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VOLATILE ANALYSIS OF WHOLE AND MINIMALLY PROCESSED FRUITS AND VEGETABLES FOR PHYSIOLOGICAL AND SAFETY ANALYSIS

By

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VOLATILE ANALYSIS OF WHOLE AND MINIMALLY PROCESSED FRUITS AND VEGETABLES FOR PHYSIOLOGICAL AND SAFETY ANALYSIS

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Solid phase micro extraction (SPME) sampling and rapid gas chromatography (GC) in combination with time-of-flight mass spectrometry (TOF-MS) was used for volatile analysis of whole strawberry, cut carrot and broccoli florets to assess the physiology and microbiology of these produce. Volatiles of strawberry stored under refrigerated air (2°C) changed as the fruit senesced. Esters were found to be the dominant volatile class. The production of methyl acetate was found to negatively correlate with loss in firmness, suggesting that the production of this volatile may be related to cell wall metabolism of the fruit. For cut carrot, the dominant volatile compounds were the terpenes. Most terpenes detected decreased markedly during the first 3 days of evaluation. Alpha phellandrene was the only terpene to increase as the produce aged. In broccoli, there was not a single group of volatiles which amply predominated during storage. The types of volatiles found included terpenes, aldehydes, fatty acid derivatives, sulfides, ketones and benzoids. For *E. coli* and *S. typhimurium*, 1-propanol may have the potential to be used as a fingerprint compound for *E. coli* in produce packages while heavier alcohols such as dodecanol, octadecanol and nonadecanol appeared to be characteristic for *S. typhimurium.* Today, with the availability of rapid, accurate and more sensitive volatile analysis techniques, it is worthwhile to use volatiles produced by plants and microorganisms as a research asset. Volatile analysis may be a useful tool to assess some physiological events that take place in plants and microorganisms that need yet to be fully understood.

DEDICATION

To Jenny, Laura and Nona: they own my inside.

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FOREWORD

In the United States and across the world, governments are calling for tougher standards of sanitation and hygiene to be applied in the fresh produce industry that previously were found only in processed foods. The Food and Drug Administration (FDA), for instance, is planning on putting food safety guidelines in place before the year 2000 for high risk fruits and vegetables. Federal authorities now have begun to acknowledge that the microbial problems in the produce industry are not like other food groups: they are more varied and complex because of the number of commodities and growing practice and handling. Because of this higher awareness of the nature of the problem of pathogens on produce, authorities are more willing to recommend produce trace back systems, quicker approval of technologies such as irradiation, more research and a comprehensive sanitation and hygiene program for the produce industry.

These concerns in conjunction with a rapidly changing consumer base and exploding global trade present challenges for scientists, government and food industries at the levels of fundamental and applied research. In this dissertation, volatile analysis of whole and minimally processed fruits and vegetables has been used to try to assess the physiological condition of plant productss and tests for human pathogen presence. Volatile emissions by fruits, vegetables, microorganisms and their combination were assessed for their potential for the development of sensing technologies in the food industry.

INTRODUCTION

Quality control for millions of packages fruit and vegetable products which pass through the hands of farmers, shippers, fresh product storage operators, processors, processed product storage operators, retailers and the consumers is limited at best. The 'use by' dates, printed on shipping boxes and packages, although helpful, cannot identify packages with poor quality product. Sensing devices that are able to assess the physiological status of the product and/or the presence of biological hazards at various stages in product delivery are needed and should enhance reliability in product quality and safety.

At present, enzyme-linked immuno-sorbent assays (ELISAs) are available to test for the presence of a number of human pathogens (Samarajeewa et al., 1991). However, ELISA tend to be slow, subject to variable results and are not amenable to automation. For a facility that may be shipping dozens of product lines to dozens of locations per day, sampling under these conditions would not be prudent and would reveal nothing relative to quality (e.g., flavor, tissue integrity, state of senescence). A remote inspection biological sensor is being developed to detect *E. coli* and *Salmonella* in contaminated meat in real time (SatCon Technology Corp., Cambridge, MA). This technique uses a laser spectrographic combination. It has a laser source that is reflected off and interacts with pathogens on a carcass surface, then returns. Because pathogens have a very specific signature, the system can make an identification and give a general indication of the number of organisms present in the packages. However, this technology while promising, has yet to be implemented and its applicability and reliability out of the laboratory is yet to

be tested.

Recent developments in extremely rapid gas chromatography coupled with time-of-flight mass spectrometry (GC-MS), have made feasible real-time assessment of biological materials. The emanation of specific volatiles, which can serve as fingerprint or signature-type compounds for some physiological processes such as senescence or the presence of microorganisms and may offer significant opportunities for quality assessment, are poorly characterized at best.

The field of gas chromatography and mass spectrophotometry will likely continue to advance and become more accessible, which will make possible detection of volatiles with sufficient rapidity to permit the on-line monitoring of volatiles with the intention of relating the changes to quality and safety of produce. Work is under way in the development of sniffers for volatiles or classes of volatiles at the part-per-billion level (Hetzroni et al., 1995). Volatile sensors for industrial process control systems are already in use. In addition, the field of biosensor applications continues to expand. With regard to volatile emanations from plant products and microorganisms, there already exists a biosensor for alcohols (Chematics, Inc., North Webster, IN.). Thus, volatile sensing in the headspace of packaged products has the potential to be performed as an in-package biosensor or via automated, rapid instrumentation. It would be of a great value to develop a sensor to detect the presence of *E. coli* and/or other microorganisms in headspace packaging, using fingerprint signature-type volatiles.

A volatile detection system requires the development of a database relating the physiological state of the product to changes in its volatiles profile. This database does

not exist in any meaningful form for lightly-processed plant products. To a great extent, senescence-related volatiles primarily refer to the biosynthesis of the gaseous hormones ethylene and methyl-jasmonate and the generation of lipoxygenase pathway products, which include aldehydes, alpha- and gamma-keto fatty acids, divinyl ether fatty acids and others. While ethylene is a signal molecule for the induction of ripening and senescence (Abeles et al., 1992) its presence may not be particularly closely tied to tissue degradation. Jasmonates offer more promise, perhaps, being more specifically tied to senescence and a downstream product of lipoxygenase activity (Parthier and Sembdner, 1983; Reinboth et el., 1994). Other lipoxygenase products such as aldehydes, offer much potential in that they are produced in copious amounts and are directly linked to the loss of tissue integrity (Galliard, T., 1978; Hildebrand et al., 1988; Hildebrand, 1989; Thompson, 1988). Further characterization of volatile profiles is needed.

The effect of a polymer barrier on the retention of volatiles such as those noted here is not well understood. Little information is available on the permeability of polymers to organic volatiles. To a large extent, this is a function of the difficulty of making such measurements. The permeability of LDPE to hexanal as well as a number of other plant volatiles has been measured (Deng and Beaudry, unpublished data; Song et al., 1996). Hexanal (a lipoxygenase product) and hexyl acetate (an apple flavor compound) permeability was found to be 50 to 100-fold higher than the permeability to O_2. Despite its high permeability, LDPE serves as a sufficient barrier to apple flavor volatiles to permit their detection in sealed packages using only a glass syringe with a volume of 50 μL. Importantly, polymers of ever increasing selectivity and capacity are being developed that

may be able to preferentially retain classes of compounds such as those related to aroma.

The available information regarding volatiles emitted from plants and microorganisms is sufficient to suggest that one might be able to successfully relate specific volatiles to the physiological and quality status of whole or lightly processed products. The development of detection systems for volatiles appears to be dynamic and their future promising. In addition, the need for additional monitoring by the product industry is increasing. What remains to be done is to quantitatively and qualitatively establish the relationship between package volatiles and specific measures of quality loss and safety.

