CAPSAICINOID: STUDIES ON CHEMICAL DEFLOWERING FOR ENHANCING HARVESTABLE CAPSAICINOID PRODUCTION AND MECHANISMS FOR CAPSAICINOID-SPECIFIC METABOLISM IN PEPPER FRUIT

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CAPSAICINOID: STUDIES ON CHEMICAL DEFLOWERING FOR ENHANCING HARVESTABLE CAPSAICINOID PRODUCTION AND MECHANISMS FOR CAPSAICINOID-SPECIFIC METABOLISM IN PEPPER FRUIT

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INTRODUCTION

Peppers are popular food additives in many parts of the world, valued for their sensory attributes of color, pungency and aroma. Peppers originated in South America and the first records of peppers as a food ingredient date back to 7000 BC when they were consumed by Indians inhabiting what is now Mexico (Bosland, 1994). By the late 16^{th} and early 17^{th} century, pepper cultivation and enthusiasm had spread to many parts of the world. Today chili peppers are popular seasonings, contributing in excess of 60% of the total spice trade by volume (Caterina and Julius, 2001). Peppers are quite diverse and may be classified by the trade according to end use. Peppers grown for their characteristic hot flavor are of the genus Capsicum (family Solanaceae), *C. annuum* L. var. *annuum* principally and *C. frutescens* L. to a lesser extent. Hot peppers grown in tropical areas are characterized by a stimulation of a range of distinct pungency responses. A second pepper type valued for its brilliant deep red color is paprika, evoking none to significant pungency. Both types have a distinct aroma, valuable in certain formulations. A third pepper type classified according to end use is *C. annuum* var. *annuum* , the large-sized fleshy bell pepper used as a fresh vegetable and valued for its aroma, color and crisp texture, but evokes no pungency (Govindarajan and Sathyanarayana, 1991).

The name capsicum may have been derived from the Greek “Kapso” meaning to bite, in reference to its pungency, or from the Latin “capsa” referring to the fruit pod (Govindarajan, 1985; Maga, 1975).

Hot pepper is widely used for its pungent flavor and also as a drug. The Scoville organoleptic test, invented by W. L. Scoville in 1912, is a subjective measure of chile pungency. Approximately one part per million of ‘heat’ is equivalent to 15 Scoville units (American Spice Trade Association, 1960 and Hoffman and Lego, 1983). In the original test, exact weights of pure ground chilies of different types were soaked separately in alcohol overnight. Since capsaicinoids were soluble in alcohol, the soaking extracted some of the pungent chemicals from the pod. The samples were diluted with increasing amounts of sugar water. The hotter the sample, the greater the amount of water required to dilute it until the pungency was no longer detectable to the palate. Thus, if the dilution required was 1000 units of sugar water to 1 unit of the alcohol/chili sample, the sample was said to have a pungency of 1000 Scoville units. Five tasters recorded their opinions and a majority of three of the tasters had to agree before a value was assigned.

In an eight hour period, no more than six samples could be run through the panel. Total analysis from alcohol soak to sensory evaluation of the six samples took sixteen to 24 hours and the numbers of daily tests were limited to 6 samples due to panel fatigue. The validity and accuracy of the Scoville Organoleptic test have been widely criticized because of its subjective nature. The American Spice Trade Association (ASTA) and the International Organization for Standardization have adopted a modified version. Nowadays the High-Performance Liquid Chromatography (HPLC) test is used. In this procedure, pepper pods are dried, ground and the chemicals responsible for the pungency

(capsaicinoids) are extracted, and the extract is quantitated by HPLC. The quantity of each individual capsaicinoid is determined, and a modified Scoville rating is assigned by multiplying quantity by a pungency correction factor for the capsaicinoid detected. This method allows an objective pungency analysis. Not only does this method measure the total pungency, or Scoville heat rating, but it also allows the amount of the individual capsaicinoids to be determined. In addition, many samples may be analyzed within a short period (we routinely extract and analyze 24 samples per day and some procedures allow for over 100 samples to be analyzed in a 24 hour period).

The pungency of chile peppers is measured in multiples of 100 units, from the bell pepper at zero Scoville units to the Habanero at 350,000 to as high as 577,000 Scoville units. The pungency of pure capsaicin is said to be 16,000,000 Scoville units. Various varieties of chile peppers can be ranked according to their heat or pungency level. Pungency in chili pepper varies dramatically according to variety, and may be secondarily influenced by environment, cultural practices and post harvest handling (Bosland, 1993). The total pungency in pepper fruit is determined by adding the pungency values of each individual capsaicinoid present (Krajewska and Powers, 1998).

Pungency values for different market type peppers are:

0-100 Scoville Units includes most Bell/Sweet pepper varieties.

500-1000 Scoville Units includes New Mexican peppers.

1,000-1,500 Scoville Units includes Espanola peppers.

1,000-2,000 Scoville Units includes Ancho & Pasilla peppers.

1,000-2,500 Scoville Units includes Cascabel & Cherry peppers.

2,500-5,000 Scoville Units includes Jalapeno & Mirasol peppers.

5,000-15,000 Scoville Units includes Serrano peppers.

15,000-30,000 Scoville Units includes de Arbol peppers.

30,000-50,000 Scoville Units includes Cayenne & Tabasco peppers.

50,000-100,000 Scoville Units includes Chiltepin peppers

100,000-350,000 Scoville Units includes Scotch Bonnet & Thai peppers.

200,000 to 300,000 Scoville Units includes Habanero peppers.

Around 16,000,000 Scoville Units is Capsaicin.

Capsaicinoids can be ranked according to their heat or "pungency" level in pure form:

At 8,600,000 Scoville Units is Homocapsaicin.

At 8,600,000 Scoville Units is Homodihydrocapsaicin.

At 9,100,000 Scoville Units is Nordihydrocapsaicin.

At 16,000,000 Scoville Units is Dihydrocapsaicin.

At 16,000,000 Scoville Units is Capsaicin.

(http://www.egconsult.com/Scoville.htm)

Until recently, the hottest chilli pepper ever recorded was a Red Savina Habanero which had 577,000 Scoville pungency units while in contrast Jalapeno has between 2,500 and 10,000 units. In complete contrast, the Sweet Italian Bell pepper has a pungency of '0' units (Bellringer, 2001). Recently Indian Scientists have claimed that 'Tezpur chilli' (*Capsicum frutescens* var. *nagahari*) grown in the Northeastern part of the country had the highest combined amounts of capsaicin and dihydrocapsaicin (4.28% and 1.42% w/w) contributing to a pungency rating of 855,000 (Mathur et al., 2000).

Importance

Peppers are valued on the world market for their pungency, color and aroma. In ancient times, peppers were used as a remedy for asthma, coughs and sore throats by Mayas and to treat toothaches by the Aztecs (Bosland, 1996).

Capsaicinoids are white crystalline alkaloids that melt at 64 to 65 ˚C. They are sparingly soluble in water, but are readily soluble in fats and oils (Govindarajan and Sathyanarayana, 1991; Andrews, 1995). The alkaloids are tasteless and colorless; pepper flavor, without regard to pungency is due to small amounts of several aromatic substances. The flavor is due to mixture of volatile oils that are located throughout the outer wall of the placenta. Capsaicinoids are very potent and humans can perceive as low as 10 ppm, while birds, most insects and frogs are insensitive to heat (capsaicinoids). Most of the mammals have a specific receptor (vanilloid receptor) which other vertebrates lack.

In addition to use as a food ingredient, pepper capsaicinoids have various other uses ranging from personal defense sprays to pharmaceutical uses as a pain blocking agent (Caterina and Julius, 2001). Capsaicinoids are characterized by high biological activity and their pharmacological, neurological and dietic effectiveness has been well characterized. Treatment with capsaicin induced apoptotic cell death in a dose-dependent manner in HT-29 human colon cancer cells, and there may also be a putative role in cancer chemoprevention (Kim et al., 2004). Capsaicinoids is a potent sensory stimulating agent, acting on chemogenic pain receptors, peripheral and central warmth detectors and on pulmonary aortic and carotid baroreceptors (Saria et al., 1981). Use of higher levels of capsaicinoids, show beneficial effects in the metabolizing lipids with high-fat or high-

carbohydrate food intake. Even at lower levels of capsaicinoids with a high-fat diet, significant lowering of liver and serum triglycerides and serum lipoprotein triglycerides have been observed (Govindarajan and Sathyanarayana, 1991).

The sensations of heat and pain in the mouth are the result of the stimulation of local heat receptors in the skin and mucous membranes by capsaicin. The capsaicin (vanilloid) receptor VR1 is a sensory neuron-specific ion channel that serves as a polymodal detector of pain-producing chemical and physical stimuli. Capsaicin is a trigeminal stimulant that is important in gustatory physiology (Liu and Simon, 2000). Capsaicin can also help in the mediation of pain: prolonged application of capsaicin is thought to cause the desensitization of sensory neurons responsible for pain (Goettl et al., 1997). Capsaicin can induce sweating, which may be why chillies became popular in hot dry climates. Further, it stimulates the actions of the muscles of the stomach and intestine; this improves digestion and makes chilli peppers an attractive condiment for a food that might upset the stomach (Andrews, 1984). Most importantly, it appears that the dispersal of pepper species has been aided by the action of capsaicin, preventing animals with a digestive system that can destroy chilli pepper seeds from eating them, while allowing animals who will pass the seeds to eat them with no ill effects (Robbins, 1992; Tewkbury and Nabhan, 2001).

Consumption of chilli products

Capsicums are widely used in preparation of curries, hot sauces and Mexican and Asian cuisines. Recently the popularity of these cuisines has increased. This is due in part

to the desire to reduce fat in the diet by substituting foods that do not depend upon fat for their flavor and appeal (Henderson and Henderson, 1992)

The Capsicum genus produces the most consumed spice in the world. Peppers are used for flavoring and coloring in food manufacturing, coloring for cosmetics and for imparting heat to medicines. Besides pungency, they are used to give taste, aroma, texture, color and visual appeal to ethnic foods (Uhl, 1996). From a nutritional perspective, capsicums are good sources of vitamins particularly vitamin C in green fruits and vitamin A in mature and ripened red fruits (Bosland, 1994)

Pepper production has increased considerably from the past (International Pepper Community, IPC). The production of pepper has shown continuous growth since 1998. In 1998 the production was 205,000 metric tones (mts). It increased to 218,340 mts in 1999, 254,210 mts in 2000, 305,695 mts in 2001, 341,312 mts in 2002 and 355,160 mts in 2003. However, the projection for 2004 and 2005 were around 294,159 and 299,275 mts due to production decline in Indonesia and Brazil. The United States, ranks sixth in pepper production with an estimated acreage of 105,000 and 837,000 tons per annum (Lucero, 2002). A trend towards using spices to compensate for less salt and fat in food, and an increasing popularity of ethnic foods resulted in increased demand for pepper spices to record levels in the U.S.

Classification

The genus Capsicum includes twenty seven species, out of which five are domesticated and twenty two are undomesticated (Chinthakuntla, 2005). *Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens* are considered the

domesticated species of peppers. Capsicum fruits are considered vegetables, but are berries botanically. Capsicum fruit types are usually classified by fruit characteristics, i.e. pungency, color, shape, flavor, size, and their use. Despite their vast trait differences, virtually all capsicum cultivars commercially cultivated in the world belongs to *C. annuum*.

The important varieties from the genus Capsicum are (Bosland, 1994; Govindarajan, 1985):

C. annuum: Sweet or mild types: Hungarian paprika, Spanish pimentos, Bell pepper

Pungent types: Jalapeno, Serrano, Ancho, New Mexican, Cayenne, Pasilla, Piquin, de arbol, Poblano

C. frutescens: Tabasco, MAlagueta, Habanero and Tezpur

C. baccatum: Aji

C. pubescens: Rocoto, Manzano o peron, Chambaroto

Genetic inheritance of pungency relates to inheritance of the combined ability to produce each capsaicinoid individually since different genes seem to control biosynthetic capacity independently for each capsaicinoid (Zewdie and Bosland, 2000). Capsaicinoid genes seem to move freely across different species within the Capsicum genus, and heritability of species-specific capsaicinoid profiles appears to be relatively low (Zewdie and Bosland, 2001). The quantity of each capsaicinoid accumulated during fruit development is due to unique biosynthetic activity rather than interconversion between

previously synthesized capsaicinoids since capsaicinoid profiles within a pepper fruit appear not to change during capsaicinoid accumulation (Iwai et al., 1979).

Total capsaicinoid accumulation was reported to be maximum in fruits of greenhouse plants (200 $\mu g.g^{-1}$) compared to field grown plants (120 $\mu g.g^{-1}$) after 40 to 50 days from fruit set (DFFS) (Contreras-Padilla and Yahia, 1998). This might be due to soil and climatic conditions.

Chemical deflowering

Ammonium thiosulfate has been successfully used in blossom thinning of peaches. In peaches thinning is done to increase fruit size, improve fruit quality, and reduce limb breakage. Ammonium thiosulfate acts by interfering with ovule fertilization, either by preventing successful pollination or by disturbing pollen growth (Greene et al., 1999). The best thinning results were obtained when treatment was given when blossoms were 65%-80% open rather than closer to 100%. Byers et al., (1986) indicated that ammonium thoisulfate had useful thinning activity on peach and also increased shoot growth and flowering in the subsequent season. Thinning action of ammonium thiosulfate appeared to be by desiccating stigmas and styles of flowers and thereby prevention of pollination and fruit set (Williams et al., 1995)

Webster and Hollands (1993) reported that high volume sprays at blossom time of 1.5% ammonium thiosulfate plus a non-ionic wetter effectively thinned the plum cultivar. Victoria. Two or three sprays thinned better than single spray and if applied at or after anthesis, the chemical thinned slightly better than at white bud. Blossoms sprayed at or soon after anthesis were more sensitive to ammonium thiosulfate than those sprayed earlier and it may be due to disruption of pollination and fertilization by sprays. The

difference in thinning efficacy between multiple and single sprays could be due to differences in the proportion of blossoms targeted with chemical, the amounts of chemical deposited on the blossoms, or the sensitivity of blossoms at various stages of development.

For paprika and cayenne peppers, ethephon treatments (0, 1000, 3000 and 5000 $\mu L.L^{-1}$) accelerated fruit maturation, which, if timed appropriately, increased the percentage of red marketable fruit for chillies, and decreased the percentage of green paprika fruit (Krajayklang et al., 1999). For chilli pepper plants averaging 20% fully colored red fruits at the time of ethephon application, the intensity of extractable red color in chilli fruit increased by 16% for 1000 $\mu L.L^{-1}$ ethephon, while pungency improved by 46% and 48% for 1000 and 3000 $\mu L.L^{-1}$ ethephon respectively. Ethephon at 5000 $\mu L.L^{-1}$ induced defoliation with only 6% and 12% leaf retention on paprika and cayenne plants respectively, and caused fruit skin damage on both fruit types. Khan et al., (1997) reported that the total dry mass of harvested fruit of paprika pepper decreased linearly as ethephon rate increased from 1000 to 4000 $\mu L.L^{-1}$ (sprayed three times) approximately 30 to 45 days prior to harvest, but the red marketable fruit, as a percentage of total harvested fruit mass increased linearly with ethephon rate. A single application of ethephon at about 2000 to 3000 $\mu L.L^{-1}$ approximately 5 to 6 weeks prior to once-over mechanical harvest was recommended as a controlled abscission agent to increase the percentage of harvested red fruit in paprika pepper.

Biochemical pathway for capsaicinoid synthesis

Capsaicinoids are amides of vanillylamine and C9 to C11 branched chain fatty acids. More than twelve different compounds have been reported in nature : The five

major capsaicinoids are Capsaicin (CAP), Dihydrocapsaicin (DC), Nordihydrocapsaicin, Homodihydrocapsaicin and Homocapsaicin (Suzuki and Iwai, 1984), the structure of which are vanillyl amides of 8-methylnon-trans-6-enoic acid, 8-methyl nonanoic acid, 7-methyl nonanoic acid, 9-methyl decanoic acid and 9-methyldec-trans-7-enoic acid, respectively (Fig.1). Among them CAP and DC are major components of the majority of Capsicum species. Bennett and Kirby (1968) reported the following composition of the capsaicinoids in whole fruit components of *C. annuum*: capsaicin-69%, dihydrocapsaicin-22%, nordihydrocapsaicin-7%, homocapsaicin-1% and homodihydrocapsaicin-1%.

Tracer experiments using DL-phenyl-[3-14C]alanine or L-valine-[U-14C] showed that the formation and accumulation sites of capsaicinoids were the placenta (Iwai et al., 1979) and to a smaller extent in the fruit pericarp (Suzuki et al.,1980). During fruit development, the epidermal cells elongate and develop osmophilic granules that accumulate capsaicinoids. The capsaicinoids are thought to be synthesized in the glandular areas of the epidermis and the interlocular septum of the fruit (Zamski et al., 1987). At the intracellular level, the capsaicinoids are found in the vesicular and vacuolar fraction of the cell (Fujiwake et al., 1980). Zamski et al. (1987) reported that the placenta of hot peppers have blister-like surfaces, while the placental surfaces of non-pungent peppers appear smooth under a scanning electron microscope. Transmission electron microscopic studies indicate that the capsaicinoids are synthesized within the inner compartment of the endoplasmic reticulum which exhibits a puffed appearance following prolonged capsaicinoid synthetic activity. The capsaicinoid-containing vesicles are thought to be derived from the puffed ER that migrate through the cytoplasm and fuse

with the plasmalemma. These protrusions may play a role in the secretion of the capsaicinoid droplets into the outer layers of the cell wall.

