% and 0% capsaicin /g dry weight, respectively.

INTRODUCTION

The chili pepper belongs to the family Solanaceae, which also includes the eggplant and tomato and has about 90 genera containing some 2,000 species of herbs, shrubs and small trees (Heiser, 1976). The term " Capsicum " refers to the fruit of numerous species of the genus *Capsicum* comprising over 200 varieties. They range from the very hot 'Jalapeno' to heatless 'Bell' peppers (Andrews, 1984). Their fruits vary widely in size, shape, flavor and sensory heat. *C. annuum* is the most widely cultivated pepper in the world and practically all of the fresh, processed, dried and frozen peppers on grocer's shelves belong to this species. *Capsicum* fruits are used throughout the world because of their unique flavor and pungency, and also have widespread use as a drug (Newman, 1953).

The primary pungent principle from *C. annuum* was first purified and obtained in a crystalline form from the crude extract by Thresh (1846), who named the compound capsaicin. Nelson (1919), determined its structure as the amide of vanillyl amine and isodecenoic acid (Figure 3, Chapter 1). Capsaicin and dihydrocapsaicin, which are responsible for 90% of the total pungency (Iwai, et al. 1979), are the most abundant principles of hot pepper (Kosuge and Furata, 1970). The *C. annuum* Scotch Bonnet, with a heat value of 250,000 - 300,000 Scoville unit, is considered to be the most pungent

pepper available on the market. It is grown widely in Jamaica for its pungency and flavor, and presently cultivated in the United States (Garrett, et al. 1991).

A variety of chemical and instrumental methods are available to identify and quantify capsaicinoids in peppers. Colorimetric and spectrophotometric analysis (Bajaj, 1980; Ramos, 1979), chromatographic methods such as thin layer chromatography (TLC) (Pankar and Magar, 1977), high pressure liquid chromatography (HPLC) (Attuquayefio and Buckle, 1987; Hoffman, et al. 1983; Mary, 1984; Saria, et al. 1981; Weaver and Awde, 1986), gas chromatography (GC) (Krajewska and Powers, 1987; Todd and Perum, 1977), gas chromatography coupled with mass spectrometry(GC-MS) (Masada, et al. 1971) are some of the most commonly used techniques for separation and identification of capsaicinoids. However, HPLC is considered to be the most reliable and rapid method. Also, HPLC has the advantage over GC because derivatization is not required for quantification.

During the past decade supercritical fluid extraction has been employed widely for the extraction of variety of compounds from different matrices in areas such as polymer fractionation, monomer purification, spice extraction and coffee decaffeination (Eldridge, et al. 1986; Friedrich and List, 1982; Hawthron, et al. 1988; Johnston and Penninger, 1989; Krukonis and Kurnik, 1985; McHugh and Krukonis, 1986; Mcnally and Wheeler, 1988; Stahl, et al. 1980).

A gas becomes a supercritical fluid at temperatures and pressures above its critical point (Figure 1). Carbon dioxide , commonly used as a supercritical fluid, exhibits a critical temperature and pressure of 31.1^{0}C and 73.8 atm, respectively. In the range above its critical temperature and pressure, carbon dioxide is in the supercritical state and can dissolve a variety of compounds. Supercritical fluid extraction (SFE) exploits the unique properties of gaseous solvents above their critical states to fractionate mixtures of compounds in a single step (McHugh and Krukonis, 1986). At the critical state, they have the dissolving powers of a liquid while retaining such properties of a gas as high diffusivity and low viscosity. In other words, supercritical fluids possess a liquid-like density, but also exhibits gas-like transport properties of diffusivity and viscosity. Therefore, supercritical fluids are able to dissolve a variety of compounds, even those with high molecular mass and low volatility (Gouw and Jentoft, 1972).

Carbon dioxide is probably the most widely used supercritical fluid because its critical temperature (T_c =31.1°C) makes it an ideal solvent for extracting materials that are thermally labile. Also, CO_2 is non-toxic, non-flammable, and environmentally preferred over organic solvents. The extraction conditions of 275 atm and 35°C were generally chosen to achieve a density of 0.93 at its supercritical state.

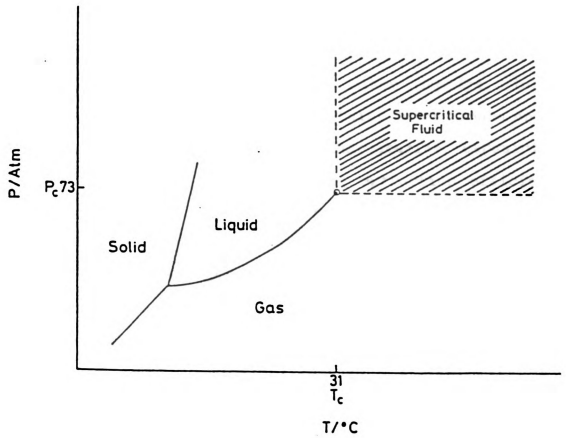

Figure 1: Supercritical CO_2 phase diagram
P_c- Pressure, T_c- Temperature

Supercritical extraction also can be performed at varying conditions, e.g., changing temperatures at constant pressure or changing pressures at constant temperature. Other supercritical fluids, such as NH_3 and N_2O, can also be employed depending on the nature of the compounds to be extracted (Rizvi, et al. 1986).

The objective of this work was to compare the pungency of 'Scotch Bonnet' pepper vs. other peppers, including Jalapeno, Chili, Cayenne, Bell Captain, Sweet Banana and Forti F_1 peppers, to isolate and characterize capsaicin and dihydrocapsaicin, and compare the extraction efficiency of supercritical CO_2 fluid with organic solvents.

MATERIALS AND METHODS

General experimental: Vacuum liquid chromatography (VLC) was performed on the silica gel (Analtech silica gel 60 Å pore size, 35 - 75 micron particle size). Preparative thin layer chromatography (Prep. TLC) was performed on silica gel Uniplates (Analtech silica gel GF-254, 0.5 mm, 20 X 20 cm, 2000, 1500, 500, 250 microns). Column chromatography was performed using a 3.3 cm X 33 cm column containing silica gel (Analtech silica gel 60 A pore size, 35 - 75 microns particle size), and thin layer chromatography (TLC) on silica gel (Aldrich silica gel G F-254, 0.250 mm layer, 2.25 μ mean particle size). Unless specified, the developed plates were

viewed in an ultraviolet fluorescence analytical cabinet (Spectroline Inc.) at 366 nm and 254 nm, respectively, or sprayed with 50% H_2SO_4, then heated to charc the compound.

Low pressure C-18 column chromatography was performed using an LC-SORB glass column (Chemco, 3.2 cm X 50 cm) packed with C_{18} silica gel and equipped with a low-pressure pump (Model 81-M-2, Chemco), rheodine injector (Chemco) and a UV-IS 200 detector at 280 nm (Sanki Laboratories Inc.).

Supercritical fluid extraction (SFE) of peppers was achieved on a Dionex - 703 SFE equipped with 8cm X 32 ml extraction cells (Dionex Co. USA).

High performance liquid chromatography (HPLC) was performed using an HPLC system consisting of a Waters Automated Gradient Controller equipped with a Waters Model U6K injector, Shodex Degasser, Waters Model 590 pump and two Waters M-6000A pumps. The guard column was Nova-Pak (Waters Associates) with removable C-18 cartridge. The column was Waters Radial - Pak C_{18} cartridge, 4 μ particle size, 5 X 10 mm inserted in a Waters RCM 8 X 10 radial compression module (RCM). Detection was at 280 nm with a Waters 490 programmable multiwavelength U.V. detector. Data acquisition was carried out on a Waters 740 data module. The mobile phase was acetonitrile : water (1:1). The flow rate was 0.75 ml/min.

Melting point was determined on a Kofler hot stage melting point apparatus (Bristoline Co. USA) and was uncorrected.

Proton (^{1}H)and carbon (^{13}C), ^{1}H - ^{1}H decoupling and DEPT nuclear magnetic resonance spectra were obtained on a Varian VXR - 300 spectrometer (Varian Co. USA), 300 Mhz for proton and 75 Mhz for carbon, and a Varian VXR - 500 spectrometer, 500 Mhz for proton and 125 Mhz for carbon. Electron impact mass spectra (EIMS) were obtained on a Jeol model JMAX 505 mass spectrometer at 70 eV. (Jeol JMS Co. USA). Ultraviolet (UV) absorption analysis was conducted on a Shimadzu UV-Visible model UV-260 spectrophotometer.

Plant material: The matured fruits of *C. annuum* Scotch Bonnet, Mayata F1, Hybrid Bell Captain, Cuba and Sweet Banana were collected from pepper plants grown in the greenhouse of the Department of Horticulture, Michigan State University, East lansing, Michigan. Other types of *C. annuum* pepper used were Chili purchased from Meijer Inc., Lansing, Michigan, Cayenne purchased from Kroger Co., Okemos, Michigan, Jalapeno purchased from Horrock's Farm Market, Lansing, Michigan. All peppers were lyophilized by a DURA-DRY FTS - Tray Lyophilizer (FTS SYSTEM Inc. USA), and stored in sealed plastic bags at - $20^{0}C$ prior to extraction.