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CHAPTER I

LITERATURE REVIEW

Modified atmosphere packaging (MAP) effects include extension of shelf-life of produce and enhancement of distribution and storage systems. The use of MAP, however, has implications for changing microbiological activity, visual quality and product taste/flavor. GC-mass spectrometry (GC-MS) and solid phase micro extraction (SPME) sampling technology are analytical techniques available that may allow us to better monitor and evaluate these aspects of MAP. By doing so, we should be able to gain a better undestanding of the physiology, physicochemistry and biochemistry of senescing fruits and vegetables. We may also be able assess produce safety and quality. Important goals in this area include analysis and control of microbial development in produce packages, design of hazard analysis critical control point (HACCP) programs, quality modeling, and development of biosensors .

Senescence Physiology of Fruit and Vegetables

The idea that death of plant tissue or an organism might be actively induced by endogenous factors that are a natural part of organism development has yet to be contradicted. Nooden (1988) stated that senescence is related to certain internal changes that increase the probability of death with increasing chronological age. Senescence could also be defined as the deteriorative processes that are natural causes of death of plants or plant parts (Thimann, 1980). Thomson and Platt-Aloia (1987) suggest that the ultrastructural changes during senescence include first, a degradation of the contents of the protein bodies followed by the fusion of the vacuolated protein bodies into large vacuoles. Associated in time with these transitions, the cisternal endoplasmic reticulum

becomes tubular, resulting in the fragmentation of the tonoplast membrane of the vacuole followed by a complete loss of cellular compartmentation.

Dalling (1987) suggests that the phenomenon of plant senescence, regardless of whether a particular organ is evaluated or the plant as a whole, is invariably associated with the progressive loss of soluble and insoluble protein. Within a cell, the amount of any protein is determined by the balance between synthesis and degradation of that protein. Because of its abundance and metabolic importance, there is more information on ribulose-bisphosphate carboxylase (RuBPCase) than on other proteins. Brady (1988) studied the relationship of senescence and some physiological changes in bean *(Phaseolus vulgaris* L.) and reported that in *P. vulgaris* senescent tissue there is a decrease in chlorophyll content, total protein, and rRNA biosynthesis.

Senescence of higher plants are genetically as well as environmentally regulated processes intimately associated with hormonal interactions. Of the well-recognized plant hormones known to date, ethylene is unique in that it is a simple, gaseous hydrocarbon that is a principal feature of processes that enhance plant senescence (Mattoo and Aharoni, 1988). In addition, cytokinins play an important role in controlling many of the processes that contribute to plant senescence (Nooden, 1980; Nooden and Leopold, 1978; Staden et al., 1988; Thimann, 1980). In fruits, chlorophyll loss and the conversion of chloroplast to chromoplasts is frequently accompanied by a decrease in cytokinin (Staden et al., 1988). Fruit of the nonripening *rin* tomato variety have a high cytokinin content at the full-green stage, and this persists, whereas in the normal ripening fruit, cytokinins decrease as the fruits change color (Davey and Staden, 1978) . Second to ethylene, abscisic acid is the

most well studied of the senescence promoters (Nooden, 1988). Poovaiah (1988) also suggested that calcium distribution plays a critical role in cell function and that a high level of free calcium in the cytoplasm is injurious to the cell. At such a level, calcium reacts with inorganic phosphate to form an insoluble precipitate.

Kuo and Parking (1989) reported that lipid peroxidation plays a role in the developmental process of senescence in fruits and vegetables. The peroxidation of linolenic acid is the only known source of ethane, which is not metabolized further (Lyons and Breidenbach, 1987). Thus, ethane production could be a good index for assessing senescence if we take into consideration other physiological parameters such as changes in firmness, electrolyte leakage, chlorophyll changes, and soluble solids.

Lipoxygenase (LOX)

Lipoxygenase (LOX) (linoleate : oxygen oxidoreductase, EC 1.13.11.12) is a ubiquitous protein that catalyzes the degradation of fatty acids. Because many products of the LOX reaction are aromatic, the presence of lipoxygenase activity in many foodstuffs can affect their sensory properties, particularly during long-term storage, in both desirable and undesirable ways. LOX is a member of a class of nonheme iron-containing dioxigenases that catalyze the addition of molecular oxygen to fatty acids containing a cis, cis-1,4-pentadiene system to give an unsaturated fatty acid hydroperoxide (Siedow, 1991). LOX has been reported to be ubiquitous in plants, which means that failure to measure LOX activity in a given species likely reflects the insensitivity of the detection method used rather than absence of the enzyme activity .

In a resting LOX molecule, the single atom of nonheme iron at the active site exists in a high-spin Fe (II) state. The active form of LOX can then be generated from the resting enzyme by reaction with one equivalent of hydroperoxide product which oxidizes the active site iron to a high-spin Fe (III) state.

Given the diversity of plants in which LOX activity has been reported, it is somewhat surprising that the number of species from which the enzyme has been well characterized is limited. However, where plant LOX has been characterized to some extent, the enzyme consists of a protein whose molecular weight is in the vicinity of 95,000 with a range of 72,000 to 108,000 (Siedow, 1991). That LOX plays a role in senescence is probably the oldest of the currently extant theories about this enzyme's function. The reason for this is obvious: senescence is essentially a degradative process, which includes the loss of membrane integrity. The most prevalent source of potential LOX substrate is found among the polyunsaturated fatty acid side chains present in membrane lipids. In fruit, a pool of free fatty acids released from the membrane by action of hydrolysis of lipids via lipase during fruit senescence (Lester, 1990). Likewise, the products of LOX reaction are themselves relatively reactive and can result in further membrane deterioration and increased permeability. Further evidence of the role of LOX in senescence is the ability of the enzyme and its products to generate free radicals, a factor that seems to be of critical importance in plant senescence.

Several studies have indicated that an increase in LOX activity is a common feature of senescing plant tissues and that treatments believed to delay the onset of senescence, such as the addition of cytokinins or antioxidants, reduce the levels of

exogenous LOX activity relative to untreated controls (Pearry and Prince, 1990). However, the finding of high LOX activities in actively growing plant tissues contrasts markedly with the literature suggesting a role for LOX in senescence (Siedow, 1991). A ripening sequence has been proposed for 'Golden Delicious' which starts with the maturity-controlled degradation of fatty acids by LOX into hexenal, followed by the oxidation of the aldehyde into hexanoic acid and then into smaller carboxylic acids (Feys et al., 1980).

Methods used for the determination of LOX activity include spectrophotometry, oxygen depletion, immunology, and electrophoresis. The spectrophotometric method is by far the most commonly used and is based on measurements of the increase in ultraviolet (UV) light absorption at a wavelength of 234 nm, when LOX acts upon essential fatty acids (Surrey, 1963). The increase in absorption is related to the amount of peroxide formed, which is proportional to time and enzyme concentration. The immunological approach to measure LOX activity is based on the existence of antibodies against the enzyme, providing a very sensitive probe for the detection of the enzyme. Probes for the RNA messanger for LOX upon conserved regions within the nucleotide coding sequence of the LOX gene and make possible the development of a very sensitive assay using Northern blot techniques to ascertain the presence of the LOX transcripts (Siedow, 1991). LOX can also be detected following the course of the reaction between LOX and the unsaturated fatty acid by measuring the uptake of molecular oxygen with a Clarke-type oxygen electrode (Grossman and Zakut, 1979).