The biochemical pathway leading to capsaicinoids has two distinct origins, one of which contributes the vanillylamine aromatic component through the phenylpropanoid pathway from phenylalanine through cinnamic acid, coumaric acid, caffeic acid, ferulic acid, vanillin and vanillylamine (Fujiwake et al., 1982). The other contributes the fatty acid moiety from valine or leucine through alpha keto-isovalerate or alpha keto-isocaproate (Suzuki et al., 1981).

In the phenylpropanoid pathway (Fig. 2), phenylalanine ammonia lyase (PAL) catalyses the elimination of ammonia from L-phenylalanine to form trans-cinnamic acid, this being the first committed step of phenylpropanoid biosynthesis in higher plants (Hanson and Havir, 1981). Cinnamate 4-hydroxylase (CA-4H) catalyses the second step in wich cinnamic acid is converted to P-coumaric acid in the presence of NADPH (Russell and Conn, 1967; Stafford, 1969; Potts et al., 1974; Lamb and Rubery, 1975). P-coumaric acid 3-hydroxylase (CA-3H) catalyses the conversion of P-coumaric acid to caffeic acid, while caffeic acid-o-methyltransferase converts it to ferulic acid (Sukrasno and Yeoman, 1993). The conversion of vanillin to vanillylamine is catalyzed by an aminotransferase (Aluru et al., 1998).

The phenylpropanoid pathway is shared with other pathways of general phenylpropanoid metabolism and is common to all higher plants. This leads to the formation of a wide range of phenolic compounds including cinnamates, benzoates, flavonoids, coumarins, tannins, saponifiable cell wall phenolics and lignin–like substances (Stafford, 1974; Fry, 1982). However, the later part of the reaction sequence

from ferulic acid through vanillin and vanillylamine to capsaicinoids is only found in pepper fruits.

Holland (1989) reported that phenyl ammonia lyase and cinnamate 4-hydroxylase in phenylpropanoid metabolism are not the rate limiting steps in capsaicinoid biosynthesis. The limitations occur at or near the end of the pathway.

Valine serves as a precursor for the formation of even-numbered branched-chain fatty acids and leucine for odd-numbered branched-chain fatty acids (Suzuki et al., 1981). Valine and leucine are obtained from pyruvate, an intermediate in glycolysis. Fatty acid synthesis is turned on in chloroplasts, where the ratio of NADPH/$NADP^+$ is high. The branched-chain fatty acid pathway of the capsaicinoid biosynthetic pathway involves conversion of valine to α-ketoisovalerate which is further decarboxylated to an isobutyryl moiety. The elongation of the isobutyryl moiety leads to even numbered fatty acid. For odd numbered fatty acids, leucine is first converted to α-ketoisocaproate which is further decarboxylated to an isovaleryl moiety. The elongation of the isovaleryl moiety leads to odd-numbered fatty acids. The first step in fatty acid biosynthesis for capsaicinoid biosynthesis involves the conversion of valine to α-keto-isovalerte or leucine to α-keto-isocaproate which requires the activity of a branched chain amino acid aminotransferase enzyme (Fig. 2). In plants, fatty acid synthesis utilizes acetyl-CoA as a building block for assembly of long chain (C16-C18) fatty acids (Harwood, 1996). Acetyl-CoA is provided through the action of pyruvate dehydrogenase on pyruvate. The acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACCase). ACCase is considered to be a regulatory enzyme in leaf fatty acid synthesis. Malonyl-CoA is transferred to acyl carrier protein (ACP) via the activity of malonyl-CoA:acyl transferase (MCAT). The next

step in fatty acid synthesis involves the condensation of acetyl and malonyl groups to form long chain acyl-ACPs by the action of β-ketoacyl-ACP synthases (KAS). At least three different KAS's are required to produce the 18 carbon fatty acid. The first condensation to form a four carbon acetoacetyl-ACP is carried out by KAS III. KAS I is involved in increasing chain lengths from C4-C16 and KAS II elongates the 16 carbon palmitoyl-ACP to yield stearoyl-ACP. The biosynthesis of capsaicin and dihydrocapsaicin requires even-numbered short chain fatty acid precursors obtained from valine. The branched-chain fatty acid pathway of capsaicinoid biosynthesis requires the component enzymes of the regular unbranched fatty acid synthetic pathway. Isobutyryl-CoA is predicted to be the prime component for extension of branched–chain fatty acids leading to the formation of 8-methylnonanoic acid or 8-methyl-6-nonenoic acid. The elongation of isobutyryl-CoA using malonyl-CoA is predicted to require β-ketoacyl-ACP synthase (KAS) activity. KAS I is specifically required for the elongation of chain length from C2-C16, which is the chain length relevant to the synthesis of fatty acids required for capsaicinoids biosynthesis (Aluru, 1999). Thus it may be possible that C1-C6 carbon atoms originate from acetate and C7-C10 from valine (Markai et al., 2002).

Capsaicin, the major pungent compound of pepper fruit is an amide derivative of vanillylamine and 8-methyl-trans-6-enoic acid (Bennet and Kirby, 1968) while dihydrocapsaicin is an amide derivative of vanillylamine and 8-methyl nonanoic acid (Fig. 1). The conversion of vanillylamine and a branched fatty acid is carried out by capsaicinoid synthetase located in the vacuole (Fujiwake et al., 1980).

Capsaicinoid synthetase (CS) has two activities.

1) Acyl activating activity which activates the acyl group to a Co-A activated moiety using ATP and Mg^{2+} as cofactors

$$\text{8-methyl-nonanoic acid + ATP + CoA-SH} \xrightarrow{Mg2+} \text{8-methylnonanoyl-CoA + ADP}$$

2) Acyl transferase activity which transfers acyl-CoA derivative onto vanillylamine to yield capsaicinoid.

$$\text{8-methylnonanoyl-CoA + Vanillylamine} \longrightarrow \text{Dihydrocapsaicin}$$

CS activity in pepper fruits remained very low during the first 15 days after flowering. A slight increase was detected between days 15 and 22, followed by a sharp increase to attain a maximum at day 30 after flowering (Ochoa-Alejo and Gomez-Peralta, 1993).

Ripening is a process in which degradation of chlorophyll (unmasking of xanthophylls, anthocyanin), conversion of starch to sugars, breakdown of pectin etc., occurs with the help of enzymes making the fruit softer, juicy and sweet with volatile substances. Ripening in pepper is characterized by disappearance of lutein and neoxanthin, both characteristic chloroplast pigments, and increase in concentration of beta-carotene, antheraxanthin, violaxanthin, capsanthin, etc. Capsaicin synthesis in vivo coincides with attainment of physiological maturity and subsequent fruit ripening- a process incurring a major chemical restructuring of the walls of the cells making up the body of the fruit (Gross et al., 1986). Softening may provide a huge supply of substrates

for phenylpropanoid metabolism (vanillylamine synthesis) for capsaicin synthesis (Holden et al., 1987).

Objectives

1) To determine the effect of chemical deflowering treatment with ammonium thiosulfate at different stages of flowering for enhancing total capsaicinoid yield of pepper plants with once-over winter mechanical harvest.

2) To evaluate the fatty acid precursor substrate availability in relation to capsaicinoid-specific biosynthesis and accumulation, with specific emphasis on 8-methyl-trans-6-enoic acid for capsaicin and 8-methyl nonanoic acid for dihydrocapsaicin; characterization of fatty acid sterol esters from pepper placental lipid extract.

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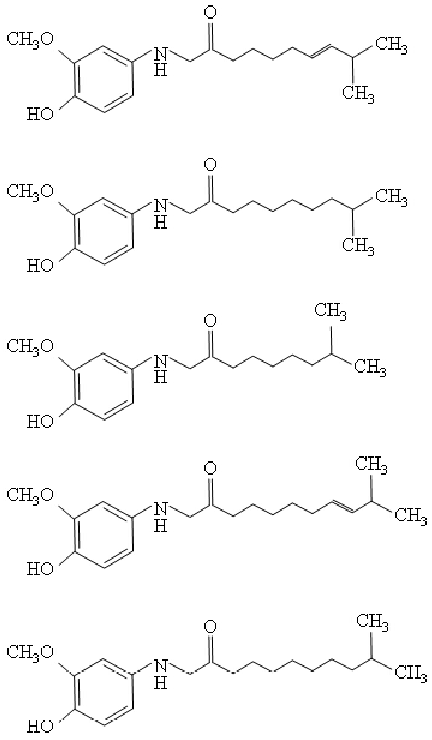
Figure 1. Structure of five major capsaicinoids in pepper



Capsaicin (CAP)

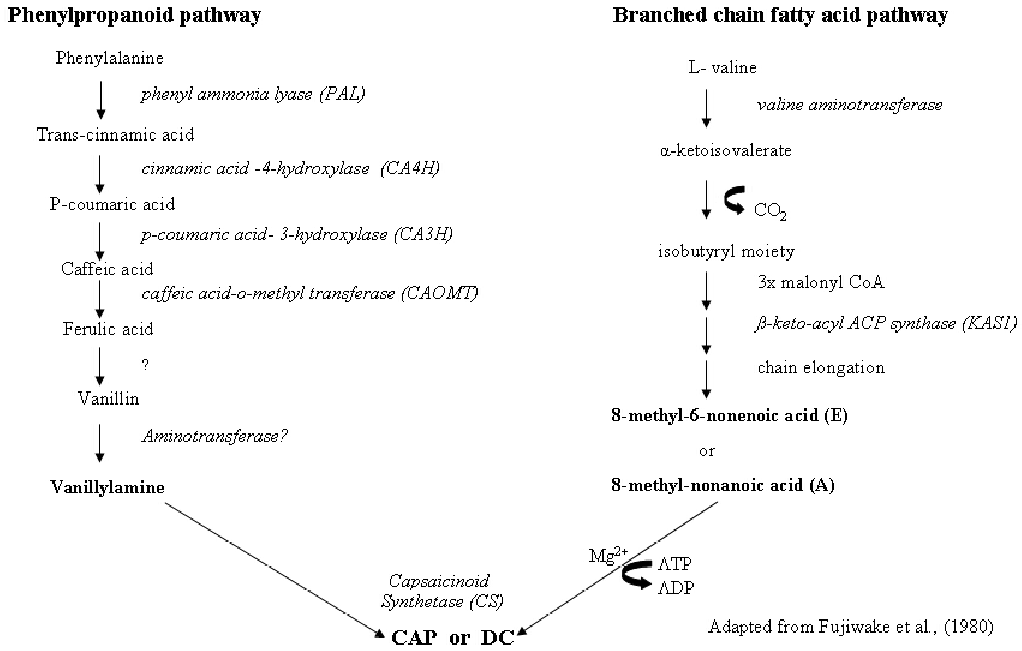
Dihydrocapsaicin (DC)

Nordihydrocapsaicin

Homocapsaicin

Homodihydrocapsaicin

Figure 2. Biosynthetic pathway of capsaicinoids



CHAPTER II

AMMONIUM THIOSULFATE DEFLOWERING TREATMENTS TO INFLUENCE MATURITY OF ONCE-OVER HARVEST YIELD AND CAPSAICINOID CONTENT OF PEPPER FRUIT

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Pungency in hot peppers (*Capsicum annuum* L. var. *annuum*) is mostly due to capsaicin (CAP) and dihydrocapsaicin (DC), contributing more than 90% of the total pungency. Loss of harvestable pungency occurs prior to once-over winter harvest due to shattering of early set fruits. Ammonium thiosulfate (ATS) treatments were applied to pepper plantings at two locations in Oklahoma (Bixby and Hydro) after first, second and third flower flushes, designated as maturity stages I, II and III respectively, either as single applications (timing 1 = stage I, timing 3 = stage II, timing 5 = stage III) or as successive applications at stages I and II (timing 2) and stages I, II and III (timing 4). Four ATS concentrations of 3%, 6%, 9% and 12%, and a control (water) were applied. Fruits were harvested after killing frost, dried and separated into red, orange, green and moldy fruits. Capsaicinoids were extracted using N, N-dimethylformamide (DMF) and analyzed by HPLC. Among 5 trials conducted on 'Plant 19' and 'Okala' at Bixby and Hydro (2002 and 2003), higher concentration of ATS (6%, 9% and 12%) was more effective than control and 3% ATS treatments, in the removal of early set flowers for 'Plant 19' Hydro, 2003 ($P \leq 0.05$) and 'Okala' Hydro, 2003 ($P \leq 0.1$). Higher ATS concentration had a positive effect on fruit yield and fruit capsaicinoid concentration and thereby capsaicinoid yield for 'Plant 19' and 'Okala' at Hydro (2003). ATS treatments

were not significantly different from control for 'Plant 19' at Bixby (2002 and 2003). Timing of spray application did not greatly influence deflowering pattern and capsaicinoid yield except for 'Plant 19' at Bixby, 2002. Fruit yield and capsaicinoid content on a per plant basis was significantly influenced by location, cultivar and year. The increase in capsaicinoid yield afforded by 6%, 9% and 12% ATS concentrations at Hydro (2003) ranged from 8 to 15 percent.

Hot peppers *Capsicum annuum* L. var. *annuum* are valued for the characteristic pungent flavor they impart in prepared foods (as direct ingredients or as extracts). Pungency is mostly due to capsaicin (CAP) and dihydrocapsaicin (DC) contributing more than 90% of the total pungency in most peppers (Bennett and Kirby, 1968; Suzuki et al., 1981). Pepper extracts have various uses ranging from personal defense sprays to pharmaceutical ingredients as a pain blocking agent (Caterina and Julius, 2001). They influence homologous pain receptors, peripheral and central heat detectors and pulmonary aortas (Saria et al., 1981). Capsaicin is a trigeminal stimulant that is important in gustatory physiology (Liu and Simon, 2000). Capsaicin can also help in the mediation of pain: prolonged application of capsaicin is thought to cause the desensitization of sensory neurons responsible for pain (Goettl et al., 1997).

When grown for extraction processing, peppers are usually machine harvested after a killing frost. Loss of harvestable pungency occurs as a consequence of the once-over winter harvest due to chemical degradation, as well as shattering, of early set fruits. A means to retain these fruit for harvest, or to eliminate them prior to capsaicinoid accumulation and avoid the loss, should enhance harvestable capsaicinoid yield. Holmes

(1981) reported that the removal of pepper fruits (*Capsicum annuum* L.) within three weeks after fruit set increased the number of flowering phase cycles (flushes), and increased fruit number over the control with no fruits removed; early deflowering of pepper, at the first two flowering phases, resulted in higher flowering, fruit set and marketable yield. Developing fruits in Zucchini squash (*Cucurbita pepo* L.) can inhibit subsequent fruit set and growth of fruit (Stephenson et al., 1988); the inhibition could arise from competition for limited assimilates, dominance due to production of growth regulators or by a combination of both (Marcelis and Baan Hoffman-Eijer, 1997).

The most commonly used blossom thinners have included hormonal blossom thinners (Ethephon and NAA). Their physiological effects for tree fruit included increased fruit size, higher yield, and early fruit maturity (Havis, 1962) as well as increased tree vigor, leaf size, flower bud numbers, and flower bud hardiness (Byers and Lyons, 1985; Byers and Marini, 1994). For paprika and cayenne peppers, ethephon treatments (0, 1000, 3000 and 5000 $\mu L.L^{-1}$) accelerated fruit maturation which, if timed appropriately, increased the percentage of red marketable fruit for chillies, and decreased the percentage of green paprika fruit (Krajayklang et al., 1999). For plants averaging 20% fully colored red fruits at the time of ethephon application, the intensity of extractable red color in chilli fruit increased by 16% for 1000 $\mu L.L^{-1}$ ethephon, while pungency improved by 46% and 48% for 1000 and 3000 $\mu L.L^{-1}$ ethephon respectively. Ethephon at 5000 $\mu L.L^{-1}$ induced defoliation with only 6% and 12% leaf retention on paprika and cayenne plants respectively, and caused fruit skin damage on both fruit types. Khan et al., (1997) reported that the total dry mass of harvested fruit of paprika pepper decreased linearly as ethephon rate increased from 1000 to 4000 $\mu L.L^{-1}$ approximately 30 to 45 days

prior to harvest, but the red marketable fruit as a percentage of total harvested fruit mass increased linearly with ethephon rate. A single application of ethephon at about 2000 to 3000 $\mu L.L^{-1}$ approximately 5 to 6 weeks prior to once-over harvest was recommended as a controlled abscission agent to increase the percentage of harvested red fruit in paprika pepper.

Non-hormonal blossom thinners usually act by scorching flower tissues and hence prevent fertilization. They rarely have any effect on flowers that are fertilized. DNOC (sodium 4, 6-dinitro-ortho-cresylate) a caustic compound, was used widely as a blossom thinning agent in apple (*Malus domestica*) (Williams, 1979). It is no longer in use due to lack of re-registration, since fruit size and return bloom was not increased in apple (Wertheim, 2000). Ammonium thiosulfate (ATS) is a liquid fertilizer containing approximately 12% nitrogen and 26% sulfur. Earlier work by Byers et al., (1986) showed that ATS had useful thinning activity on peach (*Prunus persica*). It increased peach shoot growth and unlike DNOC on apples, it also increased flowering in the following season. ATS may cause injury to petals and young leaves, but appears relatively safe for fruits (Byers, 1997). Chemical thinners like ATS leave no residue and are environmentally safe (Irving et al., 1989). In peaches ATS blossom thinning was done to increase fruit size, improve fruit quality and reduce limb breakage. Its mode of action appeared to be interference with ovule fertilization, either by preventing successful pollination or by disturbing pollen growth (Greene et al., 1999).