Separation of capsaicin and dihydrocapsaicin from Aldrich sample: The ^{1}H NMR spectrum of capsaicin (Aldrich Chemical Co.) indicated that it was a mixture of capsaicin and dihydrocapsaicin (Figure 2). The capsaicin (190.4 mg)

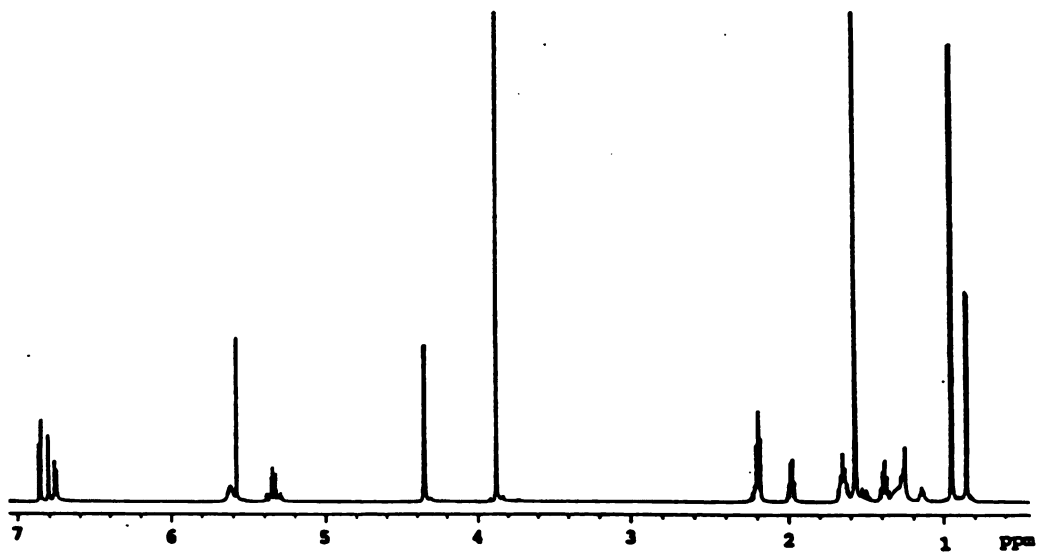

Figure 2: ^{1}H NMR spectrum of Aldrich capsaicin

(Aldrich Chemical Co.) was dissolved in 1 ml of acetonitrile and injected onto the LC-SORB glass column (Chemco, 32 mm X 50 cm) packed with C_{18} silica gel and eluted with acetonitrile/water (1 / 1). The 10 fractions, fraction I (200 ml), II (150 ml), III (20 ml), IV (15 ml), V (40 ml), VI (130 ml), VII (150 ml), VIII (200 ml), IX (210 ml) and X (250 ml) collected were analyzed by HPLC, and those having a peak with the retention time of capsaicin (fraction III, IV and V) or dihydrocapsaicin (fraction VIII) were combined separately. Removal of solvent *in vacuo* afforded the pure capsaicin (29.5 mg) and dihydrocapsaicin (4.9 mg), respectively.

Standard Curve: A stock solution of capsaicin (1,6000 µg/ml) was prepared by dissolving pure capsaicin (Aldrich Chemical Co.) in acetonitrile. Serial dilutions were made to obtain standard solutions containing 400, 200, 100, 80, 40, 20, 10, 8, 4, 2, 1, 0.8, 0.4, 0.2, 0.1 µg/ml of capsaicin, respectively. A standard curve was generated by plotting an HPLC peak area of capsaicin against the concentration of capsaicin (Figure 3).

Isolation of capsaicin and dihyrocapsaicin from 'Scotch Bonnet' pepper: The procedure for the isolation and purification of capsaicin and dihydrocapsaicin from the lyophilized 'Scotch Bonnet' pepper is shown in figure 4. The dried tissue (192 g) was blended with hexane (1L, 2 min.) and

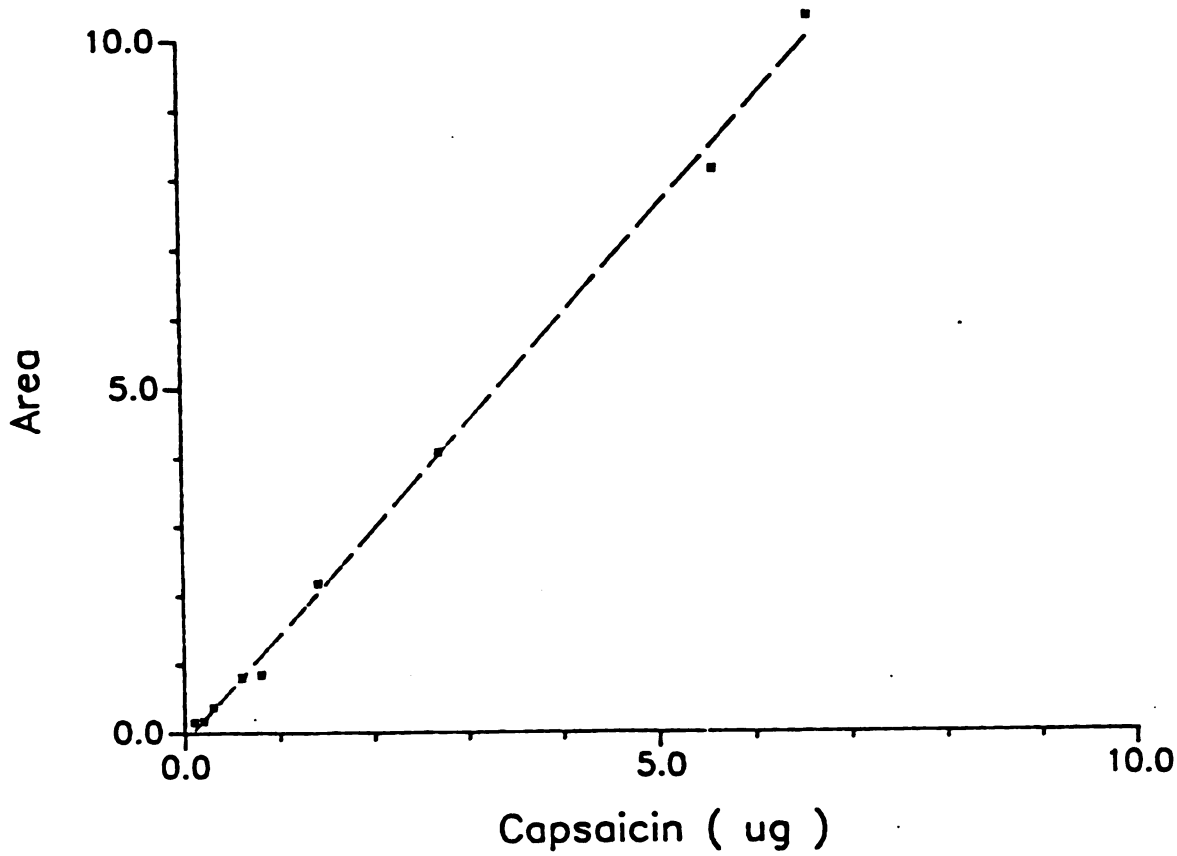

Figure 3: Standard curve of capsaicin

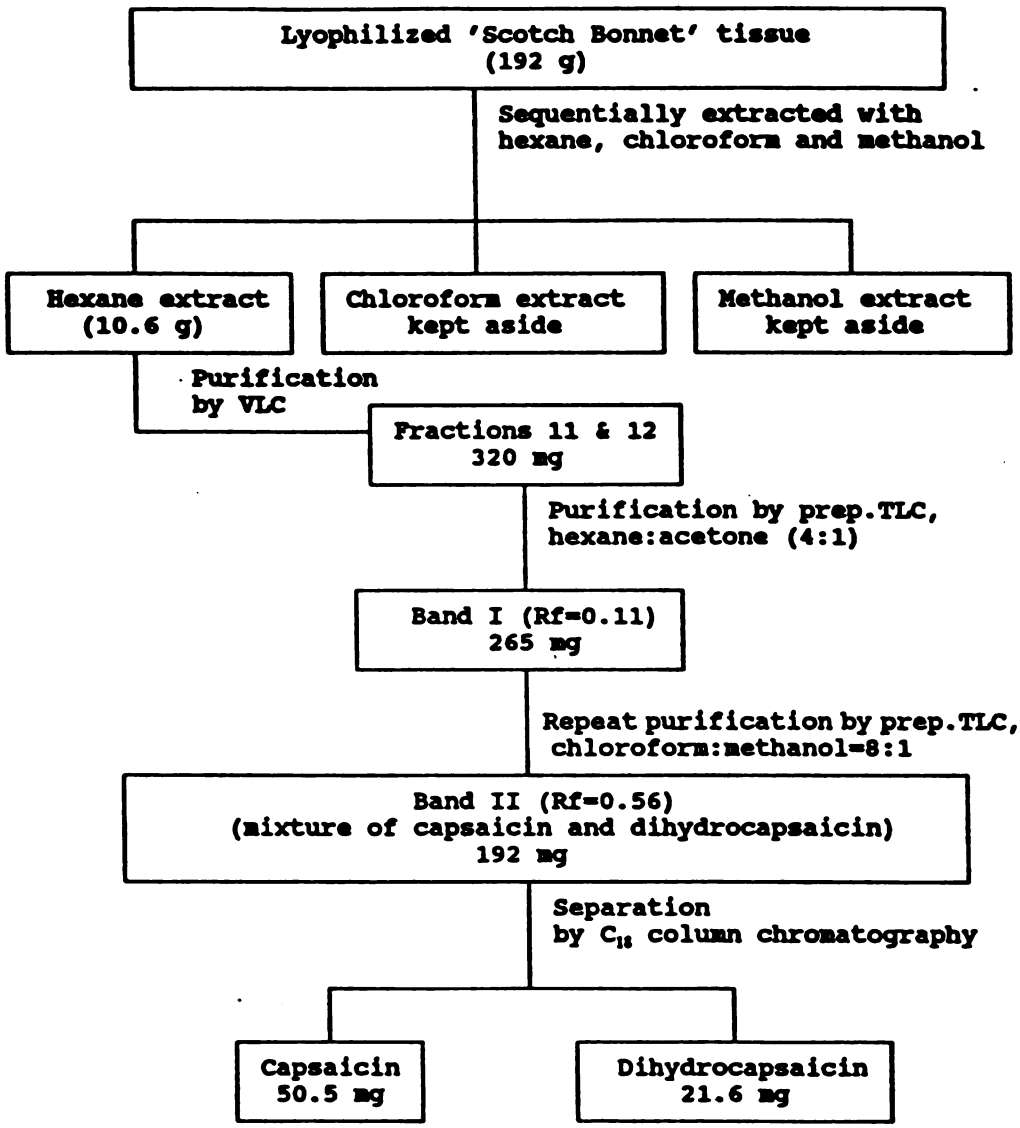

Figure 4: Isolation and purification of capsaicin and dihydrocapsaicin from 'Scotch Bonnet'

the slurry was poured into a glass column (33 mm X 33 cm) and percolated with hexane (5 L, 24 h). The extraction was continued with chloroform (500 ml X 4, 28 h) and methanol (500 ml X 5, 26 h), respectively. All extracts were evaporated *in vacuo* and afforded reddish-brown (19.1 g), brownish-red (1.5 g) and dark yellow (30.7 g) oily products from hexane, chloroform and methanol extracts, respectively. The hexane extract, containing most of capsaicin and dihydrocapsaicin among these extracts, was purified initially using vacuum liquid chromatography. A slurry of column silica (fine, 4.5 - 5.0 μm) in hexane was packed under vacuum in a sintered glass filter and washed with hexane. The hexane extract (10.6 g), dissolved in hexane (60 ml), was applied on the silica under vacuum and eluted with hexane (fraction 1 to 7, 1870 ml), hexane : acetone (4 : 1, fraction 8 to 12, 950 ml), chloroform : methanol (3 : 1, fraction 13, 500 ml) and methanol (fraction 14, 280 ml), respectively. Fractions 11 and 12 containing mostly capsaicin and dihydrocapsaicin upon TLC and taste analysis, were combined and evaporated to dryness (320 mg), then further purified by preparative TLC using hexane : acetone (4:1). The band I (R_f = 0.11), containing capsaicin and dihydrocapsaicin, was eluted with chloroform : methanol (4 : 1) and the removal of solvent *in vacuo* afforded a low melting-point creamy solid (265 mg). This was purified further by preparative TLC using chloroform : methanol (8 : 1) and the band II at R_f 0.56, containing

capsaicin and dihydrocapsaicin was removed and eluted with chloroform : methanol (4 : 1). ^1H NMR spectrum of this mixture is showed in figure 5.