Activation of the LOX pathway in plant tissue gives rise to a series of products

derived from fatty acids (Croft et al., 1993). Analysis by GC-MS of volatile products produced through the LOX pathway indicates cis-3-hexenol and trans-hexenal are produced, which arise from the C-13-hydroperoxide of linoleic acid.

Vegetable and Fruit Volatiles

A large number of volatile compounds are common at some level of concentration in most vegetables and fruits. They arise from unsaturated fatty acids, amino acids, and other generally universal components of fruits and vegetables (Buttery, 1971). Some commonly occurring compounds in vegetables and fruits include aliphatic saturated aldehydes, aliphatic monosaturated aldehydes, aliphatic diunsaturated aldehydes, aliphatic saturated alcohols, aliphatic unsaturated alcohols, aromatic hydrocarbons, sulfur containing compounds, esters, terpenes, and chemical of other classes. The main thing that differentiates fruits from vegetables is that fruits have higher concentrations of sugars and fruit volatile oils (Buttery, 1971; Teranishi et al., 1971). Common components of fruit volatile oils are aliphatic esters such as ethyl acetate, ethyl butyrate, ethyl hexanoate, butyl hexanoate, butyl acetate, hexyl acetate and branched esters such as 2-methylbutyl acetate, ethyl-2-methylbutarate and others. In many fruits, beside the background of commonly occurring compounds, some unique compounds are frequently responsible for the main aroma character of the food. Such compounds have sometimes been referred to as character impact compounds (Nurten, 1977).

In both fruits and vegetables, there are some aroma compounds present in the intact product. These compounds apparently mostly produced in the normal metabolism

of the plant by the controlled use of enzymes (Nurten, 1977; Teranishi et al, 1971). Many volatiles on the other hand, are produced only when the raw fruit or vegetable is broken open such as cutting, chewing, or blending. This is an uncontrolled effect, where enzymes in one part of the fruit are mixed with chemical components in another part normally kept separated (Schwimmer and Friedman, 1972). For example in bananas, oranges, pears, carrot, and bell peppers, at least some part of the aroma can be perceived in the intact fruit or vegetable. However, in cucumbers, some melons, and tomatoes the intact material has extremely little aroma (Buttery, 1971).

One of the most universal enzyme-driven occurrences in a wide variety of foods is the enzyme-induced oxidative breakdown of a small percentage of the unsaturated fatty acids in that food. The oxidation of unsaturated fatty acids by enzymes has been investigated (Teranishi et al., 1971). There is evidence that indicates that a number of different enzymes systems can be involved: (1) the lipoxygenase which, forms the initial peroxides; (2) isomerases which can arrange the initially formed peroxides molecule to a different peroxide; and (3) other enzymes, which can chemically change the initially formed peroxide. Each plant has its own set of enzymes, pH, and other conditions that can lead to different volatile products, starting with the same unsaturated fatty acids which are principally linoleic and oleic acids. These fatty acids are usually in the form of triglyceride, phospholipids, or lipoprotein in the plant material. Unsaturated fatty acids are an essential part of every plant cell. Fatty acid degradation products, particularly a homologous series of aliphatic saturated and unsaturated aldehydes, are some of the most common volatiles found in foods. Fatty acid fragments (aldehydes, alcohols, and ketones)

of C_6, C_7, C_8, C_9, and C_{10} chain length are particularly common (Buttery, 1971). C6 compounds (Hex-3-enol, hexanol, hex-2-enal, hexanal, and hex-3-enal always seem to be formed wherever green plant material is crushed. Apparently they are formed through enzymatic oxidation of linoleic and linolenic acids. These compounds are usually described as having "green" odors. C9 aldehydes and alcohols are produced in cucumbers. Nona-E-Z-6, 6-dienal from breakdown of linolenic acid provides most of the characteristic cucumber aroma. C7 compounds such as hept-4-en-2-ol are found in tomato and are probably formed from linoleic acid. The corresponding ketone, hept-4-en-2-one is found in banana. C8 and C10 types of compounds are both the result of breakdown of linoleic acid and are found in citrus and beans. Some other compounds such as sulfur-containing volatile compounds result from the amino acid metabolism (Buttery et al., 1976). Pentina et al. (1995) reported a possible enzymatic pathway for the production of some of the sulfur-containing compounds found in broccoli. They suggested that for a cell-free system of fresh broccoli, sulfur containing compounds are formed from L-methionine and S-methionine. Simon et al. (1980) and Kopas-Lane and Warthensen (1995) reported that volatiles produced by carrot are mostly terpenes.

Hamilton-Kemp et al. (1996) conducted studies on the metabolism of natural volatile compounds by strawberry fruit. As part of these experiments to evaluate naturally-occurring volatile compounds as fumigants to reduce microbial populations, especially pathogenic fungi on strawberry fruit, several test compounds were found to be metabolized by fruit to yield additional volatile compounds. The three principal types of volatile metabolites formed by strawberry fruit as determined by GC and GC-MS analyze

of headspace vapor samples were esters produced from aliphatic alcohols (e.g., 3-hexenyl acetate from 3-hexen-1-ol), alcohols formed by the reduction of aliphatic aldehydes (e.g., 1-hexanol from hexanal), and saturated products from reduction of carbon-carbon double bonds conjugated with an aldehyde or ketone carbonyl moiety (e.g., 2-nonanone from 3-nonen-2-one). The pathways resulting in the above metabolites in strawberry appear to involve three systems (Perez et al., 1993). The incorporation of alcohols into esters in strawberry and other fruit is due to a developmentally regulated enzyme known as alcohol acyl-CoA transferase. This enzyme is necessary for production of several esters that generate characteristic aromas associated with the fruit. The second group of metabolites, alcohols, are produced following reduction of aliphatic aldehydes to their corresponding alcohols is likely accomplished through the action of alcohol dehydrogenase (Bartley and Hindley, 1980; Hamilton-Kemp et al., 1996). This enzyme system also produces ethanol from acetaldehyde when plant tissue is placed under anaerobic conditions. A third system resulting in the reduction of carbon-carbon double bonds adjacent to carbonyl groups is not known; however, the closest analogy seems to be the reduction of unsaturated acyl moieties during the chain elongation steps of fatty acid synthesis (Hamilton-Kemp et al., 1996; Perez et al., 1993; Yamashita, 1976).

Much needs to be learned regarding the biosynthesis of volatile materials produced by plant products. From a horticultural perspective, the impact of environmental factors and cultural processes on aroma and other volatile production are at best poorly understood.

Modified Atmosphere Packaging of Fruits and Vegetables

Modified atmosphere packaging could be defined as the enclosure of food products in gas-barrier materials, in which the gaseous environment has been changed in order to inhibit spoilage processes and therefore either maintain a high quality within a perishable food during its natural life or actually extend the shelf-life (Beaudry et al., 1992; Cameron, 1989, 1994; Church and Parsons, 1994). The rationale for the application of MAP to extend the shelf life of fresh produce is that by reducing O_2 and increasing CO_2 levels, respiration is inhibited, degreening and decay is prevented, and senescence is subsequently postponed (Ben-Arie and Zutkhi, 1992; Kader et al., 1989; Vergano and Pertruit, 1993).

With modified atmosphere packaging, the atmosphere inside a package generally is low in O_2 and high in CO_2, with typical values at steady-state of 2-3% and 5%, respectively (Beaudry et al., 1992; Cameron, 1989; Church and Parsons, 1994; Gorris et al., 1996). The composition of the atmosphere is the product of the initial gas composition, the gas exchange through the packaging material and the respiratory activity of the produce (Beaudry et al., 1992; Brackett, 1989; Cameron, 1989, 1994; Church and Parsons, 1994; Gorris et al., 1996). One method of achieving modified atmosphere packages is by flushing pouches with a desired gas mixture before sealing the package. MAP can also be done by shrinking flexible films tightly around an item, a method know as shrink-wrapping or seal-packaging (Brackett, 1989; Exama et al., 1993).