Successful use of ATS as a blossom thinner has depended on ATS concentration and timing of single or multiple spray applications. ATS concentration of 1.5% at blossom time effectively thinned the 'Victoria' plum (*Prunus Americana*) (Webster and

Hollands, 1993) and 'Delicious' apple (Bound and Jones, 2004) without causing excessive damage. Higher concentrations of ATS (5%) + 5 mL/L X-77 (surfactant) at early full bloom in 'Loring' peach was as effective as manual removal of stigma with half of the style for reducing fruit set (Byers and Lyons, 1985); 37 g/L ATS in 'Cox's Orange Pippin' apple was effective by complete desiccation of the blooms and in 'Braeburn' apple by desiccation of the style (Irving et al., 1989). Multiple applications of ATS (3% and 4%) at various stages of flowering (20%, 50% and at full bloom) over-thinned and resulted in excessive leaf desiccation in 'Delicious' apple (Bound and Jones, 2004).

Sanders and Looney (1993) reported the importance of proper ATS spray timing as well as its rate to achieve selective blossom thinning in apple; ATS application after full boom and pollination resulted in lesser thinning action. Spraying of 1% ATS in 'Elstar' apple shortly after full bloom was more effective in reducing later fruit load than once fruit set occurred (Balkhoven-Baart and Wertheim, 1997). Fallahi et al., (2004) suggested that a double application of 1.5% ATS or single application of 2.5% ATS resulted in satisfactory levels of blossom thinning in 'Fuji' apple; double application of 2.5% ATS resulted in excessive thinning. Multiple applications (2 or 3 sprays) of ATS at intervals during the blossom period was reported to be more effective in thinning than a single spray in 'Victoria' plum (Webster and Hollands, 1993) and 'Delicious' apple (Bound and Jones, 2004).

ATS has never been evaluated as a deflowering agent for peppers. Our objective was to evaluate ATS for early flower removal in chilli pepper fruit. We tested a combination of ATS concentrations and ATS spray timings to evaluate the feasibility of enhancing harvestable pungency through early flower removal.

Materials and Methods

Plant material: Seeds of a pepper selection closely related to 'Okala' chili pepper ('Plant 19') a gift from Jim Motes, were seeded into Speedling seed trays (200 plants per tray) on 27 Feb. 2002 and 12 Mar. 2003 and transplanted at the Bixby Vegetable Research Station [153 km (95 miles) east of Stillwater, OK] on 30 Apr. 2002 and 8 May 2003 and at SS Farms in Hydro [217 km (135 miles) west of Stillwater, OK] on 2 May 2002 and 5 May 2003 respectively. Transplants from 'Okala' were also utilized at the Hydro location for ATS spray application trials in 2003. The 'Okala' transplants were produced commercially and were transplanted on the same day as 'Plant 19'. Plants were established with 0.46 m within row spacing and 1 m between row spacing for a plant density of approximately 21,700 plants/ha. Production inputs were provided as needed to promote optimum growth and fruit were harvested following plant death after a killing frost during early winter in November or December.

Anhydrous ATS (99% purity) was purchased from Aldrich Chemical Co. (St. Louis, MO). ATS concentrations of 3%, 6%, 9% and 12% (w/v) were prepared by dissolving 30, 60, 90 and 120 g ATS respectively in minimum quantity of water and made up to one liter. ATS was applied as a spray with a backpack sprayer fitted with a flat fan nozzle calibrated to provide a spray rate of 280 $L.ha^{-1}$ (30 $gal.acre^{-1}$). Treated rows were flanked on each side with non-treated guard rows to prevent drift effects on other treatments. Treatments were applied in a split-plot arrangement, with timing of spray application as main plot and ammonium thiosulfate concentration as sub plot. Each plot consisted of 12 plants, with the first and last plant as borders. Spray was applied at three different stages of pepper flowering, as a single application or as multiple

applications and the spray timings were assigned as 1, 2, 3, 4 and 5 as depicted in Figure 3. The pepper plant flowering stages depicted in Figure 3 illustrate relative amount of flowering, with stage I after the first full flowering flush (approximately one month after transplanting), stage II after the first and second flowering flushes (approximately two weeks after stage I) and stage III after the first, second and third flowering flushes (approximately two weeks after stage II). Single ATS sprays were applied at timings 1, 3 and 5 (developmental stages I, II and III respectively). Two sequential ATS sprays were applied at timing 2 (stages I and II), while three sequential ATS sprays were applied at timing 4 (stages I, II and III). Three ATS concentrations (3%, 6% and 9% in water) for 'Plant 19' at Hydro (2002) plus a water control, and four ATS concentrations (3%, 6%, 9% and 12% in water) plus a water control for 'Plant 19' at Bixby (2002 and 2003) and 'Okala' at Hydro (2003) were applied. The experiment contained four replications of ten harvested plants each. ATS spray application schedule for 'Plant 19' at Bixby and Hydro in 2002 and 2003, and for 'Okala' at Hydro in 2003, is presented in Table 1.

Pepper plants were killed by freezing temperature on 14 Oct. 2002 and 6 Nov. 2003 in Bixby; and on 4 Nov. 2002 and 6 Nov. 2003 in Hydro. During harvest ten whole plants per plot were cut at ground level, placed inside burlap bags and transported to Stillwater. The pepper plants of 'Plant 19' were harvested on 12, 14 and 19 Nov. 2002, and 15 Dec. 2003 at Bixby. Harvests for 'Plant 19' were conducted at Hydro on 21 Nov. and 9 Dec. 2002, and 4 Dec. 2003. 'Okala' at Hydro was harvested on 18 and 25 Nov. 2003. Peppers were hand picked from all ten plants and the composite sample was dried in a forced-air drier at 40 ˚C for up to 7 days until the fruits became brittle. After drying, peduncles were removed and peppers were sorted into three maturity categories of green,

orange and red based on color and a fourth category based on presence of mold regardless of pepper color, and weights were recorded. Green color referred to fruits which were immature and mature green. Orange color referred to fruits which were ripening. Red color referred to fruits which were ripe. Mold referred to visibly decayed fruits, mostly in ripe fruits. Pods were stored inside sealed freezer bags at -20 ˚C to await grinding just prior to extraction.

Capsaicinoid extraction and analysis

Prior to grinding, bags containing pod samples from each of the four categories were removed from the freezer and allowed to reach room temperature prior to opening to prevent moisture condensation onto the pod surface. Representative fruit were ground with a Wiley mill (Thomas Scientific, Swedesboro, NJ) to pass a 20 mesh screen, not more than a week prior to capsaicinoid extraction. The powder (0.200 g) was accurately weighed into 2 dram vials and 2 milliliters of N, N-dimethylformamide (DMF) (Fisher Scientific, PA) was added and the vial sealed with Teflon-lined screw caps. Extraction was carried out at 80 ˚C in a dry block heater for one hour. Samples were swirled every 15 minutes during the 1 hour period to assure proper mixing. Samples were then removed from the heat block, centrifuged for 5 minutes in a Speedvac centrifuge (Savant Instruments Inc. Farmingdale, NY; approximately 3,500 g_n) and supernatants were decanted into a 10 mL volumetric flask. The procedure was repeated three more times for a total of 4 extractions and the volumetrics were brought to volume with DMF. Preliminary recovery trials were conducted to establish capsaicin (CAP) and dihydrocapsaicin (DC) recoveries of 95 and 101 percent, respectively.

Aliquots of the extracts were placed into autosampler vials and utilized directly for HPLC capsaicinoid analysis using a modification of the "short run" procedure of Collins et al., (1995). Capsaicinoid separations were achieved at a flow rate of 1 mL.min^{-1} using a Dionex gradient HPLC pump GPM-2 (Dionex Corp., Sunnyvale, CA). Samples were injected with an AS-3500 automatic sample injector by overfilling a 10 µL sample loop. Separation of CAP and DC was conducted with a Waters Nova-Pak C-18 (Waters Corp. Milford, MA) radial compression column (8 mm x 100 mm) with a Nova-Pak C-18 precolumn guard cartridge. The mobile phase in reservoir A was 80% methanol: 20% HPLC grade water; the mobile phase in reservoir B was 100% methanol. Capsaicinoids were separated under isocratic conditions using mobile phase from reservoir A. The column was reconditioned after each run with a gradient to 100% methanol from reservoir B as follows; 0-13 min, 100% A; 13-18 min, 100% A to 100% B; 18-23 min, 100% B to 100% A. The column was allowed to reequilibrate to solvent A for 7 minutes prior to injection of another sample. Peaks were detected with a Shimadzu RF-511 (Shimadzu Corp., Kyoto, Japan) spectrofluorometric detector with excitation wavelength at 280 nm and emission wavelength at 338 nm in low sensitivity mode. Sample components were identified by coelution with standard CAP (8-methyl-n-vanillyl-6-nonenamide) and DC (8-methyl-n-vanillyl-nonanamide) (Sigma Chemical Co., St. Louis, MO) and quantified by comparing with external standards. Standard solutions of 200 ppm were prepared in DMF by 10-fold dilution of a 2000 ppm stock solution of CAP and DC. The stock solution was stored in the freezer at -20° C and allowed to reach room temperature prior to opening to avoid moisture condensation.

Statistical Analysis

The main effects of ATS timing, concentration and their interaction on pepper fruit yield, capsaicinoid concentration and its content were determined by analysis of variance (ANOVA) according to the Proc mixed model procedure of the Statistical Analysis System (SAS Inc., Cary, NY). The significant means were separated by LSD ($P \leq 0.05$).

Results

ATS did successfully deflower pepper plants in all years, all locations and both cultivars (Fig. 4). We observed little effect of ATS on fruit removal once flower petals began to senesce. Control pepper plants one week after stage III development (Fig. 4A) appeared normal with previously set immature fruit and the forth flowering flush was commencing. Pepper plants at the same developmental stage, one week after the third 9% ATS treatment (Fig. 4B) had numerous flowering nodes devoid of flowers or fruit and were entering their first flowering and fruiting flush.

Fruit yield ($g.plant^{-1}$), capsaicinoid (CAP and DC) concentration ($mg.g^{-1}$) and capsaicinoid content ($mg.plant^{-1}$) was significantly influenced by location ($P \leq 0.05$) and is presented by year for Bixby and Hydro in Tables 2 (2002, 'Plant 19'), 3 (2003, 'Plant 19') and 4 (2003, 'Okala'). For 'Plant 19' in 2002 the fruit yield and capsaicinoid content was highest at Bixby and capsaicinoid concentration was highest at Hydro (Table 2). In Bixby, pepper fruit yield and capsaicinoid content was highest at spray timings 1, 4 and 5 ($P \leq 0.05$); ATS concentration and its interaction with timing did not show significant effects on fruit yield, capsaicinoid concentration or its content. In Hydro, there was no significant effect of ATS concentration or timing as well as their interaction on pepper fruit yield, but an ATS concentration main effect was observed for capsaicinoid

concentration and capsaicinoid content. Fruits from the 6% ATS treatment contained higher capsaicinoid concentration and content than the 3% and 9% ATS treatments ($P \leq 0.05$) and equal content to the control.

In 2003, treatments of timing 5 (single spray at the third flower flush) and ATS concentration 12% were added for Hydro (Table 3). The fruit yield and capsaicinoid concentration pattern of the two locations followed the same trend as in Table 2 with pepper yield higher at Bixby and capsaicinoid concentration higher at Hydro. In Bixby, the ATS treatments did not show significant effects on pepper fruit yield, capsaicinoid concentration or its content. In Hydro, the capsaicinoid concentration and content were affected by ATS concentration only ($P \leq 0.05$). Fruits from the control contained lower capsaicinoid concentration and content than ATS concentration treatments above 6%, and were not different from the 3% ATS treatment. Fruit yield and capsaicinoid content was higher in 2003 than 2002 at both locations; capsaicinoid concentration was higher in 2003 than 2002 in Bixby, it was not different between years at Hydro.

For 'Okala' in Hydro (2003), the ATS treatments did not show any significant effects on fruit yield, capsaicinoid concentration or its content (Table 4). There was a cultivar effect between 'Plant 19' and 'Okala' at Hydro ($P \leq 0.05$) (Table 2 and 3); total fruit yield was higher for 'Okala' and conversely the capsaicinoid concentration was higher for 'Plant 19'. The total capsaicinoid content did not differ between the two cultivars.

Among different fruit maturities of 'Plant 19' and 'Okala' across locations and years, the average capsaicinoid concentration was similar for red (20 $mg.g^{-1}$) and orange and moldy fruits (19 $mg.g^{-1}$), and lower for green fruits (12 $mg.g^{-1}$). For 'Plant 19' there

was a location effect for the distribution of fruit maturities in 2002 (Table 5). When the total fruit yield was segregated as a percentage into color/mold groupings, Bixby and Hydro differed; red (63% vs. 67%), orange (7% vs. 12%), green fruits (1% vs. 13%) and moldy fruits (29% vs. 8%). In Bixby, there was an ATS timing effect on red fruit yield and its capsaicinoid content ($P \leq 0.05$); similar to total fruit yield and capsaicinoid content, ATS timings 1, 4 and 5 showed higher red fruit yield and capsaicinoid content than timings 2 and 3. ATS treatment effects were not observed for other maturity categories. In Hydro, ATS treatment effects did not affect maturity/mold groupings for fruit yield. However the capsaicinoid content of red fruits was significantly influenced by ATS concentration; the 6% ATS treatment was higher than 3% and 9% ATS treatments and not different from the control. ATS treatment effects were not observed for capsaicinoid content of other maturity categories.

The fruit maturities for 'Plant 19' at two locations in 2003 (Table 6) showed similar distribution pattern for fruit maturities; red (64% vs. 62%), orange (7% vs. 7%), green (20% vs. 19%) and moldy fruits (9% vs. 12%) respectively. In Bixby, there was an ATS timing effect on moldy fruit yield ($P \leq 0.05$); ATS timing 5 showed higher moldy fruit yield than timings 2, 3 and 4 and equal moldy fruit yield to timing 1. In Hydro, an ATS concentration effect was observed for red fruit capsaicinoid content, with 6% ATS concentration having higher capsaicinoid content than control and 3% ATS concentration. ATS treatment effects were not observed for fruit yield and capsaicinoid content of other maturity categories in both locations.

The distribution of fruit maturities for 'Okala' at Hydro in 2003 (Table 7) showed higher percent of red fruits (80%) than 'Plant 19' from 2002 and 2003 in both locations.

The percent of orange, green and moldy fruits were 5%, 7% and 8% respectively. There was an ATS timing effect on moldy fruit yield; ATS timing 1 was higher in moldy fruit yield than all other timings. ATS treatment effects were not observed for other fruit yield and capsaicinoid content among different fruit maturities.

Discussion

Unlike tree fruit with one annual compact flowering period, peppers flower and fruit in sequential flushes. In temperate climate, flowering and fruit development cease when plants are killed by freezing temperatures. ATS was an effective chemical deflowering agent for pepper (Fig. 4), with little effect on pollinated fruit once flower petals were senescing. This finding is consistent with earlier work relating to ATS as a deflowering agent for 'Gala' apple (Byers, 1997), where ATS caused injury to petals, leaves and shoot tips, but did not cause a deleterious effect on fruits. Apart from preventing fertilization, foliar damage may also be partially attributed to higher concentration of ATS (Balkhoven-Baart and Wertheim, 1997; Bound and Jones, 2004). We noted that ATS treatment unified later flowering, resulting in more uniform pepper maturity distribution in plants throughout the field. For 'Plant 19' at Bixby (2002), ATS sprayed once earlier at developmental stage I or later at developmental stage III, as well as sprayed at all three developmental stages increased fruit yield and capsaicinoid content over plants only sprayed at developmental stage II or at both developmental stages I and II. Webster and Hollands (1993) concluded in their report that two or three sprays of 1.5% ATS effectively thinned plum cv. Victoria; thinning efficacy between single and multiple sprays could be due to differences in the proportion of blossoms targeted with ATS, amount of chemical deposited on the blossom and also the sensitivity of blossom at

various developmental stages. Jones et al., (1998) reported the importance of timing of application and suggested that blossom as well as post-bloom thinning applications were more effective than a one time application. Since pepper plants flower continuously throughout the growing season, multiple sprays of ATS might prove to be effective in knocking off early, lower yielding fruit sets and unifying later flowering and subsequent fruit maturation.

The deflowering action of higher concentrations of ATS appeared beneficial in two out of five cases for yield and capsaicinoid content of later set fruit, and was affected by environment. In Bixby (2003), ATS treatment effects were not observed for pepper fruit yield, capsaicinoid concentration and its content in 'Plant 19' (Table 3), while in Hydro (2003), the higher ATS concentrations 6%, 9% and 12% resulted in higher capsaicinoid concentration and its content for 'Plant 19'. Higher ATS concentration of 6%, 9% and 12% also showed higher total fruit yield than control and 3% ATS concentration ($P \leq 0.1$). Similarly, for 'Okala' in Hydro (2003), the ATS concentration showed a significant effect on total capsaicinoid content ($P \leq 0.1$) (Table 4); ATS concentrations of 6%, 9% and 12% had higher total capsaicinoid content than control.