The separation of capsaicin and dihydrocapsaicin from the mixture was achieved by C_{18} column chromatography on a LC-SORB glass column (Chemco, 32 mm X 50 cm) packed with C_{18} silica gel and eluted with acetonitrile : water (50: 50, v/v). The mixture of capsaicin and dihydrocapsaicin (0.192 g) was applied on the column and 16 fractions, fraction I (150 ml), II (70 ml), III (10 ml), IV (30 ml), V (20 ml), VI (40 ml), VII (10 ml), VIII (20 ml), IX (80 ml), X (150 ml), XI (60 ml), XII (120 ml), XIII (50 ml), XIV (150 ml), XV (120 ml) and XVI (140 ml), were analyzed by HPLC. Those having a single peak with retention time 5.9 min for capsaicin (fractions III, IV, V, VI, VII and VIII) or 8.8 min for dihydrocapsaicin (fractions XII, XIII and XIV), respectively, were combined separately. The pure capsaicin (50.5 mg) and dihydrocapsaicin (21.6 mg) were obtained after the removal of solvent *in vacuo*, and had the following characteristics:

Capsaicin $C_{18}H_{27}NO_3$; mp, 65 - 66^{0}C; ^1H NMR ($CDCl_3$): δ 0.94 (6H, d, J=6.7Hz, 8′- and 9′-CH_3), 1.47 (6H, m, 2′, 3′and 4′-CH_2), 1.97 (1H, m, J=7Hz, 10′-CH), 2.19 (2H, t, J=7.8Hz, 1′-CH_2), 3.84 (3H, s, -OCH_3), 4.34 (2H, d, J=5.9Hz, benzylic CH_2), 5.33 (2H, m, 5′and 6′- vinyl CH), 5.59 (1H, s, NH), 5.63 (1H, s, phenolic -OH), 6.77 (3H, m, 3, 5 and 6- aromatic protons) (Figure 6). ^{13}C NMR ($CDCl_3$): δ 172.81 (C=O), 146.72, 145.14

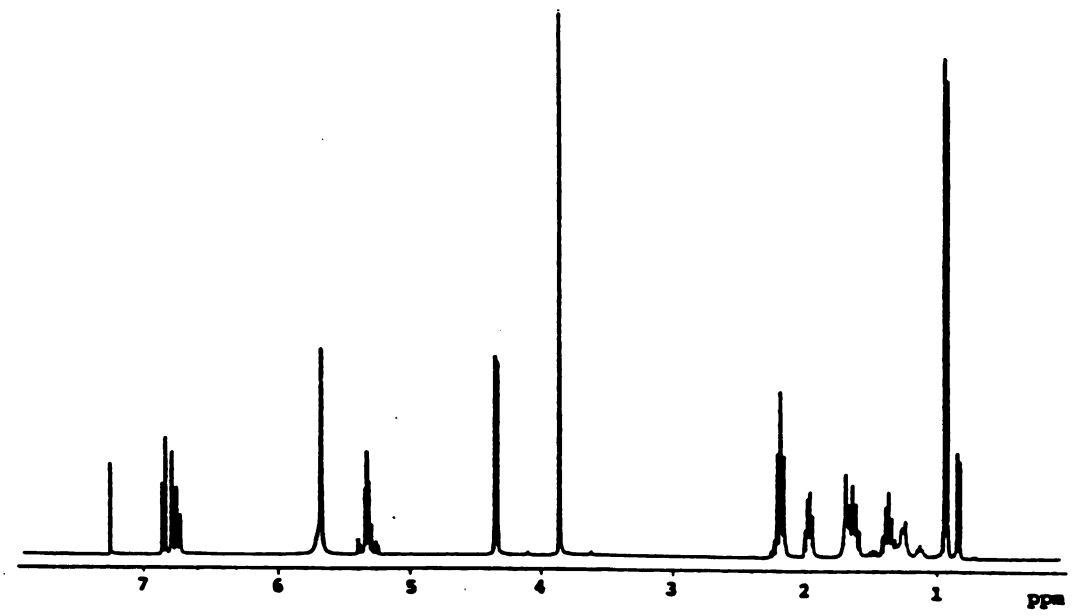

Figure 5: $_1$H NMR spectrum of the mixture of capsaicin and dihydrocapsaicin from 'Scotch Bonnet'

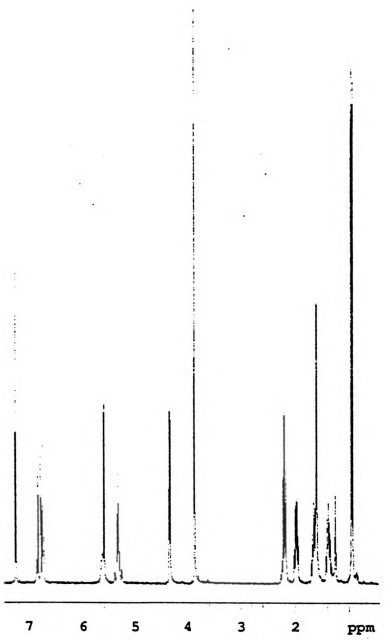

Figure 6: ^{1}H NMR spectrum of capsaicin from 'Scotch Bonnet'

(2XC-O aryl), 55.92 (OMe), 43.53 (CH_2-N), 22.63 (2XCH_3). EI-MS: m/z(% int.) 305 (M^+, $C_{18}H_{27}NO_3$, 16.4), 195 ($C_{10}H_{12}O_3$, 8.1), 168 ($C_8H_{19}NO$, 4.5), 152 ($C_8H_{10}NO_2$, 12.9), 137($C_8H_9O_2$, 100).

Dihydrocapsaicin $C_{18}H_{29}NO_3$; ^1H NMR (CDCl3): δ 0.84 (6H, d, J=5.6Hz, 8'and 9'-CH_3), 1.37 (10H, m, 2', 3', 4', 5'and 6'-CH_2), 1.58 (1H, m, 7'-CH), 2.18 (2H, t, J=7.8Hz, 1'-CH_2), 3.86 (3H, s, -OCH_3), 4.35 (2H, d, J=5.6Hz, benzylic CH_2), 5.61 (1H, s, NH), 5.63 (1H, s, phenolic -OH), 6.80 (3H, m, 3, 5 and 6-aromatic protons) (Figure 7). ^{13}C NMR ($CDCl_3$): δ 174.6 (C=O), 145.12, 146.68 (2XC-O aryl), 55.94 (Ome), 43.54 (RCH_2-N), 22.68 (2XCH_3). EI-MS: m/z(% int.) 307(M^+, $C_{18}H_{29}NO_3$, 6.1), 195 ($C_{10}H_{12}O_3$, 8.1), 168 ($C_8H_{19}NO$, 4.5), 152 ($C_8H_{10}NO_2$, 12.9), 137($C_8H_9O_2$, 100).

Supercritical CO_2 fluid extraction: The lyophilized 'Scotch Bonnet' pepper was milled and 2.4 g of the powdered tissue was packed in the 32 ml stainless steel extraction cell (1.5 cm X 20 cm). The extraction was carried out at 50^{0}C and 450 atm, and 50^{0}C and 600 atm for 30 min and 1 h, respectively. In each experiment, the extracts were collected in chloroform and the solvent was evaporated *in vacuo* to dryness prior to analysis.

Solvent extraction of peppers: The milled peppers (10 g, each) were extracted sequentially with hexane, ethyl acetate and methanol (500 ml each, 24h per solvent). The

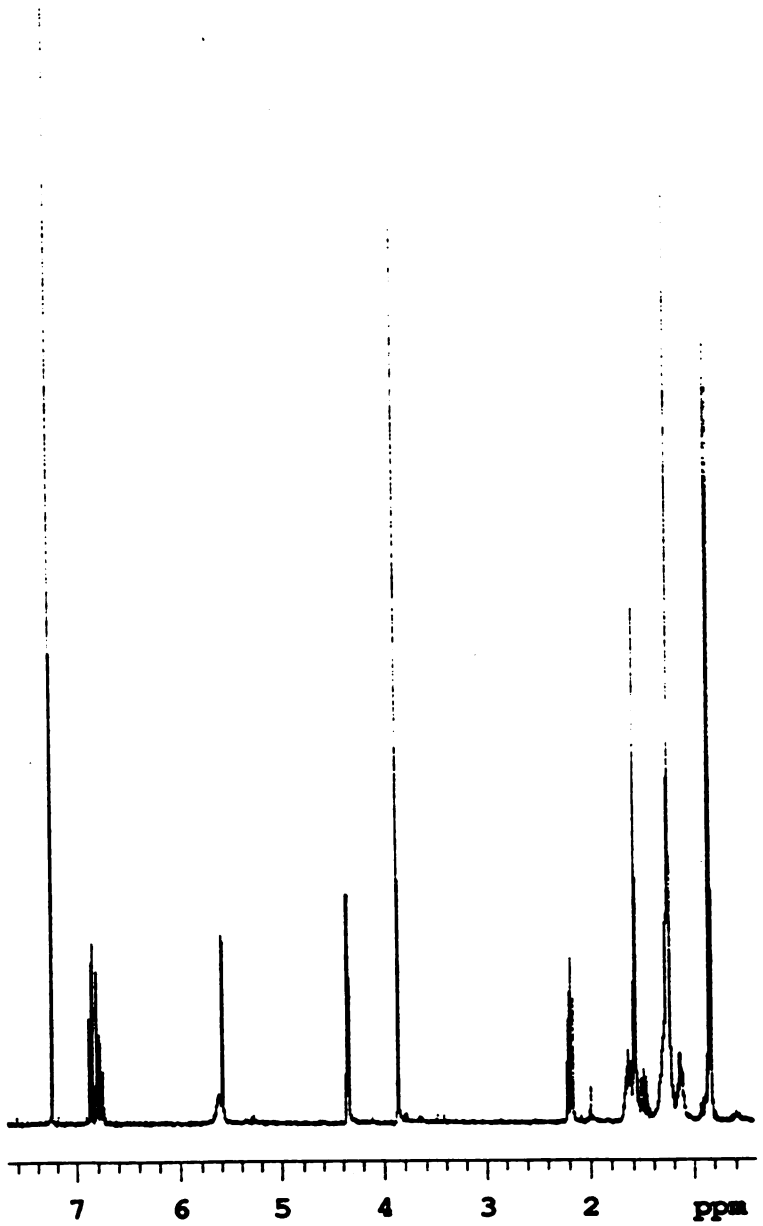

Figure 7: ^{1}H NMR spectrum of dihydrocapsaicin from 'Scotch Bonnet'

extracts were evaporated *in vacuo* to dryness and stored at 20^0C prior to the analysis.