Cameron et al. (1989) developed a model to determine O_2 consumption as a function of in-package O_2 concentration by mathematical characterization of O_2 depletion

by a tomato fruit in a closed system. They were able to develop a mathematical model to relate film permeability characteristics as a function of fruit weight to achieve desired sealed package concentrations of O_2 in modified atmosphere packages. Ben-Arie and Zutkhi (1992) were able to extend the shelf-life of `Fuyu' persimmon from 6 to 18 weeks at 1°C by modified atmosphere packaging in a low-density polyethylene (LDPE) film by retarding fruit softening and inhibiting development of peel and flesh disorders.

MAP is often used with minimally processed produce. Wiley (1994) defines minimally processed fruits and vegetable as products that contain live tissues or those that have been only slightly modified from the fresh-condition and are like-fresh in character and quality. Minimal processing includes all of the unit operations (washing, sorting, peeling, slicing, and packaging) that might be used prior to blanching in a conventional processing line. Thus, minimally processed tissues have been exposed to substantial injury due to cutting and handling (Rolle and Chism III, 1987). Unlike meat and fish, harvested fruits and vegetables are still living material and, although cut off from their normal nutrient supplies, are capable of a wide range of metabolic reactions, the most important of which is respiration (Ballantyne, 1989). Even so, Wiley (1994) states that these tissues do not exhibit the same physiological responses as normal or raw untreated intact live plant tissues. Minimally processed foods, including cut vegetables and fruits, represent one of the fastest growing segments of the fresh produce industry in the United States and across the world (Stanley, 1997; Wiley, 1994). The international-cut produce association claims that fresh-cut produce is the most promising segment of the market for fresh fruits and vegetables with a projected sale of $19 billion by 1999 relative to $5.8 billion in 1994

(Stanley, 1997).

Modified Atmosphere Packaging and Temperature

Fresh-cuts generally are much more perishable than intact products because they have been subjected to severe physical stress, such as peeling, cutting, slicing, shredding, trimming, and/or coring, and removal of protective epidermal cells. Consequently, fresh-cuts probably should be held at a lower temperature than that recommended for intact commodities (Watada et al., 1996). Despite precautions, modified atmosphere packages are often subject to a wide spectrum of external temperatures during handling, transport and subsequent retailing. Although 0°C generally is the desirable temperature for most fresh-cuts, many are prepared, shipped and stored at 5°C and sometimes as high as 10°C. Storage at this elevated level can hasten deterioration substantially because Q_{10} of biological reactions ranges from 3 to 4 and possibly as high as 7 within this temperature region (Schlimme, 1995). Respiration rates of fresh-cuts increases with temperature, and the degree of increase differs with commodities (Watada, 1996). In the 10 to 20°C storage temperature range, the Q_{10} of most fresh-cuts is greater than in the 0 to 10°C range. An increase in temperature causes a greater increase in the rate of O_2 by harvested produce than in polymer permeability creating the potential for injurious O_2 levels as temperature increases (Beaudry et al, 1992; Cameron and Talasila, 1994; Kader et al., 1989).

Beaudry et al. (1992) meassured the O_2 partial pressure of blueberry modified atmosphere packages of blueberry at which the fruit began to exhibit anaerobic CO_2

production across a range of temperatures. They found that the package lower O_2 limit increased with increasing temperature, which implies that blueberry fruit can be stored at lower O_2 partial pressure when stored at lower temperatures.

Enzymatic Considerations in Modified Atmosphere Packaging

As soon as vegetables and fruits are harvested, physiological changes occur and some of these lead to a loss in quality. Respiratory activity involving the breakdown of carbohydrates by plant enzymes continues and the changes induced, whether advantageous or deleterious, are often markedly influenced by the maturity of the plant when harvested; thus some plants organs can be stored for lengthy periods with little change in quality if harvested at the right time (Hayes, 1992).

Minimally processed fruits and vegetables with wounded tissue require special preservation methods for the purpose of extending storage life and preventing spoilage (Wiley, 1994). The effects of wounding on plant metabolism have been studied extensively in tissues prepared from bulky plant organs such as tubers and roots (Solomos, 1994). The vast literature on this subject has established that slicing or minimal processing induces profound quantitative and qualitative changes in tissues metabolism including a rise in respiration, DNA and RNA synthesis, induction of new enzymes, membrane degradation, and the appearance of novel mRNA (King and Bolin, 1989; Laties, 1978; Price and Floros, 1993; Rolle and Chism, 1987; Solomos, 1994).

Enzymes and substrates are normally located in different cellular compartments and their transfer is actively regulated, thus, processing results in destruction of cells, loss

of compartmentation for cells at the cut surface and injury stress of underlying tissues (Varoquax and Wiley, 1994). Quality loss due to lipid peroxidation and some browning reactions of cut surface and ruptured cells are dependent upon loss of compartmentation. Enzymatic peroxidation of unsaturated fatty acids results the modification of natural aromas. This peroxidation is catalyzed by LOX and leads to the formation of numerous aldehydes and ketones (Hildebrand, 1989). With regard to browning, it is an enzymatic process that occurs in bruised and cut plant tissues (Hildebrand, 1994; Mayer, 1997; Solomos, 1994; Wiley, 1994). The enzymatic reactions involved in the brown discoloration are still under investigation but are known to involve polyphenol oxidase (PPO, tyrosinase, EC 1.14.18.1). The enzymatic activities markedly depend on pH. A pH reduction of 0.5 in the natural pH of apple results in a 50% decrease in chloroplast polyphenol oxidase (PPO) activity (Varoquax, 1994).

Luo and Barbosa-Cánovas (1991) investigated enzymatic browning and its inhibition in new apple cultivar slices using 4-hexylresorcinol in combination with ascorbic acid. The quality of minimally processed apples of the cultivars 'Braeburn', 'Criterion', 'Fuji', 'Gala', and 'Jonagold' was assessed at 2.5, 12 and 20°C, and compared with traditional cultivars 'Golden Delicious' and 'McIntosh'. Among the new cultivars, 'Jonagold' had the slowest browning rate and 'Braeburn' had the fastest rate. Of the two traditional cultivars, 'McIntosh' had the faster browning rate. The temperature sensitivity of the browning varied among cultivars, where Q_{10} ranged from 1.3 to 4.4 and the apparent energy of activation (Ea) ranged from 8.0 to 80.3 kcal/mol. The browning rate dependence on temperature was as follows: Fuji < McIntosh < Criterion < Braeburn and

Gala < Golden Delicious < Jonagold.

Future of Modified Atmosphere Packaging

There remains a great need for research and innovation to determine the most appropriate gas levels for extension of shelf-life and develop methods for consistently obtaining these levels in MA packages (Cameron and Talasila, 1994). Cameron (1994) stated that new developments to expand use of MA are necessary. To that end, a sense-and-respond type of package was deemed to be the most appropriate. In their concept, a sense-and-respond system would perceive environmental conditions and/or chemical cues from the enclosed product, then change some factor such as O_2 permeability, in response to avoid the generation of unwanted conditions in the package atmosphere. An additional case can be made for sense-and-respond packages that percieve the presence of unwanted microorganisms and respond by the generation of an indicator.