Although the total fruit yield was higher in Bixby, the capsaicinoid concentration was higher in Hydro for 'Plant 19' in both years (Table 2 and 3). Higher wind velocity and lower relative humidity were observed in Hydro than Bixby (Table 8), and this environmental factor might have played a major role inducing stress and resulting in lower fruit yield and higher pungency in Hydro. This is in agreement with Bosland (1996), that the pungency in chili pepper can be secondarily influenced by weather and growing conditions. Estrada et al., (1999) also reported that environmental conditions

such as water stress might have a strong effect upon the accumulation of capsaicinoids in 'Padron' pepper due to competition between capsaicinoid biosynthesis and other phenylpropanoid metabolites. Wind stress and lower humidity can indirectly result in water stress by increasing the transpiration rate. The higher fruit yield for 'Plant 19' in 2003 over 2002 could be partially attributed to the later occurrence of a killing freeze in 2003. The difference in the distribution of pepper fruit maturities could arise from cultivar, planting time, cultural practices, growing condition, time of occurrence of freeze that kills the entire plant, etc.

Conclusions

Higher concentrations of ATS (6%, 9% and 12%) were more effective in the removal of early blooms than control and the lower 3% ATS concentration. In particular the 6% ATS concentration effectively increased the total capsaicinoid content of pepper 'Plant 19' at Hydro (2003) ($P \leq 0.05$), and 'Okala' at Hydro (2003) ($P \leq 0.1$). Higher ATS concentration appeared to unify flowering and subsequent fruit set. In two out of five cases ATS application at 6% or higher concentrations enhanced total fruit yield and capsaicinoid accumulation of later set harvestable pepper. However, there was no difference in capsaicinoid content between control and ATS concentration treatments for 'Plant 19' at Bixby (2002 and 2003); capsaicinoid content of control was higher than 3% and 9% ATS concentration, but equal to 6% ATS concentration for 'Plant 19' at Hydro (2002). Timing of ATS application had lesseffect in influencing flowering pattern, fruit set and capsaicinoid content. Although ATS concentration gave mixed results in enhancing capsaicinoid content of pepper, ATS concentrations of 6% or above, sprayed once at stage III development or three times at stage I, II and III development, may

positively influence fruit yield and capsaicinoid content, and also to minimize the risk of over thinning.

Since peppers grown commercially for extraction purposes are normally sold on a pungency basis, successful application of ATS as a deflowering agent for increasing total fruit yield and/or capsaicinoid yield may have an impact on pepper crop value. When capsaicinoid content was increased by application of 6%, 9% and 12% ATS over the control, it led to an increase of capsaicinoids of 'Plant 19' and 'Okala', Hydro, 2003, from 250 to 500 mg.plant^{-1} accounting for an increase in capsaicinoid production of from 8 to 15 percent. Since the timing of a killing freeze cannot be precisely predicted in any given season, we recommend that spray timing not exceed maturity stage III to avoid negative effects resulting from over thinning during a year with an early freeze.

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Table 1. ATS application schedule for pepper 'Plant 19' at Bixby and Hydro in 2002 and 2003, and for 'Okala' at Hydro in 2003.

Year	ATS spray timings	Bixby			Hydro					
		Plant 19			Plant 19			Okala		
		Developmental stages[z]			Developmental stages			Developmental stages		
		I	II	III	I	II	III	I	II	III
2002	1[y]	7 June	--	--	6 June	--	--	NA[x]	NA	NA
	2	7 June	19 June	--	6 June	20 June	--	NA	NA	NA
	3		19 June	--	--	20 June	--	NA	NA	NA
	4	7 June	19 June	5 July	6 June	20 June	2 July	NA	NA	NA
	5	--	--	5 July	--	--	--	NA	NA	NA
2003										
	1	24 June	--	--	26 June	--	--	26 June	--	--
	2	24 June	30 June	--	26 June	2 July	--	26 June	2 July	--
	3	--	30 June	--	--	2 July	--	--	2 July	--
	4	24 June	30 June	10 July	26 June	2 July	15 July	26 June	2 July	15 July
	5	--	--	10 July	--	--	15 July	--	--	15 July

[z]Developmental stages I, II and III correspond to the degree of plant flowering, after the first (approximately 1 month after transplanting), second (approximately 2 weeks after stage I) and third (approximately 2 weeks after stage II) flowering flushes, respectively.
[y]ATS spray timings correspond to sprays applied once at developmental stages I (spray timing 1), II (spray timing 3) or III (spray timing 5), or sequentially at developmental stages I and II (spray timing 2) or developmental stages I, II and III (spray timing 4).
[x]Cultivar 'Okala' was not included in 2002.

Table 2. Effect of ATS treatments on total pepper fruit yield (g.plant^{-1}), capsaicinoid concentration (mg.g^{-1}) and capsaicinoid content (mg.plant^{-1}) at two locations for 'Plant 19' in 2002.

	Fruit yield (g.plant^{-1})^{z}	Capsaicinoid concentration (mg.g^{-1})^{y}	Capsaicinoid content (mg.plant^{-1})^{x}
Bixby			
ATS timing 1	139.5a^{w}	17.6a	2453.4a
ATS timing 2	117.8c	16.8a	1984.9c
ATS timing 3	121.3cb	16.8a	2043.1cb
ATS timing 4	141.2a	17.6a	2495.9a
ATS timing 5	132.8ba	16.9a	2249.1ba
ATS concentration 0%	132.0a	17.4a	2300.6a
ATS concentration 3%	126.1a	16.8a	2119.1a
ATS concentration 6%	131.0a	17.1a	2243.2a
ATS concentration 9%	130.0a	17.3a	2261.5a
ATS concentration 12%	133.5a	17.2a	2302.0a
Hydro			
ATS timing 1	84.5a	20.9a	1758.1a
ATS timing 2	95.5a	21.1a	2031.7a
ATS timing 3	88.0a	20.8a	1806.1a
ATS timing 4	93.9a	21.4a	2011.6a
ATS concentration 0%	90.1a	21.7ba	1946.8ba
ATS concentration 3%	84.3a	20.2cb	1713.4b
ATS concentration 6%	94.4a	22.9a	2145.7a
ATS concentration 9%	93.2a	19.4c	1801.7b

^{z}Fruit yield in g.plant^{-1} was pepper fruit without peduncles on a dry weight basis.
^{y}Capsaicinoid concentration in mg.g^{-1} of dry pepper fruit powder.
^{x}Capsaicinoid content in mg.plant^{-1}, a product of capsaicinoid concentrations and fruit yield.
^{w}Means followed by the same letter within columns at each location do not differ significantly by LSD at $P \leq 0.05$.

Table 3. Effect of ATS treatments on total pepper fruit yield (g.plant^{-1}), capsaicinoid concentration (mg.g^{-1}) and capsaicinoid content (mg.plant^{-1}) at two locations for 'Plant 19' in 2003.

	Fruit yield (g.plant^{-1})[z]	Capsaicinoid concentration (mg.g^{-1})[y]	Capsaicinoid content (mg.plant^{-1})[x]
Bixby			
ATS timing 1	176.6a[w]	18.7a	3292.8a
ATS timing 2	187.4a	18.7a	3517.0a
ATS timing 3	180.3a	18.2a	3322.1a
ATS timing 4	186.3a	18.2a	3393.1a
ATS timing 5	191.3a	18.2a	3517.7a
ATS concentration 0%	191.2a	18.2a	3420.9a
ATS concentration 3%	181.6a	18.2a	3401.7a
ATS concentration 6%	192.3a	19.0a	3656.9a
ATS concentration 9%	179.7a	18.2a	3300.3a
ATS concentration 12%	177.1a	18.6a	3262.9a
Hydro			
ATS timing 1	146.8a	20.7a	3025.6a
ATS timing 2	155.1a	21.1a	3302.8a
ATS timing 3	152.3a	20.5a	3101.0a
ATS timing 4	148.2a	20.4a	3054.3a
ATS timing 5	151.2a	20.6a	3129.5a
ATS concentration 0%	142.1a	19.5b	2777.1c
ATS concentration 3%	144.0a	20.6ba	2953.8cb
ATS concentration 6%	157.8a	21.5a	3432.6a
ATS concentration 9%	151.5a	20.8a	3159.8ba
ATS concentration 12%	158.0a	20.9a	3290.1ba

[z]Fruit yield in g.plant^{-1} was pepper fruit without peduncles on a dry weight basis.
[y]Capsaicinoid concentration in mg.g^{-1} of dry pepper fruit powder.
[x]Capsaicinoid content in mg.plant^{-1}, a product of capsaicinoid concentrations and fruit yield.
[w]Means followed by the same letter within columns at each location do not differ significantly by LSD at $P \leq 0.05$.

Table 4. Effect of ATS treatments on total pepper fruit yield (g.plant^{-1}), capsaicinoid concentration (mg.g^{-1}) and capsaicinoid content (mg.plant^{-1}) for 'Okala' at Hydro in 2003.

	Fruit yield (g.plant^{-1})^{z}	Capsaicinoid concentration (mg.g^{-1})^{y}	Capsaicinoid content (mg.plant^{-1})^{x}
ATS timing 1	172.8a^{w}	18.2a	3118.1a
ATS timing 2	174.1a	17.7a	3071.6a
ATS timing 3	162.4a	18.1a	2921.6a
ATS timing 4	169.5a	17.9a	3033.8a
ATS timing 5	163.5a	18.2a	3017.0a
ATS concentration 0%	161.9a	17.6a	2847.8a
ATS concentration 3%	172.8a	17.5a	3026.2a
ATS concentration 6%	172.3a	18.5a	3187.2a
ATS concentration 9%	168.5a	18.2a	3069.3a
ATS concentration 12%	166.7a	18.2a	3031.6a

^{z}Fruit yield in g.plant^{-1} was pepper fruit without peduncles on a dry weight basis.
^{y}Capsaicinoid concentration in mg.g^{-1} of dry pepper fruit powder.
^{x}Capsaicinoid content in mg.plant^{-1}, a product of capsaicinoid concentrations and fruit yield.
^{w}Means followed by the same letter within columns do not differ significantly by LSD at $P \leq 0.05$.

Table 5. Effect of ATS treatments on the distribution of pepper fruit yield (g.plant^{-1}) and capsaicinoid content (mg.plant^{-1}) at two locations for 'Plant 19' in 2002.

	Fruit yield (g.plant^{-1})[z]					Capsaicinoid content (mg.plant^{-1})[y]				
	Red	Orange	Green	Mold	Total	Red	Orange	Green	Mold	Total
Bixby										
ATS timing 1	89.7b[z]	8.3a	1.2a	40.2a	139.5a	1543.7b	139.8a	9.1a	760.9a	2453.4a
ATS timing 2	69.1a	7.7a	2.5a	38.5a	117.8c	1170.5a	131.9a	16.7a	665.9a	1984.9c
ATS timing 3	75.1a	8.0a	1.6a	36.7a	121.3cb	1269.8a	145.7a	9.7a	617.9a	2043.1cb
ATS timing 4	88.6b	10.2a	1.2a	41.1a	141.2a	1528.8b	192.0a	9.9a	765.1a	2495.9a
ATS timing 5	91.6b	8.6a	1.8a	30.9a	132.8ba	1513.3b	159.8a	13.6a	562.4a	2249.1ba
ATS concentration 0%	83.4a	7.3a	1.8a	39.6a	132.0a	1449.2a	133.5a	10.4a	707.6a	2300.6a
ATS concentration 3%	79.6a	7.7a	1.8a	37.0a	126.1a	1329.7a	135.6a	12.7a	641.1a	2119.1a
ATS concentration 6%	83.5a	9.4a	1.5a	36.5a	131.0a	1400.8a	162.5a	9.5a	670.4a	2243.2a
ATS concentration 9%	81.8a	9.5a	1.7a	37.0a	130.0a	1384.8a	180.7a	12.7a	683.4a	2261.5a
ATS concentration 12%	85.8a	8.8a	1.6a	37.3a	133.5a	1461.8a	156.8a	13.8a	669.6a	2302.0a
Hydro										
ATS timing 1	55.9a	9.5a	11.6a	7.6a	84.5a	1239.7a	191.8a	154.0a	172.7a	1758.1a
ATS timing 2	62.7a	12.3a	12.3a	8.2a	95.5a	1434.1a	256.6a	167.0a	174.0a	2031.7a
ATS timing 3	57.7a	10.3a	12.5a	7.5a	88.0a	1268.3a	202.6a	169.5a	165.7a	1806.1a
ATS timing 4	62.1a	11.0a	12.7a	8.1a	93.9a	1427.9a	216.0a	188.3a	178.3a	2011.6a
ATS concentration 0%	59.0a	11.2a	12.4a	7.4a	90.1a	1362.1ba	230.6a	183.9a	170.2a	1946.8ba
ATS concentration 3%	55.3a	10.0a	11.5a	7.4a	84.3a	1211.8b	191.8a	151.2a	157.5a	1713.4b
ATS concentration 6%	62.3a	10.6a	12.6a	8.9a	94.4a	1532.2a	222.7a	184.0a	206.9a	2145.7a
ATS concentration 9%	61.8a	11.3a	12.5a	7.6a	93.2a	1263.9b	222.0a	159.7a	156.1a	1801.7b

[z]Fruit yield in g.plant-1 of different pepper fruit maturities without peduncles on a dry weight basis.
[y]Capsaicinoid content in mg.plant-1, a product of capsaicinoid concentrations and fruit yield.
[x]Means followed by the same letter within columns at each location do not differ significantly by LSD at $P \leq 0.05$.

Table 6. Effect of ATS treatments on the distribution of pepper fruit yield (g.plant^{-1}) and capsaicinoid content (mg.plant^{-1}) at two locations for 'Plant 19' in 2003.

	Fruit yield (g.plant^{-1})[z]					Capsaicinoid content (mg.plant^{-1})[y]				
	Red	Orange	Green	Mold	Total	Red	Orange	Green	Mold	Total
Bixby										
ATS timing 1	107.9a[x]	13.2a	38.7a	16.9ba	176.6a	2211.3a	212.2a	557.9a	317.6a	3346.0a
ATS timing 2	128.4a	11.7a	31.5a	15.9cb	187.4a	2583.0a	187.3a	501.6a	264.3a	3523.5a
ATS timing 3	119.2a	11.9a	34.8a	14.4cb	180.3a	2398.6a	176.8a	490.0a	258.9a	3430.4a
ATS timing 4	121.0a	14.5a	37.0a	13.8c	186.3a	2409.4a	225.2a	503.7a	259.9a	3460.4a
ATS timing 5	122.9a	13.7a	35.8a	18.9a	191.3a	2475.2a	199.2a	524.0a	319.3a	3517.7a
ATS concentration 0%	120.4a	14.6b	38.2a	18.0a	191.2a	2356.7a	220.5ba	529.8a	313.9a	3420.9a
ATS concentration 3%	114.3a	14.4b	36.5a	16.5a	181.6a	2353.6a	230.2a	530.1a	293.5a	3497.0a
ATS concentration 6%	125.3a	14.9b	36.0a	16.2a	192.3a	2627.1a	231.5a	532.4a	279.6a	3661.0a
ATS concentration 9%	119.6a	10.6a	34.7a	14.8a	179.7a	2314.2a	155.6c	527.4a	271.1a	3362.4a
ATS concentration 12%	119.8a	10.6a	32.4a	14.3a	177.1a	2425.9a	163.1cb	457.3a	261.9a	3336.6a
Hydro										
ATS timing 1	93.5a	10.7a	27.4a	15.1a	146.80a	2046.1a	232.2a	440.6a	308.9a	3025.6a
ATS timing 2	99.1a	9.7a	28.0a	18.3a	155.05a	2266.1a	207.4a	437.5a	389.3a	3302.8a
ATS timing 3	94.0a	10.7a	28.2a	19.3a	152.26a	2083.1a	239.8a	400.4a	377.8a	3101.0a
ATS timing 4	91.9a	10.4a	29.9a	16.0a	148.18a	2100.4a	216.1a	424.5a	314.1a	3054.3a
ATS timing 5	91.1a	12.6a	30.4a	17.2a	151.20a	2067.9a	259.9a	450.0a	354.6a	3129.5a
ATS concentration 0%	89.2a	10.3a	26.3a	16.4a	142.1a	1874.7c	213.0a	368.5a	321.0a	2777.1c
ATS concentration 3%	87.9a	10.8a	27.8a	17.5a	144.0a	1941.2cb	232.8a	421.5a	363.5a	2953.8cb
ATS concentration 6%	100.5a	11.0a	30.4a	15.9a	157.8a	2419.5a	239.6a	469.9a	307.4a	3432.6a
ATS concentration 9%	92.6a	11.1a	30.6a	17.3a	151.5a	2119.3cba	238.1a	458.7a	343.7a	3159.8ba
ATS concentration 12%	99.3a	11.1a	28.8a	18.8a	158.0a	2209.1ba	232.1a	434.5a	409.2a	3290.1ba

[z]Fruit yield in g.plant-1 of different pepper fruit maturities without peduncles on a dry weight basis.
[y]Capsaicinoid content in mg.plant-1, a product of capsaicinoid concentrations and fruit yield.
[x]Means followed by the same letter within columns at each location do not differ significantly by LSD at $P \leq 0.05$.

Table 7. Effect of ATS treatments on the distribution of pepper fruit yield (g.plant^{-1}) and capsaicinoid content (mg.plant^{-1}) for 'Okala' at Hydro in 2003.