Quantification of capsaicin in C. annuum cultivars: 10.0 mg each of the crude extract were dissolved in 5 ml of acetonitrile and filtered through the MILLEX-FGS filter (0.2 μm, Millipore Inc., Bedford, MA). The solutions (50 ul each) were analyzed by HPLC. The peak area for capsaicin was recorded and the percentage of capsaicin to dry weight in each *C. annuum* cultivars was calculated.

RESULTS AND DISCUSSION

Preliminary HPLC analysis of commercially available capsaicin indicated that acetonitrile/water (50/50, v/v) produced the best separation of capsaicin and dihydrocapsaicin. The HPLC chromatogram of purified capsaicin and dihydrocapsaicin from both 'Scotch Bonnet' and Aldrich capsaicin showed only single peaks at 5.9 min for capsaicin and 8.8 min for dihydrocapsaicin, respectively.

Capsaicin and dihydrocapsaicin were isolated and purified from both 'Scotch Bonnet' and Aldrich capsaicin. The mixture of capsaicin and dihydrocapsaicin obtained from the hexane extracts of 'Scotch Bonnet' was purified by C_{18} silica column chromatography, using the method used for purification

of Aldrich capsaicin, and gave pure capsaicin and dihydrocapsaicin (Figure 5, 6 and 7).

The quantification of capsaicin and dihydrocapsaicin from 'Scotch Bonnet' and Aldrich capsaicin were carried out by HPLC and the characterization by NMR and mass spectral data.

^{1}H and ^{13}C NMR spectra of capsaicin and dihydrocapsaicin from 'Scotch Bonnet' and Aldrich capsaicin were found to be identical. In the ^{1}H NMR spectra of capsaicin and dihydrocapsaicin from 'Scotch Bonnet' (Figure 6 and 7) revealed three protons at 6.8 ppm, indicative of a benzene ring with three substituents. The multiplet indicates that two of the aromatic protons are ortho coupled to each other and the third is coupled to one of the ortho coupled protons. The singlets at 5.59 and 5.63 ppm support the phenolic -OH and -NH protons in the molecule. The singlet at 3.84 ppm was assigned to an aromatic $-OCH_3$. The ^{13}C NMR spectra of capsaicin from Scotch Bonnet, shows the signal at 172.81 ppm for the carbonyl carbon. The 146.72 and 145.14 ppm were assigned for two arylic carbons. The peak at 55.92 ppm was methoxyl carbon (Ome). The signal at 43.53 ppm was assigned to CH_2-NH.

The mass spectra of both capsaicin and dihydrocapsaicin of 'Scotch Bonnet' clearly indicated a molecular ion at m/z 305 and 307, respectively. Both compounds gave base peaks at m/z 137, for the fragment $C_8H_9O_2$, and peaks at m/z 195 for $C_{10}H_{12}O_3$, at m/z 168 for $C_8H_{19}NO$, and at m/z 152 for $C_8H_{10}NO_2$.

Extraction of the lyophilized, milled fruits of 'Scotch Bonnet' using supercritical CO_2 and organic solvents afforded red oily residues in all cases. HPLC analysis indicated that the capsaicin content in the organic extract was 0.514% d.w. in 'Scotch Bonnet', 0.088% d.w. in 'Chile', 0.068% d.w. in 'Jalapeno', 0.034% d.w. in 'Cayenne', 0.047% d.w. in 'Forti F1', and zero in 'Bell Captain', 'Mayata and Sweet Banana', respectively. The capsaicin content (% d.w.) of peppers in supercritical fluid extract was 3.2% in 'Scotch Bonnet'. On the basis of the above results, the supercritical fluid extraction of capsaicin was superior to the conventional organic solvent extraction.

The capsaicinoids can be distinguished individually by their fatty acid moieties. Sticher et al. (1978) reported that the elution order of capsaicinoids on HPLC were related to their fatty acid chain length and the degree of saturation. Therefore, only a reverse-phase HPLC system can separate these compounds.

An authentic sample of capsaicin purchased from Aldrich Chemical Co. contained 67% of capsaicin and 33% of dihydrocapsaicin (Figure 2). Purification of this sample by C_{18} column chromatography yielded the pure capsaicin and dihydrocapsaicin. The pure capsaicin was used for the quantification of capsaicin in all the pepper extracts investigated.

Hexane, ethyl acetate and methanol were used

sequentially to extract dried fruits of *C. annuum* cultivars and the capsaicin content in each pepper was analyzed by HPLC.

Supercritical fluid extractions of peppers also were investigated. The supercritical fluids are unique in their dissolving capabilities and hence have been used widely in the extraction of natural products from plant and microbial tissues.

Eight pepper cultivars with different capsaicin contents were extracted with solvents. Supercritical CO_2 was used to extract 'Scotch Bonnet' pepper and the result was compared with the solvent extraction of 'Scotch Bonnet'. Figure 8 illustrates the results of capsaicin content by both supercritical CO_2 and solvent extractions. The most pungent pepper, 'Scotch Bonnet', gave the highest level of capsaicin among these peppers. The SFE extraction of 'Scotch Bonnet' gave 3.2% capsaicin /g dry weight which is about 32 times higher than that from total solvent extraction of 'Scotch Bonnet' (0.51%). 'Chile', 'Jalapeno' and 'Cayenne' peppers were found to contain 0.09%, 0.07% and 0.03%, respectively, by using solvent extraction. Capsaicin was not detected in 'Bell Captain', 'Cuba', 'Mayata F1' and 'Sweet Banana' peppers. These results indicate that the pungency of pepper is related to the amount of capsaicin present in the pepper and is in agreement with the published results (Mary, 1984; Todd et al. 1977).

Identification of capsaicin and dihydrocapsaicin from

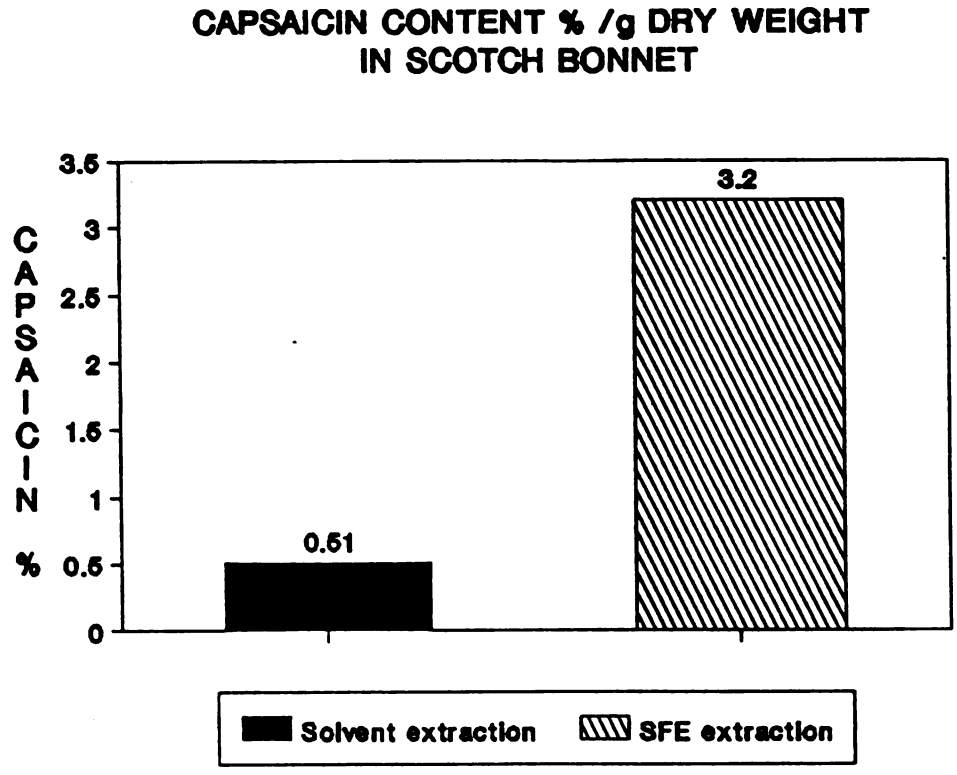

Figure 8: Comparison of SFE and solvents extraction of capsaicin from 'Scotch Bonnet'

C. annuum cultivars were achieved by comparing the retention times of the purified capsaicin and dihydrocapsaicin from Aldrich capsaicin. Only two capsaicinoids, capsaicin and dihydrocapsaicin were isolated and purified from the pepper cultivars studied. The purified capsaicin and dihydrocapsaicin from 'Scotch Bonnet' gave identical spectral data to the capsaicin and dihydrocapsaicin from Aldrich sample.

Capsaicin and dihydrocapsaicin can be distinguished clearly from their ^{1}H NMR spectra. The signal at 5.3 ppm for two olefinic protons in capsaicin (Figure 6) was absent in the ^{1}H NMR spectra of dihydrocapsaicin (Figure 7). Also, based on the ^{1}H NMR spectra of the capsaicinoids mixture obtained from 'Scotch Bonnet', the relative ratio of capsaicin and dihydrocapsaicin in the mixture was 82% and 18%, respectively (Figure 5). There were no detectable levels of other capsaicinoids in these peppers. This is the first report of the isolation, purification and characterization of capsaicin and dihydrocapsaicin from 'Scotch Bonnet' pepper.