Food Microbiology

Microbiology is the study of microscopic organisms and their activities. Within this area is the study of the distribution of the organism, their beneficial and harmful effects, especially in relation to the changes they make in their environment of which food is one particular example (Hayes, 1992; Jay, 1996). Six major groups of microorganisms are generally recognized, namely bacteria, fungi, viruses, algae, protozoa and rickettsias. The bacteria are the most important in relation to food safety but fungi also have a significant role (Fields, 1979; Frazier and Westhoff, 1977; Hayes, 1992; Ramachandra,

1980).

Because food sources are of plant and animal origin, it is important to understand the biological principles related to growth and control of the microbial flora associated with plants and animals in their natural habitats and respective roles. If one considers the types of microorganism associated with plant and animal foods in their natural states, one can then predict the general type of microorganisms to be expected on a particular food product at some later stage in its history (Banwart, 1979; Bryant, 1983; Rose, 1983). As our food are of plant and animal origin, it is worthwhile to consider those characteristics of plant and animal tissues that affect the growth of microorganisms. The plants and animals that serve as food sources have all evolved mechanisms of defense against the invasion and proliferation of microorganisms, and some of these remain in effect in fresh foods (Adams, 1898; Foster; 1989; Frank, 1922; Mossel and Ingran, 1955). By taking these natural phenomena into account, one can make effective use of some or all in preventing or retarding the microbial spoilage of the products that are derived from them. The parameters of plant and animal tissue that are inherent in the tissues are referred to as intrinsic parameters. Properties of the storage environment that affect both the foods and their microorganisms are regarded to as extrinsic parameters (Jay, 1996; Montville, 1987; Mossel and Ingran, 1955; Mountney and Gould, 1988). Intrinsic parameters include product pH, moisture content, oxidation-reduction potential, nutrient content, antimicrobial constituents and biological structures. Extrinsic parameters include temperature of storage, relative humidity of environment, presence and concentration of gases and the presence and activity of other microorganism.

The examination of foods for the presence, types, and numbers of microorganisms and/or their product is basic to food microbiology. In spite of the importance of precise identification and quantification none of the methods in common use permits the determination of the number of microorganisms in a food product (Jay, 1996; Roberts and Skinner, 1983; Sharpe, 1985). Since 1960, many modern and sophisticated physical, chemical, molecular and immunologic microbial detection methods have been developed. Many of these methods can be used to estimate numbers of cells or quantity of cellular by-products (Jay 1996; Montville, 1987; Roberts and Skinner 1983). Most of these detection methods are based on metabolic activity of microorganisms on given substrates measurements of growth response and the study of nucleic acids or combination of these phenomena (Bryant, 1983; Frazier and Westhoff, 1977; Ramachandra, 1980; Rose, 1983).

Food-borne Pathogens

Salmonellosis, staphylococcal food poisoning, and infectious hepatitis are only a few of the illnesses that have been associated with the consumption of contaminated food (Foster, 1989; Jay, 1996; Monk et al., 1995; Troller, 1983). Symptoms of nausea, vomiting, diarrhea, abdominal cramps, and malaise are most common and generally last from one to seven days. However, foodborne illness can also be fatal, particularly in very young children, the elderly, and those with weakened immune systems. Bacteria that cause foodborne illness do not necessarily make the food look, smell, or taste bad. There is no definitive way to tell simply by looking at a food whether or not it is contaminated (Montville, 1987). The best way to control pathogenic bacteria in food is to carefully

adhere to a few simple food handling principles (Hobbs, 1993; Klontz et al., 1995; Montville, 1988; Pan et al., 1997).

The bacteria *Escherichia coli* O157:H7, also known just as EC O157, is a dangerous type of *E. coli*. Appearantly healthy fresh produce or fruit juices carry the bacteria. It can be transferred from animal to animal, animal to plant and plant to animals. EC O157 can survive refrigeration and freezer storage (Hobbs, 1993, Pan et al., 1997). Thorough cooking to 70 °C, which kills EC O157 bacteria, is recommended as a safeguard against infection. Careful kitchen sanitation is also vital (Troller, 1993). Uncooked hamburger and roast beef, raw milk, improperly processed cider, contaminated water and mayonnaise and vegetables grown in cow manure have caused outbreaks. Contaminated cantaloupe, strawberries, and hard, dry sausage (salami) have been contaminated and caused illness upon consumption. Increasingly, cross-contamination at food service outlets, grocery carryouts and salad bars is causing outbreaks (Klontz et al., 1995; Monk et al., 1995; Pan et al., 1997). Transmission of the illness, particularly among children in daycare, is another problem. Since January 1993, there have been nearly 50 outbreaks in the United States . The National Center for Disease Control (CDC) estimates there may be 20,000 illnesses a year (Jay, 1996). It appears that just a few (1 to 12) of these bacteria can make you sick. After an incubation period of 3-8 days, the disease normally lasts 4-10 days. Patients may suffer severe abdominal cramps, diarrhea and sometimes bloody diarrhea. Young children, the elderly and infirm may develop complications (Rajkowski and Marmer, 1995; Ray, 1996). They can develop hemolytic uremic syndrome which can cause kidney failure, brain damage, strokes and seizures (Eley, 1996).

Clostridium botulinum is another important food pathogen that has been involved in fresh produce-related outbreaks (Doyle et al., 1997; Eley, 1996; Jay, 1996; Lilly et al., 1996;). *C. botulinum* is an anaerobic, Gram-positive, spore-forming rod that produces a potent neurotoxin. The spores are heat-resistant and can survive in foods that are incorrectly or minimally processed. Seven categories (A, B, C, D, E, F and G) of botulism are recognized, based on the antigenic specificity of the toxin produced by each strain. Types A, B, E and F cause human botulism (Jay, 1996; Ray, 1996). Types C and D cause most cases of botulism in animals. Animals most commonly affected are wild fowl and poultry, cattle, horses and some species of fish. Although type G has been isolated from soil in Argentina, no outbreaks involving it have been recognized. Four types of botulism are recognized: foodborne, infant, wound, and a form of botulism whose classification is as yet undetermined. Foodborne botulism (as distinct from wound botulism and infant botulism) is a severe type of food poisoning caused by the ingestion of foods containing the potent neurotoxin formed during growth of the organism under anaerobic conditions. The toxin can be destroyed if heated at 80°C for 10 minutes or longer. The incidence of the disease is low, but the disease is of considerable concern because of its high mortality rate if not treated immediately and properly. Most of the 10 to 30 outbreaks that are reported annually in the United States are associated with inadequately processed, home-canned foods, but occasionally commercially produced foods have been involved in outbreaks (Hobbs, 1993; Pan et al., 1997; Troller, 1983). Vegetables and seafood products have been the most frequent vehicles for human botulism. The organism and its spores are widely distributed in nature. They occur in both cultivated and forest soils,

bottom sediments of streams, lakes, and coastal waters, and in the intestinal tracts of fish and mammals, and in the gills and viscera of crabs and other shellfish. Certain foods have been reported as sources of spores in cases of infant botulism and the undetermined category; wound botulism is not related to foods (Eley, 1996; Jay, 1996; Montville, 1987).

The types of foods involved in botulism vary according to food preservation and eating habits in different regions (Eley, 1996). Any food that is conducive to outgrowth and toxin production, that when processed allows spore survival, and is not subsequently heated before consumption can be associated with botulism. Almost any type of food that is not very acidic (pH above 4.6) can support growth and toxin production by C. botulinum. Botulinal toxin has been demonstrated in a considerable variety of foods, such as canned corn, peppers, green beans, soups, beets, asparagus, mushrooms, ripe olives, spinach, tuna fish, chicken and chicken livers and liver pate, and luncheon meats, ham, sausage, stuffed eggplant, lobster, and smoked and salted fish (Lilly et al., 1996; Reddy et al., 1996).