	Fruit yield (g.plant^{-1})[z]					Capsaicinoid content (mg.plant^{-1})[y]				
	Red	Orange	Green	Mold	Total	Red	Orange	Green	Mold	Total
ATS timing 1	135.0a[x]	9.8a	12.5a	15.5a	172.8a	2515.3a	157.6a	166.2a	278.9a	3118.1a
ATS timing 2	137.9a	9.0a	13.5a	13.8b	174.1a	2482.4a	149.0a	184.3a	255.9a	3071.6a
ATS timing 3	128.7a	8.4a	12.4a	12.9b	162.4a	2392.2a	133.6a	158.4a	237.4a	2921.6a
ATS timing 4	135.0a	8.8a	12.4a	13.4b	169.5a	2474.5a	139.5a	173.2a	246.7a	3033.8a
ATS timing 5	131.4a	9.0a	11.6a	11.5b	163.5a	2419.3a	141.7a	152.3a	211.7a	3017.0a
ATS concentration 0%	127.8a	9.1a	11.2a	13.8a	161.9a	2294.1a	142.9a	150.8a	260.0a	2847.8b
ATS concentration 3%	136.2a	9.6a	12.3a	14.7a	172.8a	2402.3a	145.7a	164.3a	265.7a	3026.2b
ATS concentration 6%	136.7a	7.9a	13.8a	13.8a	172.3a	2618.8a	130.9a	190.5a	247.0a	3187.2a
ATS concentration 9%	133.8a	9.0a	12.5a	13.2a	168.5a	2464.4a	151.3a	161.3a	248.6a	3069.3a
ATS concentration 12%	133.4a	9.2a	12.5a	11.6a	166.7a	2504.1a	150.6a	167.5a	209.3a	3031.6a

[z]Fruit yield in g.plant-1 of different pepper fruit maturities without peduncles on a dry weight basis.
[y]Capsaicinoid content in mg.plant-1, a product of capsaicinoid concentrations and fruit yield.
[x]Means followed by the same letter within columns do not differ significantly by LSD at $P \leq 0.05$.

Table 8. Monthly average wind speed ($km.h^{-1}$) and relative humidity (%) data for Bixby and Hydro in 2002 and 2003 (Source: Oklahoma Mesonet).

Year	Location	Average wind speed ($km.h^{-1}$)				
		June	July	August	September	October
2002	Bixby	11.4	9.3	9.8	8.7	9.8
	Hydro	20.9	17.4	18.2	15.6	15.8
2003	Bixby	10.1	10.6	7.4	9.8	8.9
	Hydro	15.9	19.6	14.3	18.0	17.2
		Relative humidity (%)				
		June	July	August	September	October
2002	Bixby	78	79	77	74	80
	Hydro	73	73	65	68	83
2003	Bixby	77	66	74	79	75
	Hydro	73	54	62	66	65

List of Figures

Figure 3. Pepper plant developmental stages and timing of ATS application. Developmental stages I, II and III corresponded to the first, second and third flowering flushes, respectively, and were separated in time by approximately 2 weeks.

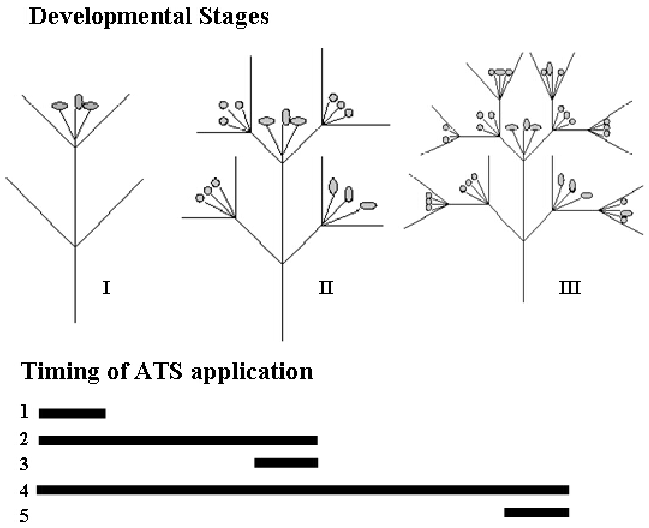
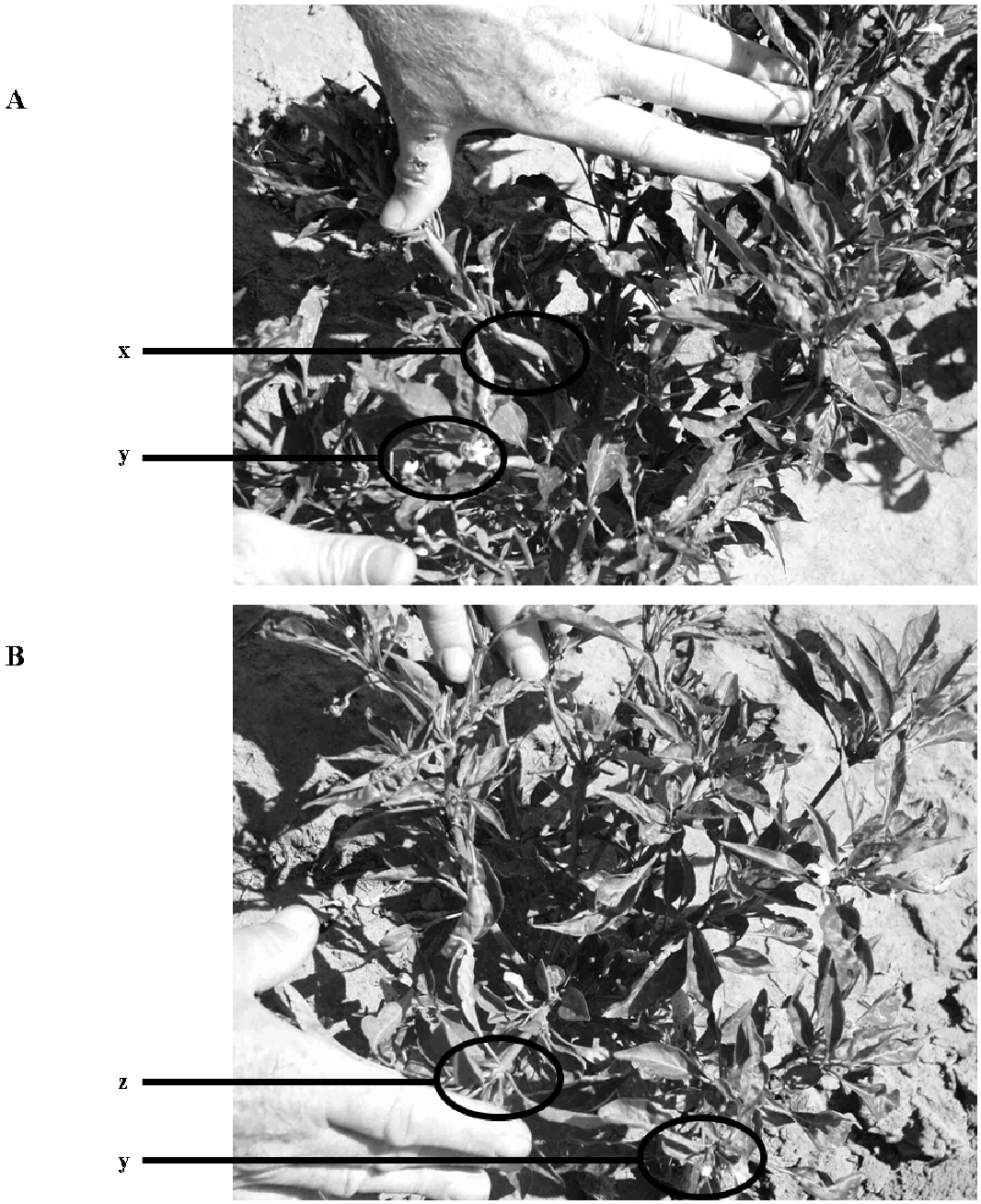


Figure 4. Effect of ATS spray application on flower removal from chilli pepper plants, one week after stage III development. Control plants (A) had numerous fruit set (x) and flowers for the forth flowering flush (y) were present. Plants (B) treated with 9% ATS sequentially at stages I, II and III (spray timing 4) exhibited flowering nodes from stages I, II and III devoid of fruit (z); flowers for the forth flowering flush (y) were present.



CHAPTER III

EVALUATION OF FATTY ACID PRECURSORS AND CHARACTERIZATION OF FATTY ACID STEROL ESTERS IN RELATION TO CAPSAICINOID ACCUMULATION IN PEPPER FRUITS

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Subject Category: Postharvest Biology

Evaluation of Fatty Acid Precursors in Relation to Capsaicinoid Accumulation in Pepper Fruits and Characterization of Fatty Acid Sterol Esters

Additional index words. *Capsicum annuum,* capsaicin, 8-methyl-6-nonenoic acid, FASE, GC, TLC, HPLC-APCI-MS, metabolism.

To investigate the mechanism of capsaicin (CAP) and dihydrocapsaicin (DC) specific accumulation in pepper [*Capsicum annuum* (L.) var. *annuum*] fruits, a series of experiments were conducted to evaluate lipid fractions containing fatty acid precursors for CAP and DC biosynthesis. The fatty acid precursors 8-methyl-6-nonenoic acid (E) for CAP and 8-methyl-nonanoic acid (A) for DC biosynthesis were extracted from fresh placenta with diethyl ether and purified using bonded phase aminopropyl column (Bond Elut). Capsaicinoids accumulated in a subsample from the same placenta were extracted with N, N-dimethylformamide (DMF) and quantitated using high performance liquid chromatography (HPLC). Extraction recovery for E and A fatty acids was greater than 90%. Free fatty acids and fatty acid sterol ester (FASE) were the only two lipid fractions containing substantial quantities of E and A. E and A in the free fatty acid fraction was higher at earlier stages of pepper fruit development and higher in green than orange than red fruits. The ratio of E to A free fatty acid precursors was always higher at all stages of fruit development. The FASE fraction was further characterized by high performance thin layer chromatography (HPTLC) as a prominent spot coeluting with cholesterol/sterol ester standards, and was devoid of free fatty acids. We verified by high performance liquid chromatography/atmospheric pressure chemical ionization – mass spectrometry

(HPLC-APCI-MS) that E and A were present in pepper placenta as FASE. Fatty acid methyl ester (FAME) analysis demonstrated that presence of E and A as free fatty acids were usually in excess of that present as FASE.

Pungency in hot peppers [*Capsicum annuum* (L.) var. *annuum*] is due to capsaicinoids which are amides of vanillylamine and C_9 to C_{11} branched chain fatty acids. Capsaicinoids are colorless, odorless alkaloids and mammalian pain receptors respond to capsaicinoid concentration as low as 0.1-1 ppm in solutions (Andrews, 1995). More than 12 different capsaicinoids have been reported in nature; the five major capsaicinoids are Capsaicin (CAP), Dihydrocapsaicin (DC), Nordihydrocapsaicin, Homodihydrocapsaicin and Homocapsaicin (Suzuki and Iwai, 1984). CAP and DC, amide derivatives of vanillylamine and 8-methyl-6-nonenoic acid (E) or 8-methyl-nonanoic acid (A), respectively (Fig. 5), are the major capsaicinoids contributing approximately 90% of total capsaicinoids in most pepper fruits. Bennett and Kirby (1968) reported the following composition of the capsaicinoids in whole fruit components of *C. annuum*: capsaicin-69%, dihydrocapsaicin-22%, nordihydrocapsaicin-7%, homocapsaicin-1% and homodihydrocapsaicin-1%. Suzuki et al., (1980) reported that the capsaicinoids were synthesized in the glandular areas of the placental outer wall. Capsaicinoid accumulation occurs over a relatively short period during fruit development and was first detected 20 days after flowering, and active accumulation was observed between 25 to 40 days (Iwai et al., 1979; Sukrasno and Yeoman, 1993). Estrada et al., (1997) reported that in 'Padron' pepper, capsaicinoids were first detected 14 days after flowering, increasing linearly until

the 35th day; peppers showed highest accumulation of capsaicinoids on the 42nd day after flowering.

All capsaicinoids are amide derivatives of vanillylamine and differ according to the specific C_9 to C_{11} fatty acids attached. The biochemical pathway leading to capsaicinoids has two distinct origins (Fig. 6). The phenylpropanoid pathway contributes the vanillylamine aromatic component from phenylalanine through cinnamic acid, coumaric acid, caffeic acid, ferulic acid, vanillin and vanillylamine (Fujiwake et al., 1982) and is common to all capsaicinoids (shaded portion in Fig. 5). The fatty acid moiety originates from valine or leucine, and determines which specific capsaicinoid is being synthesized in the fruit placenta (Suzuki et al., 1981). Valine serves as a precursor for the formation of even-numbered branched-chain fatty acids (essential for capsaicin and dihydrocapsaicin in Fig. 5) and leucine for odd-numbered branched-chain fatty acids (essential for nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin in Fig. 5).

The phenylpropanoid pathway contributing the aromatic component is shared with other pathways of general phenylpropanoid metabolism and is common to all higher plants to vanillin. The conversion of vanillin to vanillylamine is unique to chilli pepper. Much of the prior work related to regulation of capsaicinoid biosynthesis has focused on the phenylpropanoid pathway (Yeoman and Sukrasno, 1993; Ochoa-Alejo and Gomez-Peralta, 1993; Johnson and Ravishankar, 1996; Johnson et al., 1996) and comparatively little work has been done on fatty acid precursor accumulation. Using a radioisotopic technique in intact fruits of *Capsicum annuum* var. *annuum* cv. 'Karayatsubusa', Suzuki et al., (1981) reported that valine served as precursor for even-numbered branched chain fatty acids and leucine served as precursor for odd-numbered branched chain fatty acids.

The intermediates were identified as α-ketoisovalerate, isobutyryl moiety followed by chain elongation with malonyl Co-A leading to E and A fatty acid formation. Plants have type II fatty acid synthase (FAS) complex (Harwood, 1996) which includes β-ketoacyl synthase (KAS) (Yasuno et al., 2004); three KAS enzymes contribute to the elongation of the fatty acid chain until it is released by fatty acid thioesterase (FAT). In placenta, transcription of *Kas*, *Acl* and *Fat* genes were placental-specific and RNA abundance was positively correlated with degree of pungency (Aluru et al., 2003); KAS accumulation in the placenta was positively correlated with the degree of pungency.

In order to evaluate fatty acid precursors and their involvement in capsaicinoid biosynthesis, lipids must first be separated into their various component fractions. Kaluzny et al., (1985) reported a rapid method using aminopropyl bonded phase (Bond Elut) columns to separate lipids into different fractions with more than 95% recovery. In this technique, isolation of compounds is achieved by creating selectivity by serially altering the solvent (pH, polarity etc.). Using this procedure, plant lipids can be separated into free fatty acids, phospholipids, sterol esters, triglycerides, sterol, diglycerides and monoglycerides.

Fatty acids may be present in plants in the free form, or they may be esterified to glycerol (monoglycerides, diglycerides and triglycerides) or sterols [fatty acid sterol esters (FASE)]. FASE are recognized as a storage form of sterols and/or of fatty acids (Billheimer et al., 1983). FASE structures are complex in nature because of the variability in the sterol and the fatty acid moieties. The sterol moieties of FASE in many plants belong to the subclass 4,4-desmethylsterols which includes sitosterol, campesterol and stigmasterol (Dyas and Goad, 1993). Kemp and Mercer (1968) reported that the fatty acid

moieties of FASE varies among different tissues of 10 day old *Zea mays* seedlings, with linoleic acid being predominant in root, scutellum and endosperm, while palmitic acid was the major fatty acid in the shoot. Citrus chemotaxonomy based on lipid profiling, showed that the unsaturated fatty acids ($C_{18:1}$, $C_{18:2}$, $C_{18:3}$) were mainly associated with 4,4-desmethylsterols, while saturated fatty acid ($C_{16:0}$) and long chain fatty acids (C_{22} to C_{29}) were associated with 4,4-dimethylsterols (Nordby and Nagy, 1974).

For the identification and analysis of cholesterol/sterol esters (CE/SE) from complex lipid mixtures, several methods including thin-layer chromatography (TLC) (Kishimoto, 2001), gas chromatography (GC) (Kalo and Kuuranne, 2001), high performance liquid chromatography (HPLC) (Ferrari et al., 1997), and high performance liquid chromatography/Atmospheric Pressure Chemical Ionization – Mass Spectrometry (HPLC-APCI-MS) (Rozenberg et al., 2003) have been reported. Although TLC can be used to purify the FASE fraction, separation based on difference in sterol or fatty acid moieties cannot be achieved (Caboni, 2005). GC analysis of intact FASE is difficult because of the high boiling point (Rozenberg et al., 2003). Acid or alkaline hydrolysis of FASE to form free sterol and fatty acid methyl esters can be relatively easily achieved, and the components can be analyzed by GC (Phillips et al., 2002). The exact FASE distribution, however cannot be determined since sterols and fatty acids are measured independently. Analysis of SE by reversed phase HPLC on C18 columns with evaporative light scattering detector have been reported (Ferrari, 1997). Mezine (2003) reported that the less hydrophobic hexyl-phenyl column was effective in elution of SE with acetonitrile and water (90:10) as the mobile phase.

The objective of this study was to evaluate the fatty acid precursor substrate availability in relation to capsaicinoid-specific biosynthesis and accumulation, with specific emphasis on 8-methyl-6-nonenoic acid (E) for capsaicin and 8-methyl nonanoic acid (A) for dihydrocapsaicin. Another objective was to characterize the nature of the FASE fraction using TLC and HPLC-APCI-MS, in order to validate the presence of FASE as putative storage constituents in relation capsaicinoid biosynthesis of the pepper placenta.