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CHAPTER THREE

INVESTIGATION OF BIOACTIVE COMPOUNDS IN *CAPSICUM ANNUUM* CULTIVARS

ABSTRACT

The major compounds responsible for the pungency of capsicum were capsaicin and its analogues. Supercritical fluid and organic solvent extraction of *Capsicum annuum* cultivars afforded extracts containing the pungent principles, capsaicin and dihydrocapsaicin. Bioassays of extracts and pure capsaicin and dihydrocapsaicin were carried out on fungi, bacteria, nematodes and mosquito larvae. The hexane extracts of fruits of *Capsicum annuum* Sweet Banana, Maya F1, Bell Captain, Jalapeno, Chile, Cayenne and Scotch Bonnet were found to be active against fungi, bacteria and mosquito larvae at 250 ppm. The hexane extract of 'Scotch Bonnet' fruits showed strong mosquitocidal activity on fourth instar *Aedes aegypti* larvae at 50 ppm. Purification of this hexane extract afforded a pure compound, bonnetenol, that gave 100% mortality on *A. aegypti* larvae at 0.1 ppm in 24 h. Bonnetenol was characterized by mass spectral, ^{1}H, ^{13}C, ^{1}H - ^{1}H decoupling and DEPT nuclear magnetic resonance analyses. Bonnetenol was confirmed to be Z-hept-3-en-1-ol.

INTRODUCTION

The principal compounds responsible for the pungency of chile peppers are capsaicinoids, amino acids of vanillylamine and C_9 - C_{11} branched fatty acids (Figure 3, Chapter 1) (Hoffman, et al. 1983). Capsaicin and dihydrocapsaicin are the predominant compounds in chile peppers, contributing 90% or more of the total heat value (Iwai, et al. 1979).

There are no reports on the biological activity of compounds extracted from *C. annuum* cultivars, against fungi, bacteria, nematodes and insects. Supercritical fluid and organic solvents were used to extract capsaicinoids from *C. annuum* cultivars (Chapter 2). Capsaicin and dihydrocapsaicin were isolated and purified by VLC, prep.TLC and a reverse - phase C_{18} column chromatography (Chapter 2). All extracts of peppers, pure capsaicin and dihydrocapsaicin were assayed separately for nematicidal, fungicidal, bactericidal and mosquitocidal activities.

MATERIALS AND METHODS

General experimental: Vacuum liquid chromatography (VLC) was performed on the silica gel (Analtech silica gel 60 A pore size, 35 - 75 micron particle size). Preparative thin layer chromatography (Prep. TLC) was performed on silica gel

Uniplates (Analtech silica gel GF-254, 0.5 mm, 20 X 20 cm, 2000, 1500, 500, 250 microns). Column chromatography was performed using a 3.3 cm X 33 cm column containing silica gel (Analtech silica gel 60 Å pore size, 35 - 75 microns particle size), and thin layer chromatography (TLC) on silica gel (Aldrich silica gel G F-254, 0.250 mm layer, 2.25 μ mean particle size). Unless specified, the developed plates were viewed in an ultraviolet fluorescence analytical cabinet (Spectroline Inc.) at 366 nm and 254 nm, respectively, or sprayed with 50% H_2SO_4, then heated to charc the compound.

Low pressure C-18 column chromatography was performed using an LC-SORB glass column (Chemco, 3.2 cm X 50 cm) packed with C_{18} silica gel and equipped with a low-pressure pump (Model 81-M-2, Chemco), rhoeadine injector (Chemco) and a UV-IS 200 detector at 280 nm (Sanki Laboratories Inc.).

Supercritical fluid extraction (SFE) of peppers was achieved on a Dionex - 703 SFE equipped with 8cm X 32 ml extraction cells (Dionex Co. USA).

High performance liquid chromatography (HPLC) was performed using an HPLC system consisting of a Waters automated gradient controller equipped with a Waters Model U6K injector, Shodex Degasser, Waters Model 590 pump and two Waters M-6000A pumps. The guard column was Nova-Pak (Water Associates) with removable C-18 cartridge. The column was Waters Radial - Pak C_{18} cartridge, 4 μ particle size, 5 X 10 mm inserted in a Waters RCM 8 X 10 radial compression module

(RCM). Detection was at 280 nm with a Waters 490 programmable multiwavelength U.V. detector. Data acquisition was carried out on a Waters 740 data module. The mobile phase was acetonitrile : water (1:1). The flow rate was 0.75 ml/min.

Melting point was determined on a Kofler hot stage melting point apparatus (Bristoline Co. USA) and was uncorrected.

Proton (^{1}H)and carbon (^{13}C), ^{1}H - ^{1}H decoupling and DEPT nuclear magnetic resonance spectra were obtained on a Varian VXR - 300 spectrometer (Varian Co. USA), 300 Mhz for proton and 75 Mhz for carbon, and a Varian VXR - 500 spectrometer, 500 Mhz for proton and 125 Mhz for carbon. Electron impact mass spectra (EIMS) were obtained on a Jeol model JMAX 505 mass spectrometer at 70 Ev. (Jeol JMS Co. USA). Ultraviolet (UV) absorption analysis was conducted on a Shimadzu UV-Visible model UV-260 spectrophotometer.

Plant material: The matured fruits of *C. annuum* cultivars Scotch Bonnet, Mayata F1, Hybrid Bell Captain, Cuba and Sweet Banana were collected from pepper plants grown in the greenhouse of the Department of Horticulture, Michigan State University, East Lansing, Michigan. Other types of *C. annuum* used were Chili purchased from Meijer Inc., Lansing, Michigan, Cayenne purchased from Kroger Co., Okemos, Michigan, Jalapeno purchased from Horrock's Farm Market, Lansing, Michigan. All peppers were lyophilized by a DURA-DRY FTS - Tray Lyophilizer

(FTS SYSTEM Inc. USA), and stored in sealed plastic bags at -20^{0}C prior to extraction.

Supercritical CO_2 fluid extraction: The lyophilized Scotch Bonnet pepper was milled and 2.4 g of the powdered tissue was packed in a 32 ml stainless steel extraction cell (1.5 cm X 20 cm). The extraction was carried out at 50^{0}C and 450 atm, and 50^{0}C and 600 atm for 30 min and 1 h, respectively. In each experiment, the extracts were collected in chloroform and the solvent was evaporated *in vacuo* to dryness prior to analysis.

Solvent extraction of peppers: The milled peppers (10 g each) were extracted sequentially with hexane, ethyl acetate and methanol (500 ml each, 24h per solvent). The extracts were evaporated *in vacuo* to dryness and stored at 20^{0}C prior to the analysis.

Isolation of bioactive compounds from 'Scotch Bonnet': Lyophilized and milled 'Scotch Bonnet' tissue (139.9 g) was packed in a glass column (20 mm X 37 cm) and extracted with hexane (2L, 24 h) at room temperature. Removal of solvent *in vacuo* afforded an oily dark red residue (11.1 g)(Figure 1). Because only the hexane extract was active against mosquito larvae, it was further purified by VLC. A slurry of column silica (fine, 4.5 - 5.0 um) was packed under vacuum in a sintered glass filter and washed with hexane. This hexane

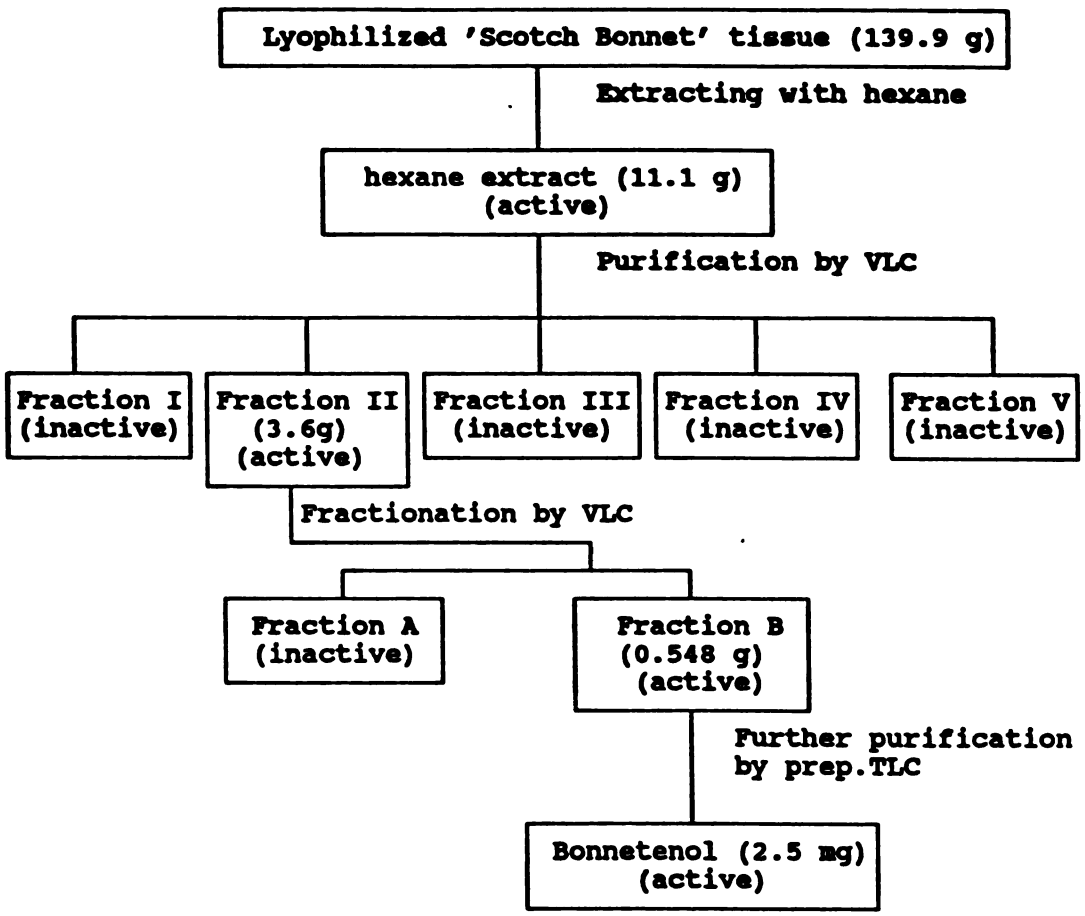

Figure 1: Isolation and purification of bonnetenol from 'Scotch Bonnet'

extract (11.1 g) then was applied on silica as a hexane solution and eluted sequentially with hexane (fraction I, 280 ml), hexane : acetone (9 : 1, fraction II, 700 ml), hexane : acetone (4 : 1, fraction III, 450 ml), chloroform : methanol (4 : 1, fraction IV, 200 ml) and methanol (fraction V, 400 ml). Only fraction II (3.6 g) showed mosquitocidal activity. Further fractionation of this fraction by VLC afforded two fractions, eluted with hexane : acetone (4 : 1, fraction A, 470 ml) and hexane : acetone (1 : 1, fraction B, 410 ml), respectively. Upon TLC analysis, fraction B (1.011 g, R_f= 0.28) was active on mosquito larvae. Preparative TLC of this fraction (0.548 g) (hexane : acetone, 6 : 1) gave three bands, which were eluted with chloroform : methanol (4 : 1). Band I (233.8 mg) at R_f=0.45 (light yellow) gave strong activity on mosquito larvae and on further purification by preparative TLC afforded pure bonnetenol. The chemical identification of bonnetenol was achieved by ^1H, ^{13}C, ^1H - ^1H decoupling and DEPT NMR experiments, along with MS and UV analysis.