Since botulism is foodborne and results from ingestion of thet toxin of *C. botulinum*, determination of the source of an outbreak is based on detection and identification of toxin in the food involved (Doyle et al 1997; Ray, 1996; Reddy et al., 1996). The most widely accepted method is the injection of extracts of the food into passively immunized mice (mouse neutralization test). The test takes 48 hours. This analysis is followed by culturing all suspect food in an enrichment medium for the detection and isolation of the causative organism. This test takes 7 days (Eley, 1996; Reddy et al., 1996).

Listeria monocytogenes has been associated with such foods as raw milk, supposedly pasteurized fluid milk, cheeses, ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (all types), and raw and smoked fish (Fenlon et al., 1996). Its ability to grow at temperatures as low as 3°C permits multiplication in refrigerated foods. The methods for analysis of food are complex and time consuming. The present FDA method, approved in September, 1990, requires 24 and 48 hours of enrichment, followed by a variety of other tests. Total time to identification is from 5 to 7 days. In the near future specific nonradiolabled DNA probes should allow a simpler and faster confirmation of suspect isolates. When the recombinant DNA technology is fully applied it may even permit 2-3 day positive analysis (Fenlon et al., 1996; Jay, 1996).

Salmonella spp. is one of the most dangerous food borne bacteria. It is a rod-shaped, motile bacterium. Nonmotile exceptions are *S. gallinarum* and *S. pullorum*. Salmonella is nonspore-forming and Gram-negative. There is a widespread occurrence in animals, especially in poultry and swine. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces, fresh produce, raw meats, raw poultry, and raw sea foods, to name only a few (Doyle et al, 1997; Eley, 1996).

Salmonella typhi and the paratyphoid bacteria normally cause septicemic and produce typhoid or typhoid-like fever in humans (Doyle et al., 1997; Jay, 1996). Other forms of *Salmonella* generally produce milder symptoms. Fresh produce, raw meats, poultry, eggs, milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces and

salad dressing, cake mixes, cream-filled desserts and toppings, dried gelatin, peanut butter, cocoa, and chocolate are sources or carrier of the bacteria. Various *Salmonella* species have been isolated from the outside of egg shells (Bean et al., 1990; Doyle, 1990; Farber, 1991). The organism can also exist inside the egg, in the yolk. This and other information strongly suggest vertical transmission, i.e., deposition of the organism in the yolk by an infected layer hen prior to shell deposition. Foods other than eggs have also caused outbreaks of *S. enteritis* disease (Bryant, 1983; Montville, 1987).

Methods for the determination of the presence of *Salmonella sp.* have been developed for many foods having prior history of *Salmonella sp.* contamination. Although conventional culture methods require 5 days for presumptive results, several rapid methods are available which require only 2 days (Fenlon et al., 1996; Jay, 1996).

Spoilage of Fruit and Vegetables

Although spoilage in fruits and vegetables can be induced by autolytic enzymes, it is caused usually by the activities of microorganism (Brackett, 1994; Charambous, 1986; Hayes, 1992). Spoilage microorganisms are an important aspect that has to be considered in minimally processed fruits and vegetables. All food exposed to air at room temperature will spoil unless preserved in some way. Eliminating microbiological causes of spoilage are key targets for the maintaining of the quality of fresh produce (Brackett, 1994; Gorris et al., 1996). There are three types of microorganisms that cause food spoilage: yeasts, molds and bacteria (Jay, 1996; Lund, 1971). Bacteria, yeast, and molds cause 15% of postharvest losses of fresh produce (Harvey, 1978). There are two types of

yeasts that cause spoilage: true yeast and false yeast. True yeasts metabolize sugar in an aerobic environment, producing alcohol and carbon dioxide gas in a process known as fermentation (Pitt and Hocking, 1997). False yeast grows as a dry film on a food surface, such as on pickle brine. False yeast occurs in foods that have a high sugar or high acid environment.

Molds grow in filaments forming a tough mass which is visible as `mold growth'. Molds form spores which, when dry, float through the air to find suitable conditions where they can start the growth cycle again (Jay, 1996 Pitt and Hocking, 1997). Both yeasts and molds can thrive in high acid foods like fruit, tomatoes, jams, jellies and pickles (Splittstoesser, 1973). Both are easily destroyed by heat. Processing high acid foods at a temperature of 100°C (212°F) in a boiling water canner for the appropriate length of time destroys yeasts and molds (Cuppers et al., 1997; Jay 1996).

Bacteria may grow under a wide variety of conditions. There are many types of bacteria that cause spoilage. They can be divided into: spore-forming and nonspore-forming (Bean et al., 1990; Doyle, 1990; Farber et al., 1989; Jay 1996). Bacteria generally prefer low acid foods like vegetables and meat (Bean et al., 1990; Fowler et al., 1976; Garg et al., 1990). In order to destroy bacteria spores in a relatively short period of time, low acid foods must be processed for the appropriate length of time at 116°C (240°F) in a pressure canner (Giese, 1991; Harvey, 1989; Garg, 1990). Minimally processed fresh fruits and vegetables are media in which microorganisms can grow (Addy and Stuard, 1986; Nguyen and Carlin, 1994). Modified atmosphere packaged-produce have been involved in outbreaks because of the consumption of

products contaminated by pathogens. The potential contamination points along the produce chain include unclean irrigation sources, unsanitary conditions for harvesting and field packing, low chloride levels for packinghouse washes, repacking operation sanitation, processing operation controls, and cross-contamination in restaurant kitchens, salad bars or consumers kitchens (Waterfield, 1996). Outbreaks of food-borne illness in produce have grown in recent years, although no one knows for sure just how prevalent the problem is.

The low pH (<4.5) of most fruits means that spoilage is caused mainly by fungi (Jay, 1996; Rose, 1983). On the other hand, the pH range of most vegetables varies between 5.0 and 7.0 and thus spoilage can be caused by either fungi or bacteria although the former are again the most important group. In terms of their spoilage characteristics, fungi are often somewhat arbitrarily divided into two groups: the plant pathogen which infect the plant before harvesting and the saprophytic fungi which attack the commodity after harvesting (Defigueiredo, 1976). An important fungal spoilage organism is *Penicillium sp.* many species of which are able to attack fruit. Perhaps as much as 30% of all fruit decay can be attributed to this genus (Montville, 1987). Many fruit-vegetables such as tomatoes, cucumbers, apple, strawberry and vegetables such as potatoes, tomato and beetroot are also susceptible. Another important disease is *Rhizopus* soft rot which affect a wide range of fruits and vegetables particularly during transit under poor refrigeration (Fields, 1979; Rose, 1983). Harvested strawberries and potatoes are often attacked by *Rhizopus sexualis L.* and spoilage is indicated by soft, mushy areas with greyish mycelium evident in the affected areas (Hayes, 1992). *Botrytis cinerea* Pers. is

another very important fungi particularly in fruits (Snowdon, 1990). Botrytis can attack a wide range of fruits including apples, appricots, stawberries, blueberries, cherries, citrus, melons, nectarines, pears, and many others. The fungal survive as sclerotia in the soil and on plant debries, and under favorable conditions asexual spores are formed. This fungus can cause heavy losses and is particularly important in pears. It is found in all producer countries, for example UK, Italy, Germany, Switzerland, Norway, Australia, South Africa and the USA. Another fungi able to cause spoilage in fresh produce is *Colletotrichum spp*. This fungi causes serious losses of strawberries, tomatoes and avocado (Gupton and Smith, 1987; Maas and Howard, 1985).