Materials and Methods

Plant material: Pepper plants from crosses of parents with high CAP : DC ratio of 2.5 as female parent and high capsaicinoid concentration (CAP + DC in excess of 20 $mg.g^{-1}$) as male parent were selected from field plantings at the Oklahoma Vegetable Research Station in Bixby in the 2001 and 2002 growing seasons. Representative fruit samples were collected from plants marked with a phenotype of determinate flowering habit and thin slender fruits, and capsaicinoids and CAP : DC ratios were determined. Pepper plants were selected from selfed progeny in field plantings at Bixby from 2002 through 2005, on the basis of total capsaicinoid concentration (high, intermediate and low) and CAP : DC ratio (high, intermediate and low). The selected plants were trimmed prior to digging from field plots, placed into burlap bags and transported to greenhouse facilities in Stillwater, OK. They were then planted in plastic grow bags containing the potting mixture “Ready Earth”. Slow release fertilizer “Osmocoat” (20:20:20 NPK) was applied monthly and soluble fertilizer was applied when required. Watering was done as needed. On an average, maximum temperature of 32 ˚C and minimum temperature of 21 ˚C was maintained in the greenhouse.

Plants were grouped into six categories of combined capsaicinoid concentration ($mg.g^{-1}$) (high -14 $mg.g^{-1}$ or above; intermediate - 6 to 14 $mg.g^{-1}$; low - less than 6 $mg.g^{-1}$) and CAP : DC ratio (high - 2 or above; intermediate - 1 to 2; low - below 1). The plant selections were placed on benches in a completely randomized experimental design and pruned every year to promote new growth, flowering flush and fruit set. Pods were selected from plants at different physiological stages of development and maturity based on visual observation: i) Unripe - green, physiologically mature ii) Ripening - orange to light red coloration iii) Fully ripened - fully red colored.

At the time of harvest, fruits of the three maturity categories were obtained from one plant of a particular category and transferred on ice to laboratory facilities and stored in a cooler at 4 ˚C. Individual fruits without peduncles were then weighed and the placenta was separated from the pericarp and seed, weighed and dried at 40 ˚C for 12 hours. A separate sample of whole fruits for capsaicinoid analysis were also weighed and dried at 40 ˚C for 72 hours. The dried placental material and whole fruit were ground to a fine powder using a mortar and pestle, mixed thoroughly and stored in a brown bottle at -20 ˚C.

Capsaicinoid extraction for high performance liquid chromatography (HPLC): Capsaicinoids were extracted from fruit and separated placental powders with N,N-Dimethyl-formamide (DMF) to quantify the actual CAP+DC and their ratio. Approximately 200 mg of whole fruit powder or 100 mg of placenta powder was transferred into a 2 dram vial, 2 mL of DMF was added and the vial was sealed with Teflon-lined screw caps. Extractions were carried out at 80 ˚C in a dry block heater for one hour. Samples were swirled every 15 minutes during the 1 hour period to assure

proper mixing. The samples were then removed from the heat block, centrifuged for 5 minutes using a Speed-vac centrifuge (Savant Instruments Inc. Farmingdale, NY; approximately 3,500 g_n) and supernatants were decanted into a 10 mL volumetric flask. The procedure was repeated 3 more times for a total of 4 extractions and the volumetrics were brought to volume with DMF. Preliminary recovery trials using this procedure produced capsaicin (CAP) and dihydrocapsaicin (DC) recoveries of 95 and 101 percent, respectively. Aliquots of the extracts were placed into autosampler vials and utilized directly for HPLC capsaicinoid analysis using a modification of the "short run" procedure of Collins et al., (1995). Capsaicinoid separations were achieved at a flow rate of 1 mL min^{-1} using a Dionex gradient HPLC pump GPM-2 (Dionex Corp., Sunnyvale, CA). Samples were injected with an AS-3500 automatic sample injector by overfilling a 10 µL sample loop. Separation of CAP and DC was conducted with a Waters Nova-Pak C-18 (Waters corp. Milford, MA) radial compression column (8 mm x 100 mm) with a Nova-Pak C-18 precolumn guard cartridge. The mobile phase in reservoir A was 80% methanol: 20% HPLC grade water; the mobile phase in reservoir B was 100% methanol. Capsaicinoids were separated under isocratic conditions using mobile phase from reservoir A. The column was reconditioned after each run with a gradient to 100% methanol from reservoir B as follows; 0-13 min, 100% A; 13-18 min, 100% A to 100% B; 18-23 min, 100% B to 100% A. The column was allowed to reequilibrate to solvent A for 7 minutes prior to injection of another sample. Peaks were detected with a Shimadzu RF-511 (Shimadzu Corp., Kyoto, Japan) spectrofluorometric detector with excitation wavelength at 280 nm and emission wavelength at 338 nm in low sensitivity mode. Sample components were identified by coelution with standard CAP (8-methyl-n-

vanillyl-6-nonenamide) and DC (8-methyl-n-vanillyl-nonanamide) (Sigma Chemical Co., St. Louis, MO) and quantified by an external standard method, by comparing with 200 ppm standards run twice for every six samples. Standard solution of 200 ppm was prepared in DMF by dilution of a 2000 ppm stock solution of CAP and DC. The stock solution was stored in a freezer at -20˚ C and allowed to reach room temperature prior to opening to avoid moisture condensation.

Lipid extraction: Approximately 50 mg powder of placental material for each color grouping/plant combination was transferred into six 2 dram vials for lipid extraction. The lipid extraction involved a modified procedure of Method 920.85 of AOAC. Two milliliters of anhydrous diethyl ether was added and stirred with a magnetic stirrer for 20 minutes, at room temperature inside a fume hood. Samples were then centrifuged in a Speed-vac for 15 minutes and the supernatant was transferred into a pre-tared 2 dram vial and dried under nitrogen gas. The extraction was repeated three more times and the final lipid extract weight (approximately 10 mg) was recorded.

Aminopropyl Column Lipid Separation. Column #1: The lipid extract was separated into different classes using bonded phase aminopropyl columns (Bond Elut; Varian Inc., Lake Forest, CA) with metallic frits (Kaluzny, 1985). To the lipid extract vial, 500 µL of chloroform was added and vortexed. It was then transferred to a preconditioned (with 2 mL hexane immediately preceding use) aminopropyl column #1 (Fig. 7). Preconditioning of the column was essential to ensure interaction between the solid phase and the sample extract (Flurkey, 2005). The different solvent mixtures and volumes of solvent used for our procedure are listed in Table 9. Just after elution of the lipid extract in chloroform, solvent A (chloroform - propanol 2:1, 4 mL) was passed through the column to elute all

neutral lipids including capsaicinoids. More than 95% of the capsaicinoids from the lipid extract were eluted with solvent A. Free fatty acids were eluted using solvent B (2% acetic acid in diethyl ether, 12 mL). The volume of solvent B was increased from 4 mL indicated in the original method (Kaluzny et al., 1985) to 12 mL to increase the recovery of E and A to more than 90% (data not shown). The free fatty acid fraction was dried under nitrogen gas to await derivatization into fatty acid methyl esters (FAME) for analysis or use for HPTLC or HPLC-APCI-MS analyses as will be described. Preliminary experiments demonstrated that no capsaicinoids could be detected from column #1 eluates past solvent A, in the neutral lipid fraction. The neutral lipid/capsaicinoid eluate from solvent A was further separated on column #2.

Column #2: After drying the neutral lipid fraction (solvent A fraction from column #1) under nitrogen gas, it was brought up in 500 µL hexane with vortexing, applied to column #2 and rinsed with an additional 500 µL of hexane (preconditioned with 2 mL hexane immediately preceding use). This eluate, plus an additional 12 mL of hexane (solvent H) was used to elute the "cholesterol ester" fraction (containing placental sterol esters). Then solvent T (1% diethyl ether, 10% methylene chloride in hexane, 4 mL) was used to elute "triglycerides", solvent C (5% ethyl acetate in hexane, 4 mL) was used to elute the "cholesterol" fraction, solvent D (15 % ethyl acetate in hexane, 4 mL) to elute "diglycerides" and solvent M (chloroform - methanol 2:1, 12 mL) was used to elute "monoglycerides", which also eluted capsaicinoids. Preliminary trials indicated that our deviation from the original procedure, adding 12 mL of solvent H and solvent M, instead of 4 mL used in the original method, was necessary to recover > 90% and 80% of the indicated sample components respectively.

Fatty acid methyl esters (FAME) procedure: The eluates from solvents B, H, T and D were dried under nitrogen gas, and 600 nmoles of capric acid (CA) was added as the internal standard for low carbon fatty acids (E and A) and 600 nmoles of heptadecanoic acid (HDA) was added as internal standard for higher carbon fatty acids (palmitic, stearic, oleic, linoleic and linolenic acids). Samples were methanolysed according to the procedure of Maness et al. (1995). Two hundred microliters of methanolic HCl (3% HCl in methanol, prepared by adding 0.5 mL acetyl chloride to 10 mL methanol) and 50 µL methyl acetate (as water scavenger) was added. The vials were sealed with Teflon-lined caps and incubated in a dry block heater for 2 hours at 90 ˚C. After 15 minutes, the contents of the vial were mixed by vortexing to assure equilibrium of the eluates into single phase during methanolysis. After 2 hours at 90 ˚C, vials were cooled to room temperature and 5 drops of tertiary butanol was added to co-evaporate with HCl during drying under nitrogen gas. FAME was dissolved in 700 µL of hexane and 1 µL aliquots were utilized for gas chromatography.

Gas chromatography was conducted using a Tracor model 540 gas chromatograph (Tracor Instruments, Austin, TX) equipped with GC 411V autosampler (Dynatech Precision Sampling Corp., Baton Rouge, LA), split injection port (J and W Scientific Inc, Folsom, CA; split ratio of 50:1) and flame ionization detector. Separations were performed using a DB 23 fused silica capillary column (30 m x 0.25 µm film thickness: J and W Scientific Inc., Rancho Cardova, CA) with helium carrier gas at a linear flow rate of 20 $cm.sec^{-1}$. The initial column temperature was 50 ˚C for 2 minutes. Low carbon FAME's ($< C_{14}$) were separated using a linear temperature program from 50 to 110˚ C at 3 ˚C per minute. A second linear temperature program from 110 to 180 ˚C at 5 ˚C per

minute was used to separate HDA and higher carbon fatty acids (C_{16} and C_{18}). The column temperature was held at 180 ˚C for 5 minutes and a third linear temperature program from 180 to 240 ˚C at 5 ˚C per minute was achieved with a final hold at 240 ˚C for 5 minutes. Individual FAME peaks were identified by co-elution with authentic standards (8-methyl-6-nonenoic acid and 8-methyl-nonanoic acid, Maybridge plc, Cornwall, United Kingdom; CA, HDA and higher carbon fatty acids, Sigma Chemical Co., St. Louis, MO). Peak areas were obtained using a Spectra Physics 4270 integrator (Spectra-Physics Inc., San Jose, CA) and quantified relative to capric acid as internal standard for E and A and to HDA as internal standard for $C_{16,}$ $C_{18,}$ $C_{18:1,}$ $C_{18:2}$ and $C_{18:3}$ fatty acids.

Solvent M, which contained the capsaicinoids from the neutral lipid fraction (Column #2), after drying under nitrogen gas was brought up in 1mL DMF and further diluted 3:1 with DMF to avoid overloading the HPLC column and capsaicinoids were quantified using HPLC as previously described.

Thin Layer Chromatography (TLC): The different lipid fractions from aminopropyl bonded phase column #2 and free fatty acids from column #1 were run on a normal phase high performance thin layer chromatography (HPTLC) plate (150 µm layer, 10x20 cm; Analtech Inc., Newark, DE). The solvent or mobile phase consisted of 80:20:1 mixture (v/v/v) of hexane, diethyl ether and acetic acid. Prior to running the samples, the TLC plate was washed in the solvent and activated at 100 ˚C for 30 minutes to remove moisture. The eluates pre-dried under nitrogen gas were dissolved in 100 µL of chloroform. One microliter of sample was spotted using 1 µL disposable pipettes (Microcaps) (Drummond Scientific Co., Broomall, PA) 1 cm from the bottom edge of the

plate and spaced 2 cm apart. TLC standard 18-5 (10 mg.mL^{-1}; Nu-Check Inc., Elysian, MN) containing free fatty acids, cholesterol, triglycerides and cholesterol esters, and samples were spotted; standard mixture of plant SE (CoroWise^{TM}; 20.7 µg.mL^{-1}; Cargill Co., Minneapolis, MN) was spotted separately. The plate was then placed in a developing chamber containing a shallow layer of the mobile phase, and allowed to develop until the solvent reached 1 cm below the top of the plate. Plates were then removed from the chamber, air dried at room temperature inside a fume hood for 5 minutes, and dipped in a solution containing 3% cupric acetate in 15% phosphoric acid and charred by heating on a hot plate. Developed plates were documented by scanning on a document scanner.

High Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization – Mass Spectrometry (HPLC-APCI-MS): Three replicates of eluate from solvent H fraction of red, orange or green samples were dissolved together in 1200 µL of tetrahydrofuran (THF)-acetonitrile (ACN) (80: 20 v/v) (Thomas and Rudel, 1987). Twenty five micromolar concentration of cholesterol decanoate (CE) (13.5 µg.mL^{-1}) and CoroWise^{TM} plant SE (20.7 µg.mL^{-1}) standards were also dissolved in THF-ACN mixture. HPLC-APCI-MS was conducted using an Agilent 1100 series LC/MSD (Agilent Technologies, Palo Alto, CA) equipped with a quadrupole mass selective detector (MSD). Using a modified procedure of Mezine et al., (2003), 10 µL of samples and standards were injected and chromatographed with a Gemini phenyl-hexyl HPLC column, 150 x 4.6 mm i.d., 5 µm particle size (Phenomenex, Torrance, CA) at a flow rate of 0.6 mL.min^{-1}. The column was maintained at a temperature of 35 ˚C. The mobile phase used in reservoir A was 90.5% THF: 9.5% ACN; the mobile phase in the reservoir B was HPLC grade water. The separation was achieved under linear gradient condition; 0

min, 92% A and 8% B; 0-2 min, 92% A and 8% B to 95 % A and 5 % B; 2-4 min, 95 % A and 5 % B to 100 % A; 4-10 min, 100 % A. The cholesterol/sterol fragments and their fatty acid moieties were detected by APCI-MS in positive and negative mode respectively. The optimized settings of the mass selective detector was as follows: fragmentator voltage, 145 V; gain, 10; threshold, 150; stepsize, 0.2; drying gas flow, 12 $L.min^{-1}$; nebulizer pressure, 45 psi; drying gas temperature, 300 ˚C; vaporizer temperature, 300 ˚C; capillary voltage, 4000 V; corona current, 4 µA and 15 µA for positive and negative mode respectively;. The mass selective detector was set to scan between m/z values of 50 and 700.

Results

The placental lipid ether extract included capsaicinoids as well as other lipid fractions. Capsaicinoids ($mg.g^{-1}$) were extracted more efficiently with DMF compared to ether. While preliminary data showed that the aminopropyl column #1 eluate (solvent A) recovered more than 99% capsaicinoids in the lipid ether extract, only 65% of capsaicinoids were extracted with ether compared to the DMF extraction protocol. Recovery of capsaicinoids through aminopropyl column #2 (solvent M) was greater than 80% of solvent A fraction from column #1 and approximately 55% compared to DMF extraction (Table 10). The capsaicinoid content was at least 4 to 8 times higher in pepper placenta than whole fruit (Table 10); the CAP : DC ratio of whole fruit and placenta were essentially the same. The capsaicinoid content of red fruits was usually higher than green fruits. The aminopropyl column #1 efficiently separated E and A free fatty acid precursors (solvent B) from the capsaicinoids (solvent A), since no capsaicinoids could be detected in solvent B fractions. Separation of capsaicinoids from other lipid fractions

was essential to avoid overestimation of E and A lipid fractions, since approximately 10 percent of the E and A fatty acids from capsaicinoids were converted into FAME during methanolysis (data not shown).

E and A fatty acids ($\mu g.g^{-1}$) were predominantly detected in two lipid fractions; free fatty acids (solvent B from column #1) (Table 11) and FASE (solvent H from column #2) (Table 12). E and A free fatty acids were found in greater concentration in green than orange than red fruits in placental lipid extracts; they were variable, but usually lower in the FASE fraction compared to the FFA fraction. The E : A ratio of FFA and FASE (Table 11 and 12) was generally higher than the CAP : DC ratio (Table 10) for the same placental extract, irrespective of the capsaicinoid content of the whole fruit. The E : A ratio of FFA and FASE followed a similar pattern, with higher content of E than A. For most plants, the E : A and CAP : DC ratios declined in unison. The higher carbon fatty acids as FFA (Table 11) and as FASE (Table 12) showed variation in the concentration of fatty acids at different stages of pepper fruit development. The relative abundance of fatty acids in the FFA fraction in descending order were E, plamitic, stearic, A, linolenic, linoleic and oleic acids. The relative abundance of fatty acids in FASE fraction, in descending order, were linoleic, linolenic, E, A, palmitic, oleic and stearic acids.