Bonnetenol: $C_7H_{14}O$, showed UV absorption at 202 (ϵ = 3109), 256 (ϵ = 1080) and 273 (ϵ = 166) nm, respectively (Figure 2). ^1H NMR ($CDCl_3$): δ 0.96 (3H, t, J=7.5Hz, 7-CH_3), 1.26 (2H, m, 5-CH_2), 1.45 (2H, m, 6-CH_2), 1.54 (1H, s, exchanged with D_2O, -OH), 1.72 (2H, m, 2-CH_2), 4.31(t, J=6.7Hz, 1-CH_2), 7.52 (d,d, J=3.4, 5.8Hz, olefinic H), 7.71 (d,d, J=3.4, 5.8Hz, olefinic H)(Figure 3a and 3b); ^1H-^1H decoupling NMR spectrum is showed

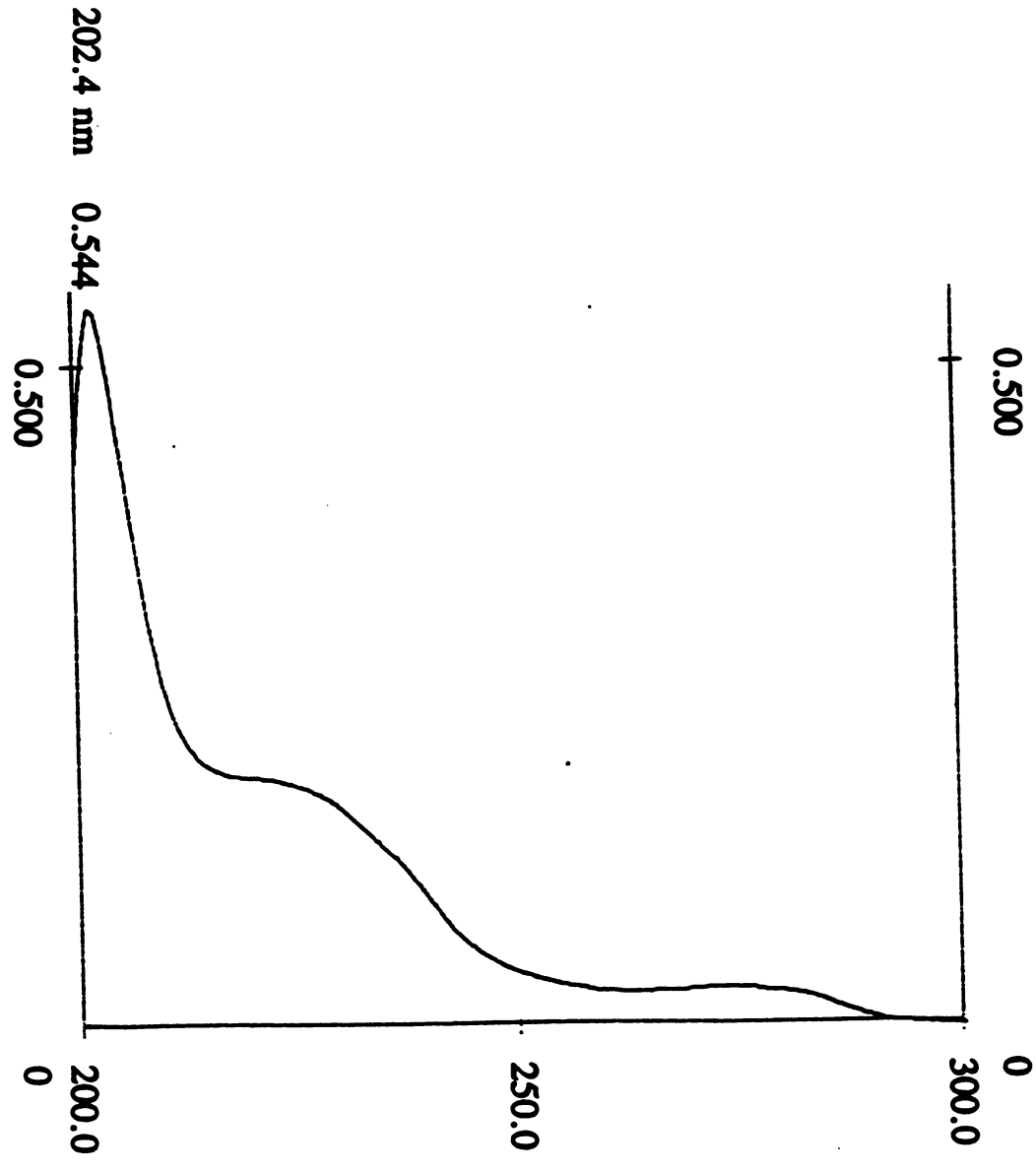

Figure 2: UV spectrum of bonnetenol

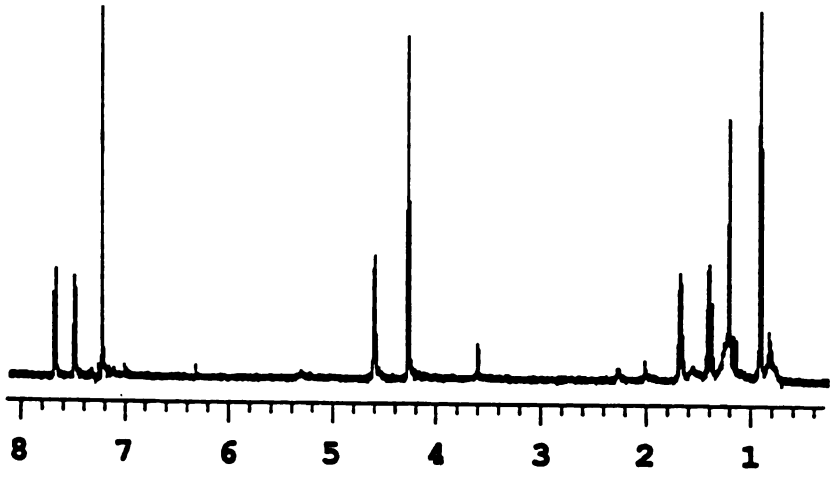

Figure 3a: ^{1}H NMR spectrum of bonnetenol with D_2O exchange

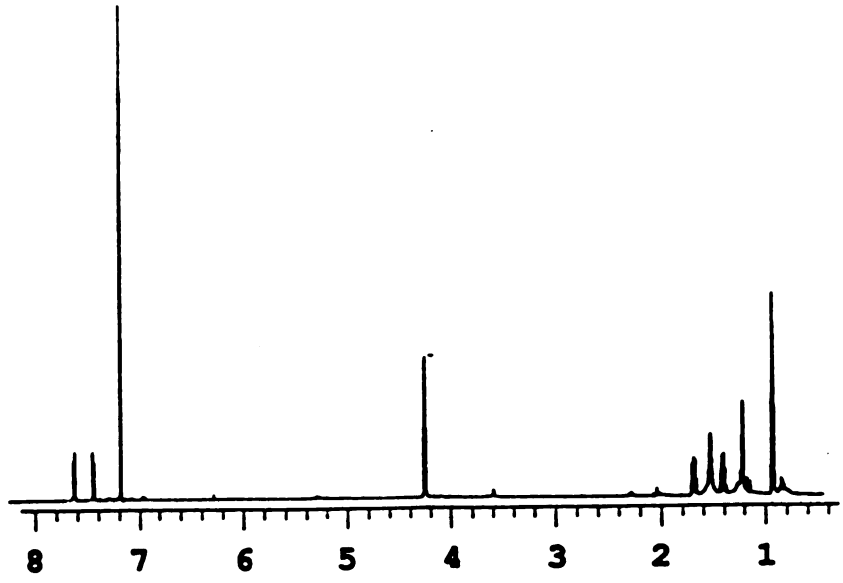

Figure 3b: ^{1}H NMR spectrum of bonnetenol

in figure 4. ^{13}C NMR ($CDCl_3$): δ 13.71 (C-7), 19.17 (C-6), 29.68 (C-5), 30.56 (C-2), 65.56 (C-1), 128.83 (C-4), 130.89 (C-3)(Figure 5); ^{13}C DEPT NMR spectrum is showed in figure 6.

EI (DI) MS: m/z(% int.) 113 ($C_7H_{13}O$, 14.7), 111 ($C_7H_{11}O$, 38.6), 97 (C_7H_{13}, 62.6), 83 (C_6H_{11}, 62.3), 69 (C_5H_9, 72.3), 57 (C_4H_9, 100) (Figure 7).

Microorganisms: *Aspergillus flavus, Candida albicans, Fusarium oxysporum, Fusarium monoliniforme, Streptococcus aureus, Staphylococcus epidermidis* and *Escherichia coli* were grown on plates containing 20 ml of Emmons agar (EM)(made by dissolving 10g of neopeptone, 20g of glucose, 18g of agar in 1 liter distilled water). *Phomopsis occulta, Phomopsis viticola, Aspergillus flavus, Botrytis sp., Rhizoctonia sp.* and *Gloesporum sp.* were grown on potato dextrose agar (PDA) plates (made by dissolving 39g of potato dextrose agar in 1 liter distilled water). All media were autoclaved at 120°C and 15 atm for 20 min.