Although bacteria are of little importance in the spoilage of fruit some 35% of microbial spoilage losses of fruits and vegetables can be attributed to them (Brackett, 1994; Gorris et al, 1996). The bacteria responsible are chiefly members of the genera *Erwinia* and *Pseudomonas* (Jay, 1996). The most common spoilage form is bacterial soft rot which affect most vegetables and some fruit-vegetables such as tomatoes and cucumbers; soft rot bacteria can also cause pre-harvest infection (Fowler and Foster, 1976; Garg et al., 1990; Lund, 1971). The soft rot bacteria, of which *Erwinia carotovora* L. is the most important, are present on the plant at harvest and they usually gain entry through damaged tissue; even the injury created by cutting lettuce and cabbage from the stem can provide an infection site for bacteria causing soft rots (Dennis, 1987; Garg et al., 1990). Growth of such bacteria is so rapid that molds are unable to compete and are not normally isolated from commodities infected with bacterial soft rot (Geeson, 1979). Pectic enzymes are readily formed by the bacteria producing soft, mushy tissues and

within a few days there is major tissue breakdown (Rose, 1983). In the case of potatoes the whole tuber may collapse, with tomatoes the outer skin may remain intact whilst the entire contents have changed to turbid liquid and with leafy vegetables slimy masses are produced (Bryant, 1983; Hayes, 1992; Montville, 1987; Ramachandra, 1980).

Many microorganisms gain access to or contaminate plant material during harvesting or subsequent handling. Therefore, it is desirable to use equipment that is as clean as possible and to minimize mechanical damage of plant material (Banwart, 1979; Bryant, 1983; Fields, 1979; Jacob, 1989; Mountney, 1988). Many external organisms can be removed from fruits and vegetables by being washed in water although washing can reduce the storage life of vegetables if they are inadequately drained afterward (Defigueiredo and Splittstoesser, 1976; Hayes, 1992). A suitable storage environment is essential so that physiological and microbiological deterioration are minimized. Storage is normally under chilled conditions (5°C) but certain commodities such as potatoes and cucumbers are best stored at 7-10°C. *Pseudomonad*s are usually responsible for bacterial spoilage of vegetables stored at 2°C while *Erwinia* are more common at slightly higher temperatures (Dennis, 1987; Garcia-Villanova et al., 1987; Jacob, 1989).

Changes to existing processes (eg. in source of raw materials) and the development of new processes such as MAP and preservation methods may all create new niches for emerging food spoilage and pathogenic microorganisms (Farber, 1991; Fowler et al., 1976; Garcia-Villanoava et al., 1987; Hintlian et al, 1986). In the last five years, substantial contribution has been made to understand the ways in which the structure of complex foods influences microbial growth as well as the effect of local chemical

environments on the partition of antimicrobial compounds between the aqueous, oil and micellar phases of food.

Mathematical approaches have been used in the development of food models to predict the activity of food microorganisms. These are dynamic models that quantify the physiological state of the cells and information is used for modeling growth of spoilage organisms in food. For example, the relation to survival kinetics has also been assessed for processed food by combining engineering equations for heat flow with microbial models to allow the extent of growth or inactivation to be predicted in three dimensional foods when they are subject to thermal gradients (Geeson, 1979). Advanced mathematical modeling is fundamental to research to study the effect of food structure on microbial growth and further be used to predict both local difficult-to-measure states and more global effects. Rapid methods to assess food spoilage and microorganisms are badly needed.

Microbiology of Modified Atmosphere-Packaged Produce

As processors move away from traditional preservation methods such as heat treatment and brine to packaged fresh produce to meet customers' demands, it is not always possible to rely on the accepted principles concerning microbial growth, survival and death. Consumers have recently become more aware of the importance of fruits and vegetables in maintaining health (Bracket, 1994). Consequently, consumers are purchasing and eating more fresh produce and demanding a greater variety of these products in the marketplace. This has forced food processors to take advantage of

modern technology and transportation to satisfy the consumer's demand (Bracket, 1994; Wiley, 1994). Process innovation requires a complementary understanding of how food-borne organisms react to new processes or storage conditions (Pitt and Hocking, 1997). Key aspects for technological innovation in the fresh produce industry are: increased consumption of convenience foods particularly growth in the cook/chill market; consumer demand for food that have undergone less processing; export potential for fresh foods and increased consumer awareness of safety issues (Hao and Brackett, 1993). In the recent past, conventional wisdom suggested that a refrigeration temperature from 2.2 to 4.4°C would generally control outgrowth of most pathogens including pathogenic psychrotrophic types of microorganism (Wiley, 1994). However, there is considerable evidence that the low temperature or refrigerator temperature treatment is not sufficient to control some psychrotrophic organisms.

Microflora responsible for the spoilage of minimally processed fruits and vegetables include a large number of fungi and bacteria species (Varoquax, 1994). MAP has been known to limit the growth of certain microrganisms (Dennis, 1987). Different gases have been assessed for their impact on the microflora of pre-cut produce (Clark and Takacs, 1980). However, microorganisms differ in their sensitivity to gases normally used in modified atmospheres. Nitrogen is often used in modified atmospheres but is primarily used to displace O_2 and has little other direct effect on microorganisms (Brackett, 1994). On the other hand, CO_2 has both direct and indirect effects on microorganisms. The impact of CO_2 will depend on the organism in question, the concentration of gas, and temperature (Clark and Takacs, 1980). The general effects of

CO_2 are related to displacement of O_2, reduction of pH, and interference of cellular metabolism. When the O_2 concentration becomes low enough, modified atmospheres can select for facultative or obligate anaerobes (Brackett, 1994).

Another issue in MAP system has been the alarm of possible hazards related to the film material itself (Hartman et al., 1990; Manura et al., 1990). There is presently a great deal of concern for the potential health risks due to the consumption of food products contaminated with packaging material residues. When using these packaging materials for food preparation at high temperatures (the boiling point of water) such as those encountered in microwave ovens, the problem may become exacerbated by the thermal leaching of the packaging material residues from the packaging film and eventual diffusion into the food products (Hartman et al., 1990; Manura et al., 1990). This concern for the contamination of food products by leaching of residual components from the packaging materials will continue to be of major concern for both the scientific community and the food industry especially with the increased use of recycled papers and plastics. There are technologies available to detect volatiles emitted by wrapping films which could compromise the safety of the packaged product. Manura (1991) conducted a study to detect and identify low levels of volatiles and semi-volatiles present in commercially available microwave safe plastic food wraps, and to determine the viability of this technique for the analysis of other packaging material analysis. He found that plastic food wraps release detectable material residues, when food are exposed to higher temperatures.

Detection and Identification of Microorganisms in Food

Analytical microbiology is a relatively new discipline that has arisen on the interface between analytical chemistry and microbiology (Fox and Black, 1992). In the 1960's and 70's microbial chemistry established the composition of many major structural components of bacteria (Fox and Black, 1992; Roboz, 1992). These structures include peptidoglycans, liposaccharides and teichoic acids. Among physicochemical techniques, mass spectrometry often offers the unique combination of great speed, high specificity, and excellent sensitivity (Berkeley et al., 1992; Board et al., 1992). These features are helpful for detection and characterization of microorganisms when the public health is threatened by contaminated food (Fenselau, 1992). Lipid biomarkers can be recovered from isolates and environmental samples by single-phase chloroform/methanol extraction, fractionation of the lipids on salicilic acid, and derivitazation prior to analysis by GC-MS (White et al., 1992). Bacterial polysaccharides contain a diverse collection of unique sugar monomers that can serve as markers to identify specific bacterial species or genera (Fox and Black, 1992).