The eluate from solvent H of aminopropyl column #2, previously termed the cholesterol ester (CE) fraction (Kaluzny et al., 1985) was run on HPTLC with known standards (Fig. 8). A prominent spot from samples coeluted with CE and CoroWise™ plant SE standard (both CE and SE exhibited a prominent spot at the same location). There was no spot corresponding to free fatty acids in this column eluate. The noticeable

tailing in this fraction may be indicative of hydrocarbons, as would occur along with esterified fatty acid sterol esters. The eluate in the triglyceride fraction (solvent T) had a lighter spot for both FASE/CE and triglyceride, while the cholesterol fraction (solvent C) had a prominent spot for triglyceride and no cholesterol/sterol spot. The diglyceride fraction (solvent D) showed a faint spot corresponding to diglyceride that would elute in this position (standard not shown in Fig. 8). The free fatty acid fraction from column #1 (solvent B) showed a prominent spot for free fatty acids and a lighter spot corresponding to phospholipids and triglycerides.

The mass spectrum of CE and CoroWise™ plant SE standards showed characteristic cholesterol/sterol fragment ions in the positive scan mode and their corresponding deprotonated fatty acid fragment ions in the negative scan mode; the samples and standards did not show intact cholesterol/sterol esters in either positive or negative scan mode. For the CoroWise™ plant SE standard, the positive scan mode showed three different sterol fragment ions *m/z* 383, 395, 397 corresponding to campesterol, stigmasterol and sitosterol respectively; the negative scan mode showed the fatty acid fragment ions *m/z* 255, 277, 279, 281 corresponding to the deprotonated palmitic acid, linolenic acid, linoleic acid and oleic acid respectively. The CoroWise™ plant SE standard also exhibited these fatty acids by FAME-GC (data not shown) in approximately the same prevalence as seen in negative scan mode for the HPLC-APCI-MS analyses. For pepper placental eluates from solvent H fraction, the positive scan mode showed at least four different putative sterol fragment ions *m/z* 383, 395, 397, 409 corresponding to campesterol, stigmasterol, sitosterol and lanosterol respectively (Fig. 9); the negative scan mode showed the putative fatty acid fragment ions *m/z* 169,

corresponding to the deprotonated 8-methyl-6-nonenoic acid and *m/z* 171 and 172 corresponding deprotonated and protonated form of 8-methyl-nonanoic acid respectively (Fig. 10); the deprotonated form of long chain fatty acids were also observed in the same eluate at approximately the same proportion as previously observed by FAME-GC.

Discussion

One of the objectives of this study was to quantify fatty acid precursors for capsaicinoid biosynthesis in the pepper placenta at different stages of fruit development in relation to capsaicinoid accumulation. Since capsaicinoids in the lipid fraction yielded approximately 10 % of their respective fatty acids during FAME methanolysis (data not shown), they were separated from the free fatty acids to avoid fatty acid contamination of those samples from incomplete methanolysis of capsaicinoids. Capsaicinoids in the neutral lipid fraction (solvent A) from column #1 were further separated from lipid fractions on column #2, eluting only in the last column eluate (solvent M) (Table 10). FAME methanolysis of solvent H (the CE/SE fraction) yielded E and A fatty acids by GC. Preliminary studies using authentic E and A fatty acid standards provided evidence that no FFA eluted in this fraction.

The prominent spot on HPTLC for solvent H coeluted with both CE and SE standards (Fig. 8) and the HPLC-APCI-MS confirmed the presence of sterol fragment ions of FASE and no peaks corresponding to cholesterol fragment ions of CE. The absence of free fatty acids on HPTLC further strengthened that E and A fatty acids observed from FAME methanolysis of solvent H fraction were from fatty acid moieties esterified to the sterols in the native sample. Although HPTLC can separate total sterol ester fraction, it cannot identify individual sterol and fatty acid moieties (Caboni, 2005).

HPLC-APCI-MS analysis was used to identify the nature of the sterol esters (Mezine et al., 2003). The presence of sterol fragments in the positive scan mode and deprotonated E and A fatty acid fragments in the negative scan mode gave information about the presence of FASE components in the solvent H eluate. Similarly, precursor ion electrospray MS-MS of sterol fragment ions of sterol esters was observed in the determination of sterol ester proportions in butterfat and vegetable oils by Kalo and Kuuranne (2001). The identity of E and A fatty acid fragment ions was verified by running intact 8-methyl-6-nonenoic acid (E) and 8-methyl nonanoic acid (A) standards which showed the same *m/z* values for both fatty acids in negative scan mode only. However an intact sterol ester was not observed for either pepper placental eluate or authentic plant SE standards.

The presence of a light FASE spot on HPTLC from the triglyceride fraction (solvent T) of aminopropyl column #2 might be due to incomplete elution of the sample into solvent H (Fig. 8). Further a light spot for triglyceride in the triglyceride fraction and a prominent spot in the later eluted cholesterol/sterol fraction of aminopropyl column indicate that triglycerides predominantly eluted with solvent C rather than solvent T.

The total capsaicinoid content was 4 to 8 times higher in the placenta than the whole fruit (Table 10), as has been found previously (Suzuki et al., 1980). The CAP : DC ratio was similar between whole fruit and placental extracts. The free fatty acid precursor was found to be always higher in green than orange and red ripe stage. Since capsaicinoid accumulation occurs over a relatively short period (25 to 40 days after flowering) during fruit development (Iwai et al., 1979; Sukrasno and Yeoman, 1993) the decline in E and A fatty acids as fruit matured would be consistent with increased use in metabolism.

Although the fatty acid content of FASE was lower than FFA, their E : A ratios were similar. This indicates that 8-methyl-6-nonenoic acid (E) was always present in excess of 8-methyl nonanoic acid (A) in both FFA and FASE. This was also supported by corresponding higher biosynthesis/accumulation of capsaicin over dihydrocapsaicin in the pepper placenta.

Conclusions

In relation to capsaicinoid (CAP and DC) accumulation, the E and A free fatty acid precursor quantitation at different stages of pepper fruit development showed greater fatty acid availability at earlier fruit developmental stage, green greater than orange and red; higher E to A ratio of FFA precursors was observed at all developmental stages. HPTLC gave evidence that the unknown compound coeluted with CE/SE fraction (solvent H) by aminopropyl column #2 might be fatty acid sterol esters, which was later verified by HPLC-APCI-MS. While it is unclear what the biological function of FASE was in pepper placenta, other authors have claimed FASE function as storage of plant sterols (Billheimer et al., 1983) and perhaps of fatty acids. The composition of sterol moieties varied between different tissues of *Musa sapientum* (Knapp and Nicholas, 1969) due to selectivity in the sterol esterification process at different tissues and based on their functions (Dyas and Goad, 1993). Similarly, variation in the fatty acids esterified to sterols in different tissues was reported by Kemp and Mercer (1968) in *Zea mays*. To date, there has been no report on the quantitation of 8-methyl-6-nonenoic acid (E) and 8-methyl nonanoic acid (A) fatty acid precursors in relation to capsaicin and dihydrocapsaicin accumulation in pepper fruit. Further, this is the first report on E and A fatty acid sterol esters in pepper placenta, which might be a storage form of 8-methyl-6-

nonenoic acid (E) and 8-methyl nonanoic acid (A), the precursors of capsaicin and dihydrocapsaicin biosynthesis. Since the positive scan mode of APCI-MS showed four different sterol fragment ions, it is unclear which sterol moieties were esterified to E and A fatty acids. Further investigation is needed to study the association of a particular sterol moiety to E and A fatty acids in pepper placenta.

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Table 9. Solvents used in the bonded phase aminopropyl column separation of pepper placental lipid extract.

	Solvents	Solvent Volume (mL)	Lipid eluted
A	Chloroform : 2-propanol (2:1)	4	Neutral lipids
B	2% acetic acid in diethyl ether	12	Free fatty acids
H	Hexane	12	Cholesterol esters/fatty acid sterol esters (?)
T	1% diethyl ether, 10% methylene chloride in hexane	4	Triglycerides
C	5% ethyl acetate in hexane	4	Cholesterol/sterol
D	10% ethyl acetate in hexane	4	Diglycerides
M	Chloroform : methanol (2:1)	12	Monoglycerides (CAP + DC)

Table 10. Extraction efficiency of capsaicinoid from pepper wole fruit and placenta by DMF vs. ether, and recovery of capsaicinoids in solvent M fraction of column #2 .

Plant	Maturity stages	DMF extraction				Ether extraction			
		Whole fruit		Placenta		Placenta			
		Capsaicinoid ($mg.g^{-1}$)[z]				Capsaicinoid ($mg.g^{-1}$)[y]			
						Solvent A		Solvent M	
		CAP+DC	CAP:DC	CAP+DC	CAP:DC	CAP+DC	CAP:DC	CAP+DC	CAP:DC
1	Red	18.7	2.8	67.5	2.3	37.2	1.9	35.9	1.8
	Orange			62.9	2.8	41.0	1.6	42.2	1.6
	Green	11.9	2.7	48.5	3.0	34.1	2.2	26.7	2.3
2	Red	16.8	2.1	74.6	2.2	71.1	1.8	60.7	1.9
	Orange			67.2	2.0	53.1	1.6	37.6	1.7
	Green	18.5	2.0	82.7	2.0	48.5	1.7	50.6	1.5
3	Red	5.9	2.0	41.5	1.9	34.3	2.6	26.8	2.2
	Orange					24.2	1.9	13.8	1.9
	Green	5.7	4.0	41.5	1.9	23.5	1.4	28.2	1.5
4	Red	22.9	1.6	101.8	1.6	78.6	1.3	65.9	1.3
	Orange			97.0	1.6	60.2	1.2	57.9	1.2
	Green	12.9	1.8	87.0	1.6	58.5	1.2	50.9	1.2
5	Red	21.4	1.6	112.3	1.4	79.1	1.2	59.8	1.4
	Orange					63.2	1.0	51.8	1.1
	Green	9.8	1.5	97.7	1.4	64.8	1.2	56.7	1.3
6	Red	13.6	1.5	73.8	1.7	57.2	1.5	49.8	1.7
	Orange					62.1	1.5	47.5	1.4
	Green	12.4	1.8	65.1	1.5	62.0	1.3	51.2	1.5

[z]Capsaicin and dihydrocapsaicin content ($mg.g^{-1}$) of dry ground pepper placenta or whole fruit by DMF extraction .
[y]Capsaicin and dihydrocapsaicin content ($mg.g^{-1}$) of dry ground pepper placenta by ether extraction present in solvent A fraction (neutral lipids) of column #1 and the solvent M fraction of column #2.

Table 11. Free fatty acid precursors E and A, and other long chain fatty acids (µg.g^{-1}) among different maturity stages of pepper fruit placenta.

Plant	Maturity stages	Free fatty acids (µg.g^{-1})[z] E	A	E:A	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
1	Red	348.0±38.9(5)[y]	96.6±2.6(5)	3.6	NA[x]	NA	NA	NA	NA
	Orange	561.1±32.0(5)	199.9±2.7(5)	2.8	NA	NA	NA	NA	NA
	Green	760.2±54.2(4)	205.9±12.2(4)	3.7	NA	NA	NA	NA	NA
2	Red	415.4±44.7(3)	107.1±1.7(3)	3.9	NA	NA	NA	NA	NA
	Orange	909.6±27.2(7)	259.0±2.2(7)	3.5	NA	NA	NA	NA	NA
	Green	1055.0±68.7(5)	340.4±25.2(5)	3.1	NA	NA	NA	NA	NA
3	Red	593.0±39.7(4)	149.6±3.0(4)	4.0	632.2±15.1(10)	367.2±5.7(10)	243.3±16.8(5)	269.6±30.6(5)	115.2±6.5(5)
	Orange	1232.9±44.3(7)	519.1±13.2(7)	2.4	760.6±49.5(9)	399.6±8.8(9)	278.8±22.1(5)	636.7±39.5(5)	150.8±11.3(5)
	Green	2275.6±93.0(8)	698.9±21.6(8)	3.3	566.2±9.2(10)	389.1±4.5(10)	88.8±3.7(7)	170.5±10.8(7)	76.6±3.4(8)
4	Red	436.9±31.3(5)	228.6±13.5(5)	1.9	NA	NA	NA	NA	NA
	Orange	573.0±45.6(6)	291.0±15.2(6)	2.0	NA	NA	NA	NA	NA
	Green	767.2±11.8(6)	258.9±5.8(6)	3.0	NA	NA	NA	NA	NA
5	Red	845.2±31.6(7)	391.2±16.7(7)	2.2	780.8±25.8(7)	463.2±6.2(7)	336.2±30.9(6)	301.1±44.9(7)	521.2±40.4(7)
	Orange	720.7±77.7(4)	398.6±26.5(4)	1.8	632.6±19.3(10)	401.4±12.3(10)	153.9±17.4(4)	168.8±3.1(3)	363.1±8.4(3)
	Green	1074.7±106.1(5)	533.6±32.5(5)	2.0	684.8±11.7(10)	490.1±6.3(10)	191.5±24.9(5)	192.8±38.8(4)	215.6±36.8(5)
6	Red	304.3±7.0(5)	143.5±7.5(5)	2.1	541.4±14.6(9)	321.9±2.7(9)	301.2±10.8(5)	366.0±15.5(5)	215.3±10.3(5)
	Orange	427.4±27.6(7)	218.7±9.3(7)	2.0	493.3±8.4(9)	328.9±6.7(9)	147.3±6.1(5)	240.4±22.3(5)	223.4±7.8(5)
	Green	422.5±36.6(9)	231.7±11.6(9)	1.8	445.2±6.8(9)	291.9±4.6(9)	68.2±4.3(5)	55.6±2.3(5)	50.9±3.8(5)

[z]E and A free fatty acid precursor and other long chain free fatty acids (µg.g^{-1}) of dry pepper placental lipid extract, eluted in the solvent B fraction of column #1.
[y]Means followed by standard error with numbers in parenthesis indicates replication in each maturity stages.
[x]High carbon fatty acids were not quantitated.

Table 12. Fatty acid sterol ester (FASE; µg.g^{-1}) accumulation among different maturity stages of pepper fruit placenta.

Plant	Maturity stages	E	A	E:A	FASE (µg.g^{-1})[z] Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
1	Red	414.3±12.9(5)[y]	91.3±2.1(5)	4.5	NA[x]	NA	NA	NA	NA
	Orange	330.2±13.2(5)	69.4±1.7(5)	4.8	NA	NA	NA	NA	NA
	Green	432.2±21.0(3)	110.4±5.3(3)	3.9	NA	NA	NA	NA	NA
2	Red	353.5±9.2(6)	92.8±1.8(6)	3.8	NA	NA	NA	NA	NA
	Orange	251.1±7.7(4)	73.8±3.1(4)	3.4	NA	NA	NA	NA	NA
	Green	456.4±7.7(6)	136.2±1.9(6)	3.4	NA	NA	NA	NA	NA
3	Red	150.9±7.7(5)	50.2±3.8(5)	3.0	143.4±18.9(2)	20.2±0.8(2)	120.6±25.0(2)	326.7±15.0(2)	55.9±15.5(2)
	Orange	98.6±3.5(5)	44.3±1.7(5)	2.2	101.1±8.6(3)	17.9±1.6(3)	63.5±7.5(3)	349.2±16.8(3)	83.9±15.3(3)
	Green	203±5.1(5)	68.3±2.1(5)	3.0	54.7±5.7(3)	8.5±1.8(3)	23.4±4.4(3)	264.1±22.9(3)	80.4±0.9(3)
4	Red	236.8±6.6(5)	94.4±2.9(5)	2.5	134.0±3.7(3)	26.6±1.5(3)	119.1±8.8(3)	363.4±25.0(3)	265.7±3.1(3)
	Orange	272.5±7.3(6)	119.9±4.0(6)	2.3	100.1±18.5(4)	52.4±17.4(4)	57.0±10.0(4)	189.0±17.2(4)	255.9±20.8(4)
	Green	225.2±3.4(4)	99.3±1.3(4)	2.3	32.1±2.6(4)	12.4±0.7(4)	10.4±1.6(4)	108.7±15.3(4)	160.8±13.3(4)
5	Red	508.2±4.3(5)	211.6±2.7(5)	2.4	615.5±78.5(3)	93.9±13.4(3)	858.5±166.1(3)	1862.1±238.6(3)	874.7±84.5(3)
	Orange	468±22.1(6)	235.7±12.2(6)	2.0	1162.7±51.7(5)	216.4±12.6(5)	990.4±44.0(5)	4664.5±237.5(5)	2052.8±129.4(5)
	Green	550.2±24.6(6)	270.7±13.4(6)	2.0	615.5±17.2(2)	163.8±18.9(2)	340.7±41.9(2)	2630.9±166.8(2)	1433.3±82.9(2)
6	Red	243.3±6.3(5)	88.4±1.9(5)	2.8	46.8±20.2(4)	12.7±12.6(4)	16.3±32.6(4)	196.3±55.1(4)	246.7±29.7(4)
	Orange	237.9±9.0(6)	104.4±5.2(6)	2.3	69.3±6.4(2)	18.2±4.0(2)	34.4±2.2(2)	277.8±18.5(2)	274.4±6.7(2)
	Green	348.4±8.2(6)	167.6±3.2(6)	2.1	166.7±0.9(3)	48.2±1.0(3)	176.2±1.0(3)	432.3±16.3(3)	225.1±7.6(3)

[z]Fatty acid sterol esters (FASE) (µg.g^{-1}) of dry pepper placental lipid extract, eluted in the solvent H fraction of column #2.
[y]Means followed by standard error with numbers in parenthesis indicates replication in each maturity stages.
[w]High carbon fatty acid sterol esters were not quantitated.

List of Figures

Figure 5. Structure of major capsaicinoids of pepper; vanillylamine portion derived from phenylpropanoid pathway and common to all capsaicinoids is shaded and the fatty acid components are identified.