Antifungal bioassay: Known amounts of the extracts, as well as pure bonnetenol, capsaicin and dihydrocapsaicin were dissolved in DMSO, to obtain 50 mg/ml stock solution. These solutions, 5 μl each (250 μg), were applied on the plates that were lawned with the test organisms and incubated at 26°C for 2-6 days. Inoculated plates without test compounds, but with solvent DMSO, served as the control. A clear zone of

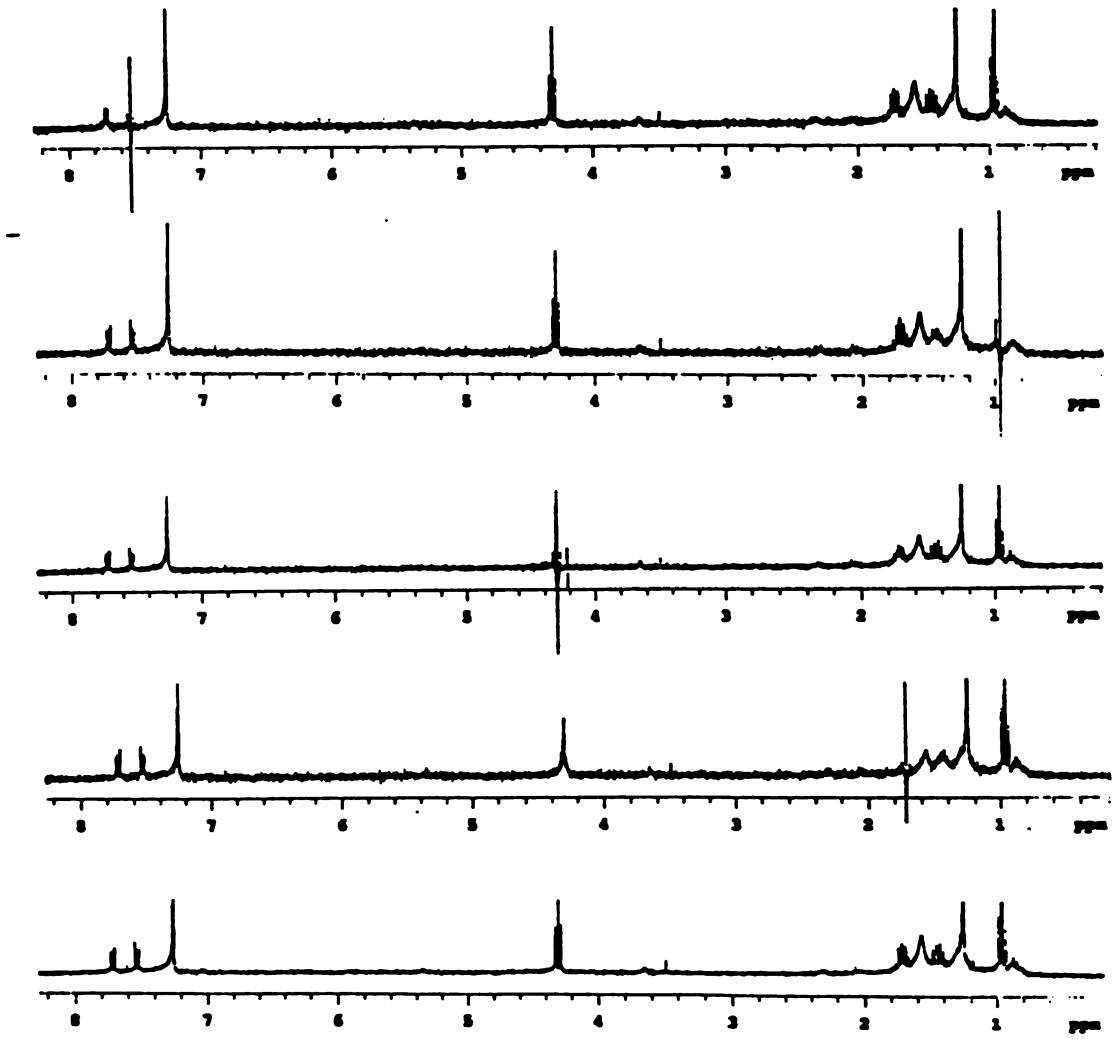

Figure 4: ^{1}H-^{1}H decoupling NMR spectrum of bonnetenol

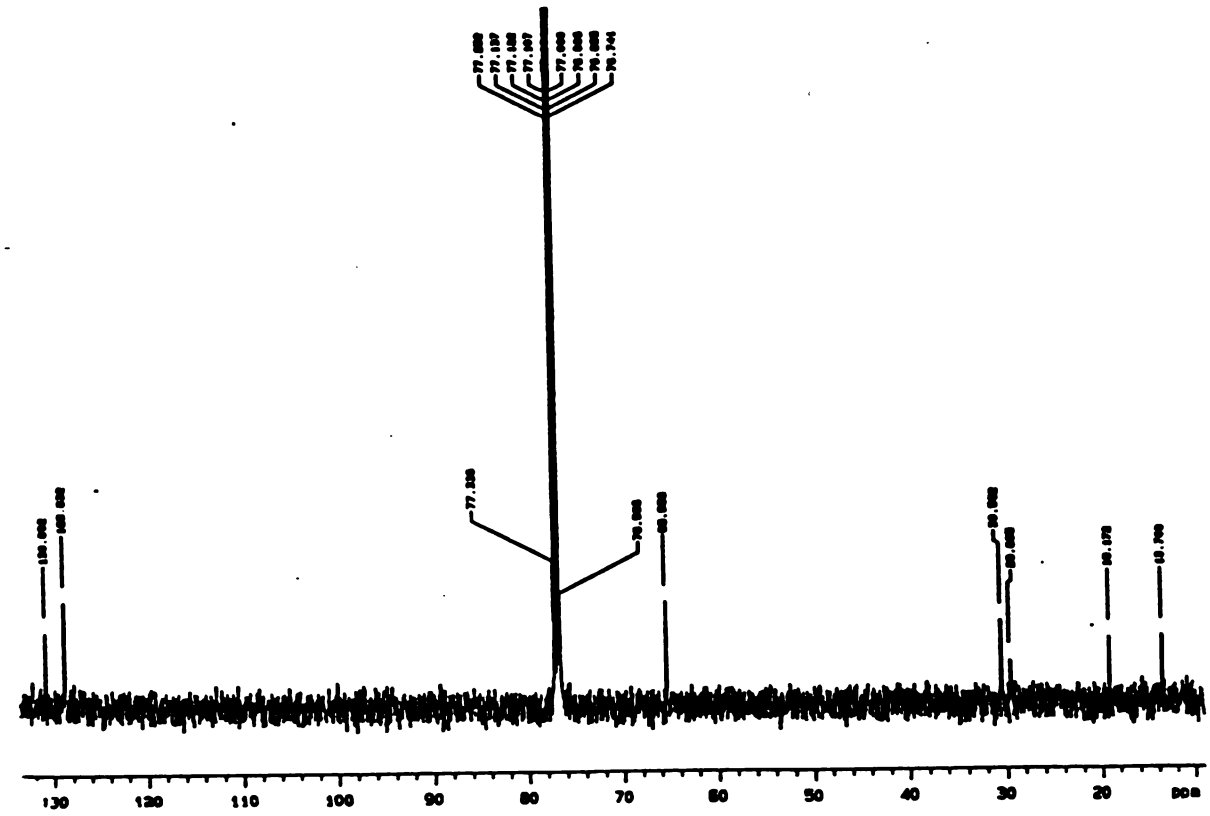

Figure 5: ^{13}C NMR spectrum of bonnetenol

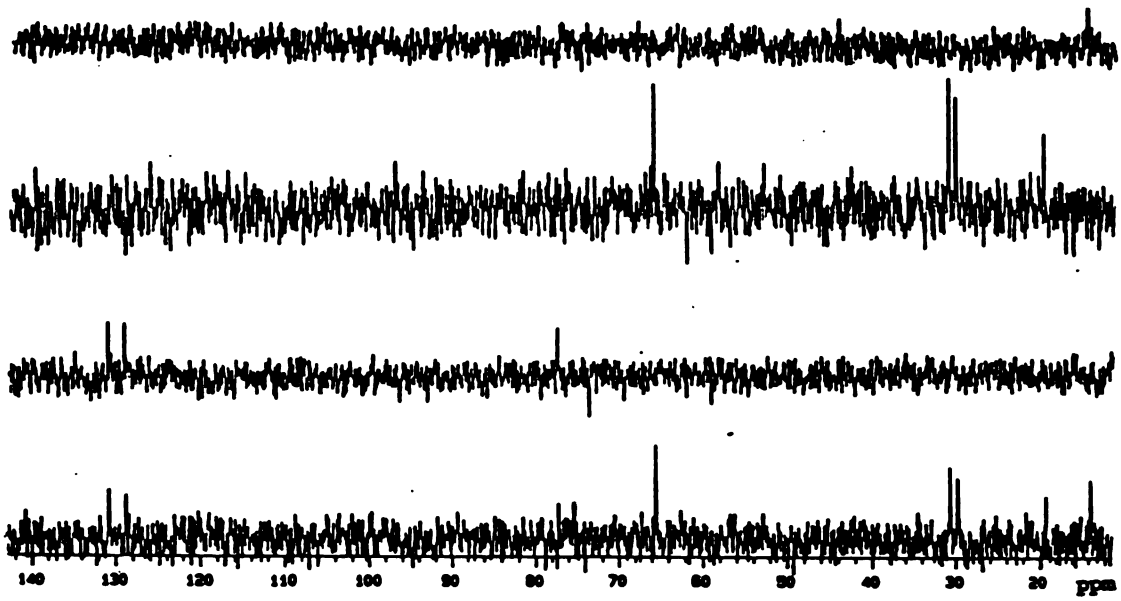

Figure 6: DEPT NMR spectrum of bonnetenol

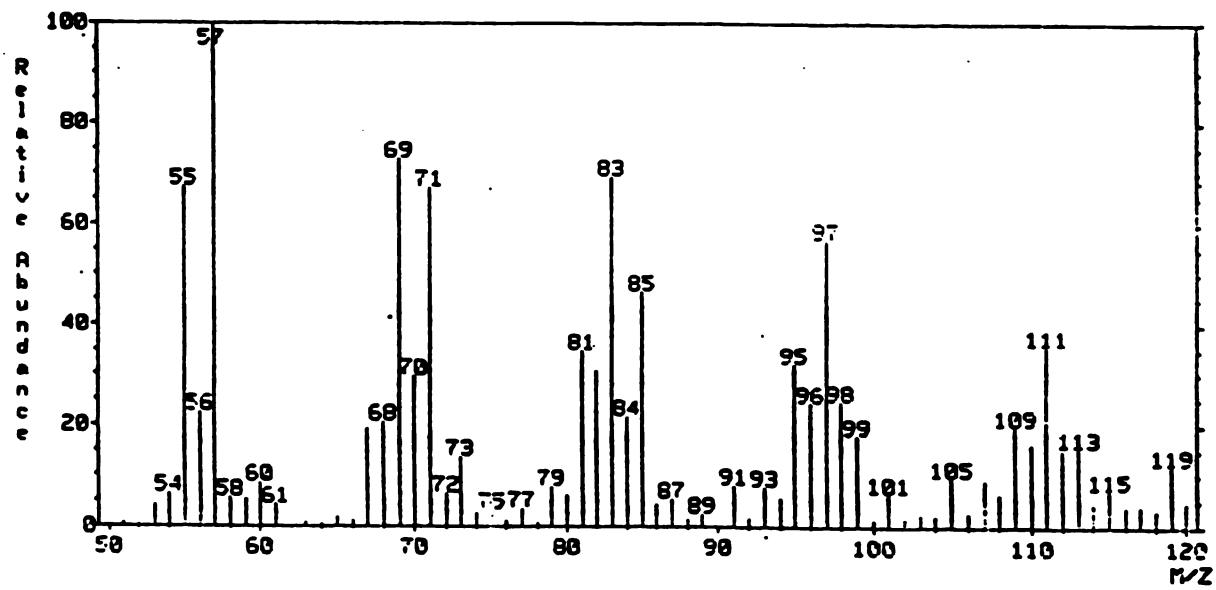

Figure 7: Mass spectrum of bonnetenol

inhibition characterized by the absence of the fungal growth around the test compound droplet was recorded after 2 - 6 days.

Antibacterial assay: The antibacterial activity of all test extracts and standard capsaicin was evaluated by the same procedure as in the antifungal assay experiments except that the test organisms were bacteria instead of fungi.

Insecticidal assay: Insecticidal activity was evaluated with 4th instar mosquito larvae, *Aedes aegypti*, reared from eggs (University of Davis California Straw) in degassed distilled water. After emergence, the larvae were fed liver powder for one week. The bioassy was conducted in glass test tubes (10 X 75 mm) each of which contained 6 - 7 mosquito larvae. DMSO solutions of the extracts (25 μl each) and pure capsaicin were added to 975 μl of distilled water with 6 - 7 mosquito larvae. The experiments were carried out in three replications at room temperature. Larvae were observed for mortality at 0.5, 1, 2, 4, 8, 10, 12, 24, 48 and 72 h intervals. Control tubes containing 6 - 7 larvae received 25 μl of DMSO alone and the mortality was recorded as in the case of test extracts.

Nematicidal activity: Nematicidal activity was tested on the free-living nematode, *Panagrilus redivivus*, reared in NG media for two weeks. 5 ml of sterile saline solution (8.5 g sodium

chloride in 1 liter distilled water) were used per plate to prepare the nematode suspension, and transferred into a sterile test tube. The nematode suspension (48 µl), containing 40 - 60 nematodes at various developmental stages was added to each well (0.7 cm d X 1.0 cm h) of a 96-well tissue culture plate. 2 µl of each extract and pure capsaicin in DMSO were added into each well. The control well contained pure DMSO. The inoculated plates were held in a container at ca. 100% humidity. Plates were observed with an inverted microscope at 40X. Mortality was recorded as the mean of three replications at each dose after 2, 4, 8, 24, 48 and 96 h.

RESULTS AND DISCUSSION

Hexane extracts of *C. annuum* cultivars showed good activity on *A. aegypti*, *C. albicans* and *S. epidermidis*, and SFE extracts of 'Scotch Bonnet' were also active on *Rhizoctonia spp.* However, ethyl acetate and methanol extracts of most *C. annuum* cultivars., as well as pure capsaicin and dihydrocapsaicin, were inactive against fungi, bacteria and mosquito larvae. No nematicidal activity was observed with any of the test compounds.

The most active compound, bonnetenol (0.0018% /g dry weight) from 'Scotch Bonnet' had a minimum inhibitory concentration of 0.1 ppm against *A. aegypti*. It was extracted

from 'Scotch Bonnet' using hexane and purified by means of VLC and preparative TLC.

Isolation and purification of pure capsaicin and dihydrocapsaicin from both Scotch Bonnet and Aldrich capsaicin were achieved by VLC, preparative TLC and column chromatographic methods. NMR and MS were used to characterize capsaicin and dihydrocapsaicin. Although capsaicin and dihydrocapsaicin are the most pungent compounds in peppers, they did not show any biological activity against the test microorganisms.

The ^{1}H NMR signal at δ 0.96 for three protons with J=7.5Hz was assigned to a CH_3 group adjacent to a methylene group. This was confirmed by ^{13}C and DEPT NMR data which indicated only one $-CH_3$ functionality in the molecule. The multiplet for two protons each at δ 1.26, 1.45 and 1.72, were assigned to three methylene groups, respectively. A triplet, which integrated for two protons at δ 4.31, was indicative of a CH_2-O functionality adjacent to a methylene group. A singlet for one proton at δ 1.54, exchanged with D_2O, supported the presence of a -OH group. Two doublets of doublets at δ 7.52 and 7.71, respectively, were assigned to two olefinic protons cis to each other as evident from their coupling constant of 6.5Hz. The smaller coupling, J=3.4Hz, was due to the cis coupling of the olefinic protons to one of the adjacent methylene groups (Figure 3a).

The ^{1}H - ^{1}H decoupling experiments provided additional

evidence for the assignments of the signals observed in the ^{1}H NMR spectra for bonnetenol. Irradiation of the triplet at δ 4.31 collapsed the multiplet at δ 1.72 to a tight doublet of doublets appearing as a triplet. The doublet of doublets at δ 7.52 and 7.71, respectively, were collapsed to a triplet each when they were irradiated separately. Decoupling the multiplet at δ 1.45 collapsed the $-CH_3$ at δ 0.96 to a singlet (Figure 4).

^{13}C NMR of bonnetenol gave only seven signals (Figure 5). The DEPT experiments indicated that two signals at 128.83 and 130.89 ppm, respectively, were olefinic carbons, one methyl carbon at 13.71 ppm, and four methylene carbons at 19.17, 29.68, 30.56 and 65.56 ppm, respectively. The signal at 65.6 ppm was assigned to the carbon with a hydroxyl group (Figure 5).

The EI (DI) mass spectrum of bonnetenol gave a single peak in the total ion current chromatogram (TIC). The mass peak at m/z 113 was attributed to the loss of one proton from the parent compound, bonnetenol. A peak at m/z 97 was characteristic of the loss of the only -OH from bonnetenol. The peak at m/z 83 and 69, differing by 14 mass units from the peaks m/z 97 and m/z 83, respectively, were due to the loss of a CH_2 fragments. The base peak at m/z 57 was assigned to the fragment C_4H_9 (Figure 7 and 8). Therefore, based on the spectral evidence, bonnetenol is confirmed to be Z-hept-3-en-1-ol (Figure 9).

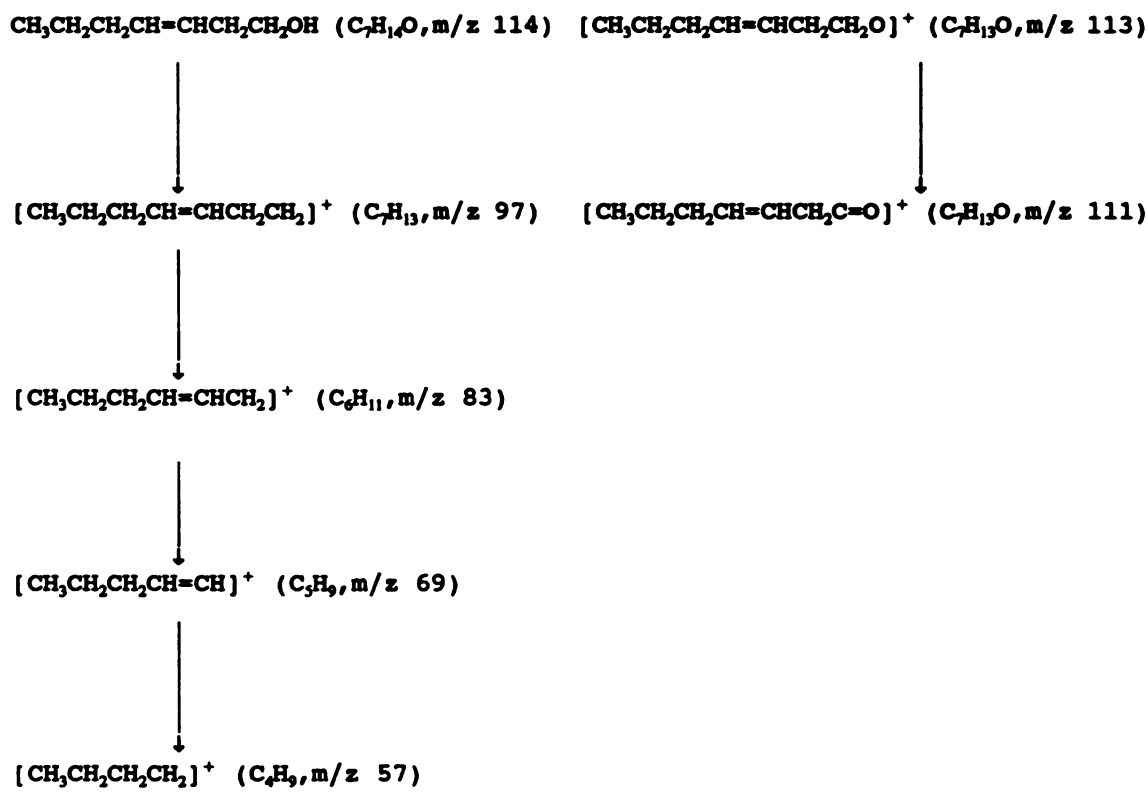

Figure 8: MS fragmentation pattern of bonnetenol

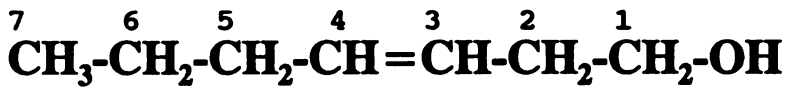

Figure 9: Structure of bonnetenol

This is the first report of the isolation and characterization of a biologically active compound from 'Scotch Bonnet'. There are no previous reports on mosquitocidal compounds in any of these *Capsicum annuum* cultivars. Bonnetenol, Z-hept-3-en-1-ol, has potential application as a mosquitocidal compound and may prove to be effective for the control of diseases such as malaria. More research must be conducted to evaluate the practical value of bonnetenol.

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