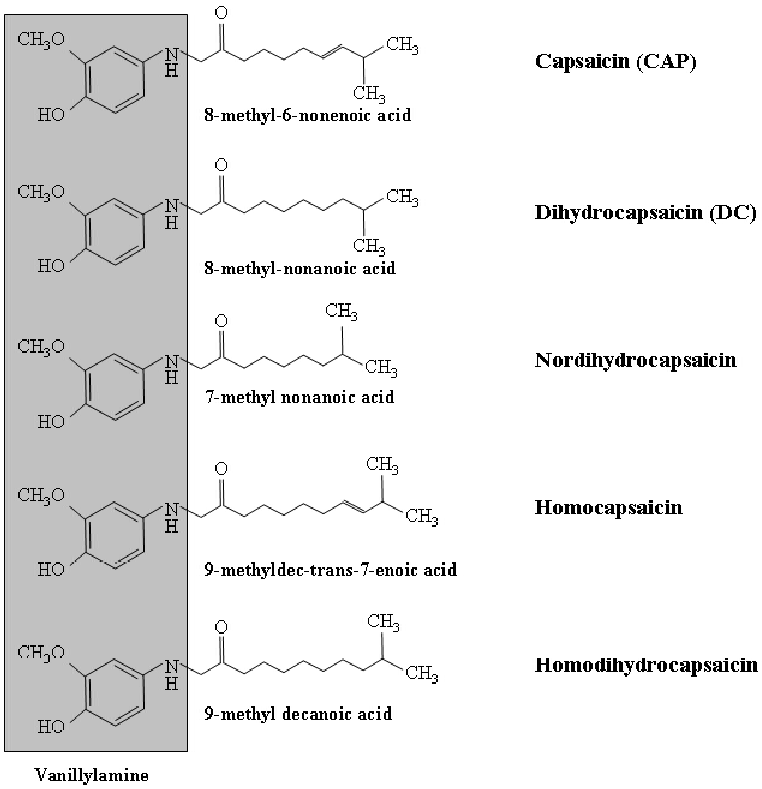


Figure 6. Biosynthetic pathway of capsaicinoids. Vanillylamine derived from phenylalanine through the phenylpropanoid pathway, and E or A fatty acids derived from valine through branched chain fatty acid pathway were condensed by Capsaicinoid synthetase (CS) forming capsaicinoids.

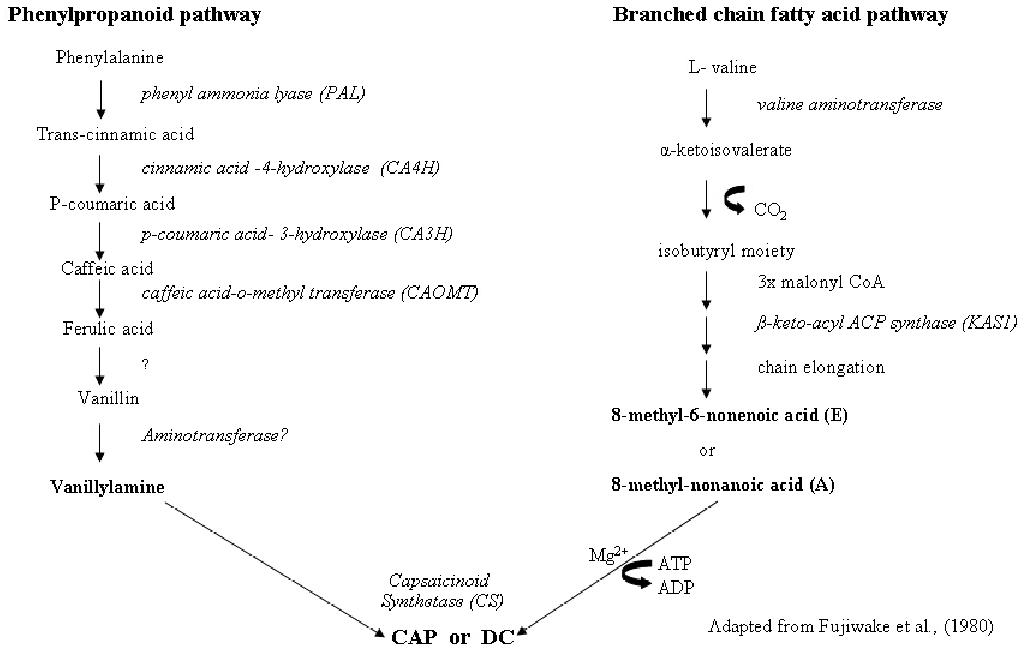


Figure 7. Diagrammatic representation of the procedure for separation of pepper capsaicinoids, FFA and FASE by bonded phase aminopropyl columns using different organic solvent mixtures. The eluates collected were dried and quantified by either HPLC or GC.

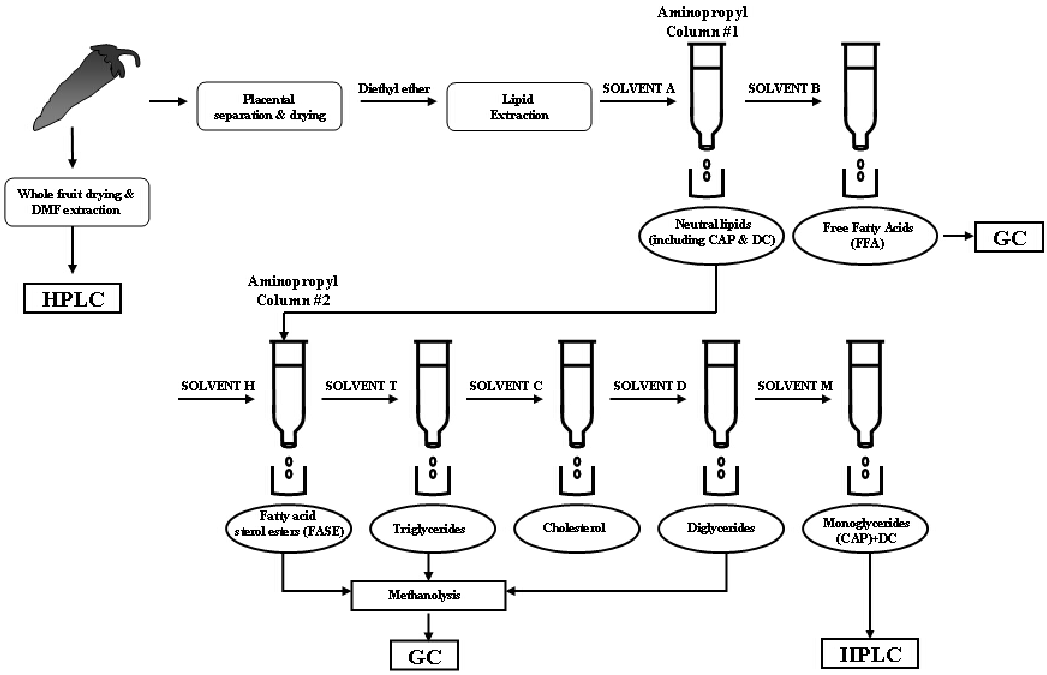


Figure 8. Thin layer chromatography of different lipid fractions of pepper placenta eluted by bonded phase aminopropyl columns. Eluates from different fractions and CoroWise^{TM} plant SE were compared with authentic TLC standards (extreme left).

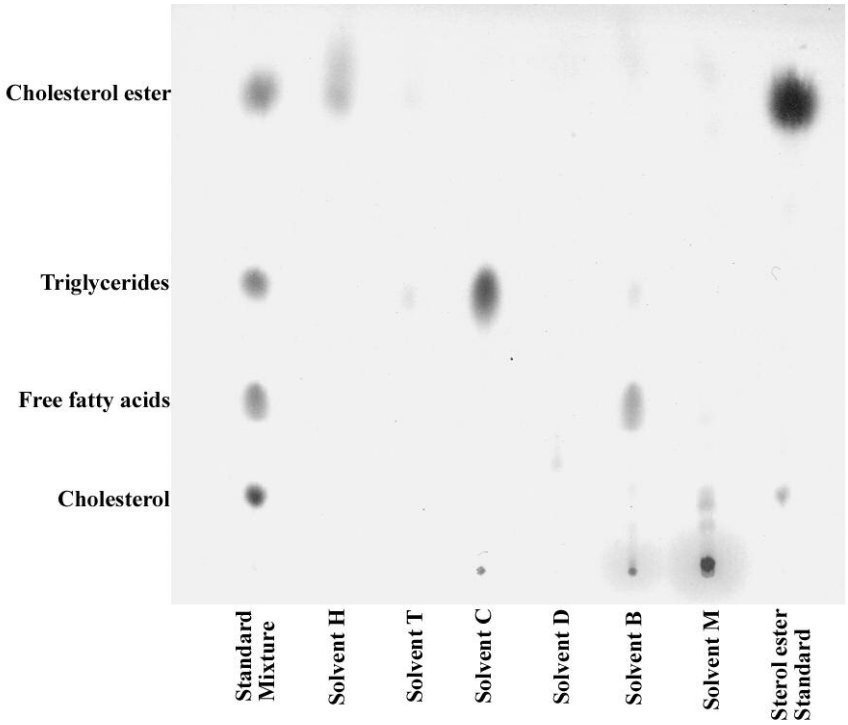


Figure 9. Mass spectrometric positive scan mode of FASE (solvent H) of aminopropyl column #2 from the pepper placental lipid extract showing sterol fragment ions.

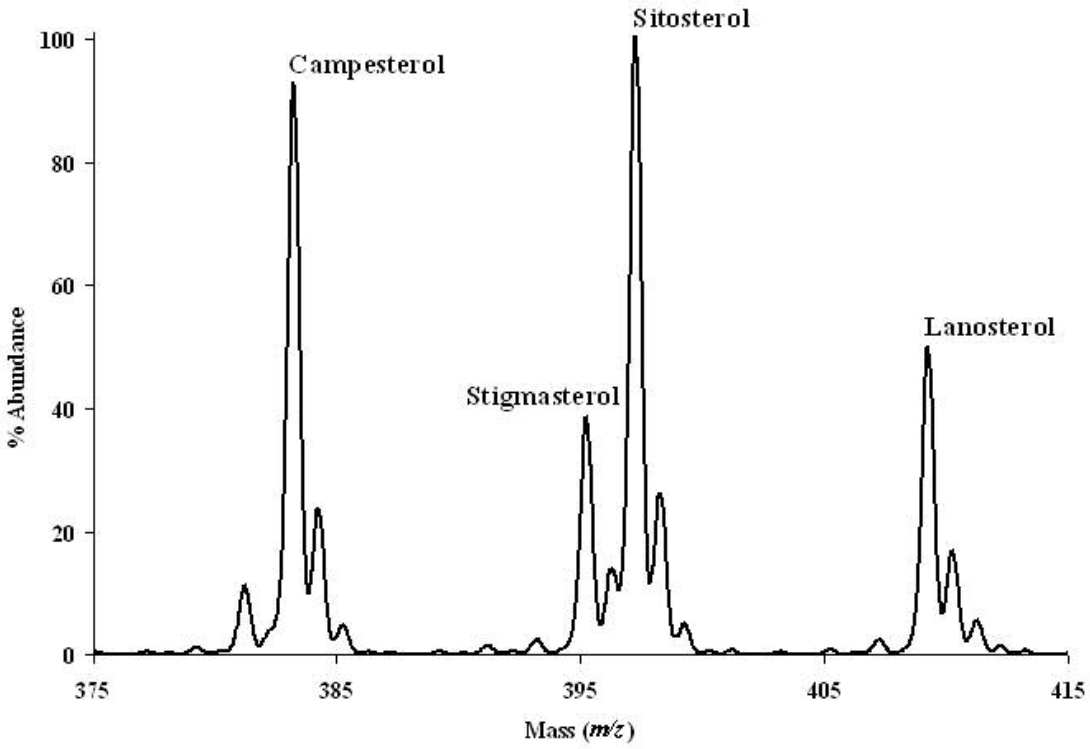
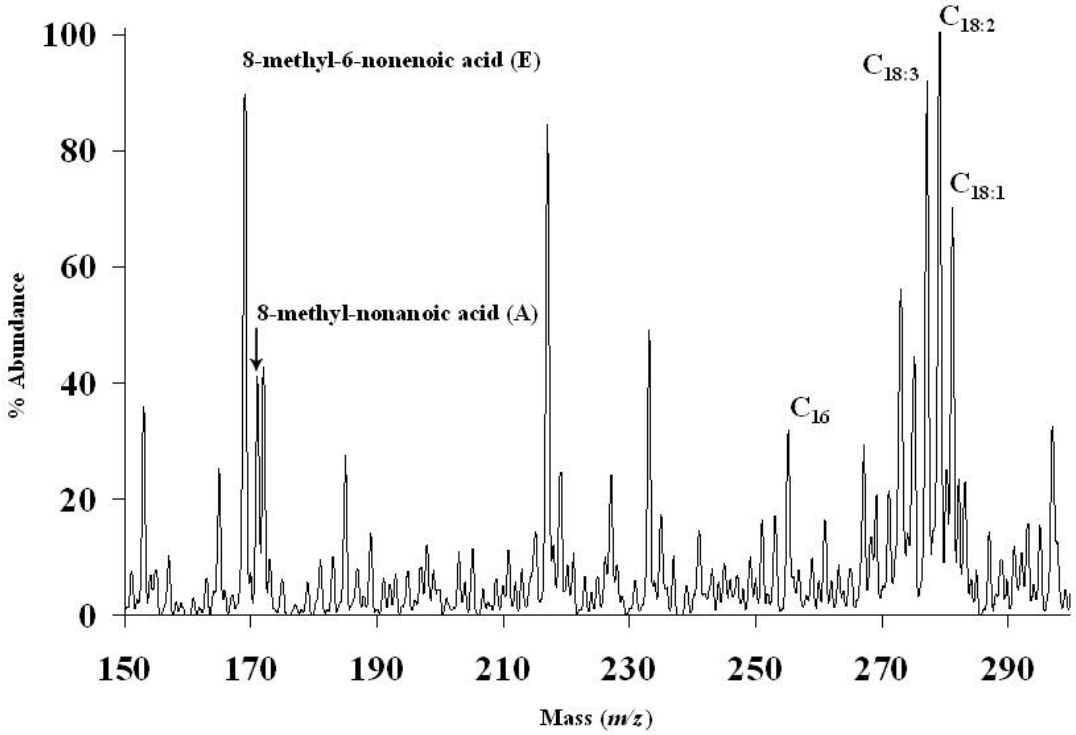


Figure 10. Mass spectrometric negative scan mode of FASE (solvent H) of aminopropyl column #2 from the pepper placental lipid extract showing fatty acid fragment ions.



VITA

Janakiraman Maruthavanan

Candidate for the Degree of

Doctor of Philosophy

Thesis: CAPSAICINOID: STUDIES ON CHEMICAL DEFLOWERING FOR ENHANCING HARVESTABLE CAPSAICINOID PRODUCTION AND MECHANISMS FOR CAPSAICINOID-SPECIFIC METABOLISM IN PEPPER FRUIT

Major Field: Crop Science

Biographical:

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Name: Janakiraman Maruthavanan Date of Degree: May, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CAPSAICINOID: STUDIES ON CHEMICAL DEFLOWERING FOR ENHANCING HARVESTABLE CAPSAICINOID PRODUCTION AN D MECHANISMS FOR CAPSAICINOID-SPECIFIC METABOLISM IN PEPPER FRUIT

Pages in Study: 94 Candidate for the Degree of Doctor of Philosophy

Major Field: Crop Science

Scope and Method of Study: Ammonium thiosulfate (ATS) was applied as a spray to field grown pepper plants at various concentrations [0, 3, 6, 9 and 12 percent (w/v)] as a single application after the first, second or third flowering flushes, or as multiple applications after the first and second or after the first, second and third flowering flush. Pepper yields were assessed after a killing frost, and capsaicinoid content was evaluated by HPLC of N, N-dimethylformamide extracts. Pepper plants from greenhouse with known and varying pungency and capsaicin (CAP) : dihydrocapsaicin (DC) ratios were harvested at mature green, turning and red maturity stages, placental tissue was excised and capsaicinoids and lipid fractions were analyzed. Short branched chain fatty acid precursors for capsaicin (8-methyl-6-nonenoic acid; E) and for dihydrocapsaicin (8-methyl-nonanoic acid; A) were evaluated in bonded phase aminopropyl column eluates, in relation to capsaicinoid content.

Findings and Conclusions: Among 5 trials conducted on 'Plant 19' and 'Okala' at Bixby and Hydro, OK (2002 and 2003) higher concentration of ATS (6%, 9% and 12%) was more effective than control and 3% ATS treatments, in the removal of early set flowers and increasing later set fruit yield and capsaicinoid content for 'Plant 19' Hydro, 2003 ($P \leq 0.05$) and 'Okala' Hydro, 2003 ($P \leq 0.1$). E and A fatty acids were predominantly found in only two lipid fractions; free fatty acids and fatty acid sterol ester (FASE) fractions. E and A free fatty acid was higher at earlier stages of pepper fruit development (higher in green than orange than red fruits). The ratio of E to A free fatty acid precursors was always higher at all stages of fruit development. The FASE fraction was characterized by high performance thin layer chromatography (HPTLC) as a prominent spot coeluting with cholesterol/sterol ester standards and was devoid of free fatty acids. High performance liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS) provided strong evidence that E and A was present in pepper placental extracts as FASE.

ADVISOR'S APPROVAL: Dr. Niels Maness