A multitude of species-specific biomolecules are needed by each microbial species to perform the functions to fill, and survive in, its niche in nature (Cole and Enke, 1992). The natural selective forces imposed on different microorganisms have required microbes to adapt and optimize specific sets of biomolecules for survival in specific environment. A few examples of these biomolecules include the lipids needed for membrane function and integrity, the respiratory quinones needed for metabolism, and the polysaccharides needed for protection from the environment (Cole and Enke, 1992; Fenselau, 1992). There are

technologies available, including chromatographic analysis, to identify and quantify a number of these biomolecules. The detection of toxins by direct sensing of the specific binding reaction of the toxin (antigen) with a specific antibody in ELISAs is a very accurate method (Doyle et al., 1997; Hobbs, 1993; Troller, 1983). However, the impossibility of doing real-time evaluation has limited its used at a wider scale (Bryant, 1983; Montville, 1987).

Food Safety and Control Systems

Reports of food-born illness associated with foods in developed countries indicate that contamination of raw products can be a health problem (Jacob, 1989). However, in a worldwide basis, most such illness is caused by foods that have been mishandled or mistreated during preparation (Klontz et al., 1995). Even though control at a single point along the production chain may be effective in combating contamination, there are considerable potential benefits in approaches which eliminate or reduce microbial loads throughout the process (Jay, 1996; Pan et al., 1997). Heavy levels of contamination could be more difficult to completely eradicate, or reduce to acceptable levels, further along the chain. The risk of cross-contamination may also increase if contaminated surfaces, equipment or food processors and handlers, come into contact with treated product (Bean et al., 1990; Garg et al., 1990; Giese, 1991).

While it is frequently not possible to entirely prevent the contamination of foods by pathogenic organisms, the safety of such foods can be assured by systematic action to: 1) minimize the contamination of raw foods whether for direct consumption or for further

processing (this includes animal husbandry and agricultural practices at the primary level), 2) destroy remaining organisms of concern in processed foods through processing operations and 3) properly formulate and use correct practices (refrigeration, freezing, hygiene etc.) in the packaging, storage and distribution of food, and in its preparation for consumption (Banwart, 1979; Giese, 1991).

Control of food-borne pathogens at the production stage can be effected systematically by: identifying the bacteria of concern, identifying the sources of contamination, and taking action to effect control of contamination at source (Giese, 1991; Mountney and Gould, 1988). It also helps to identify appropriate control points and develop processes for destroying the remaining pathogens of concern, preventing subsequent re-contamination of product by the use of proper hygiene and handling practices, and appropriate packaging materials, and/or creating an environment which is unfavorable for the growth of the pathogen(s) of concern (Klontz et al., 1995; Monk et al., 1995; Troller, 1983). Some of these practices include physical controls (such as drying, lowering water activity, or storing the product at temperatures which do not favor bacterial growth), or controlling storage times if appropriate storage temperatures cannot be guaranteed (Rajkowski et al., 1995; Ray, 1996).

Potential problems related to processed and minimally processed products are of concern to the fresh produce industry. Microbial food safety issues to date have probably not been addressed to the extent they should have, as activities have focused on pesticide residue issues (Bean et al., 1990; Garbutt, 1997; Klontz et al., 1995). Presently there are testing programs in place for coliforms, *Salmonella* and yeasts and molds in nuts. While

the issue of cross-contamination during the preparation of ready-to-eat salads is an area of particular concern (Gorris et al., 1996; Hao and Brackett, 1993; Lund, 1971), there are no similar testing programs in place.

Microorganisms, microbial metabolites and viruses of human health concern can enter the food chain on the farm or growing environment, during transportation, processing, as a result of poor hygienic practices, mishandling of food after processing and during food preparation in homes and catering establishments and retail food outlets. While a particular food may be safe at any given point, it can always be subsequently contaminated by other foods, utensils, equipment and by food handlers (Charalambous, 1986; Montville, 1987; Troller, 1983).

In raw foods, improper handling, which favors bacterial growth normally, but not always, causes spoilage organisms to grow to levels where visible signs of spoilage occur and the food is clearly unfit for human consumption (Giese, 1991; Troller, 1983). Pathogens that are present on such foods are unlikely to pose a significant risk, as the foods are normally discarded without consumption. Additionally, cooking destroys most of the pathogens of concern. For this reason, raw foods, when they are cooked and consumed immediately thereafter, usually present a low microbial risk to consumers (Jay, 1996). Processed foods are, however, different from raw foods. Processing, involving high temperatures or bactericidal activity, usually destroys most of the natural spoilage organisms that may be present on a raw food (Doyle, 1990; Garg et al., 1990; Hintlian and Hotchkiss, 1986). If the composition of the food or subsequent storage conditions (e.g. poor temperature control of chilled foods), permit the growth of pathogens which have

survived processing, processed foods can pose a hazard to consumers before they show obvious signs of spoilage.

Foods such as traditional vegetable dishes and stews which are cooked at temperatures which kill any vegetative organisms and are then immediately consumed, are arguably among the safest (Harvey, 1989; Rose, 1983). Even here there is a risk that some heat-tolerant toxins may survive the cooking process. The fresh food industry needs rapid, sensitive, selective and reliable method for the detection of the toxins or bacterial fingerprint compounds to ensure food safety (Doyle et al., 1997; Eley, 1996; Giese, 1991; Roberts and Skinner, 1983; Sharpe, 1985).

Hazard Analysis of Critical Control Points (HACCP)

HACCP is a preventive measure inspection program that assesses hazards, estimates risks, and establishes specific control measures that emphasize prevention rather than reliance on end-point testing. HACCP is a system of science-based process controls designed to identify and prevent physical, chemical and microbial hazards. The system is designed to prevent problems before they occur, and to correct deviations as soon as they are detected (Anderson, 1994; Kalish, 1991; Sarlett, 1991; Sperber, 1991). The HACCP approach is a system of process controls that is widely recognized by scientific authorities and international organizations and is used extensively in the food industry to produce products in compliance with health and safety requirements. HACCP started in the 1960's, when National Aeronautic and Space Administration (NASA) was looking for a way to guarantee that the food for astronauts on space flights was totally safe (Sperber, 1991).

They gave the task of producing "Zero Defect" food to the Pilsbury Corporation who responded by developing the system of Hazard Analysis Critical Control Points or HACCP. From this original model has sprung most of our modern thinking about food safety, whereby we look systematically for potential risks and then identify appropriate control and monitoring systems, concentrating on those deemed critical to the safety of the product. HACCP has today been the subject of an enormous amount of study (Sperber, 1991; Weilgold et al., 1994). It has been incorporated into the World Health Organization/Food and Agriculture Organization standard, the Codex Alimentarius and is now required of all food businesses in Europe under EC Directive (Dean, 1990; Kalish, 1991; Sperber, 1991; Weingold et al., 1994). By using a HACCP system, control is transferred from end product testing (i.e. testing for failure) into the design and manufacturing of foods (i.e., preventing failure). There will, however, always be a need for some end product testing, particularly for on-going verification of the HACCP process. The HACCP system has seven principles:

1. Conduct a hazard analysis. Prepare a list of steps in the process where significant hazards can occur and describe the preventive measures.
2. Identify the critical control points (CCPs) in the process.
3. Establish critical limits for preventive measures associated with each identified CCP.
4. Establish CCP monitoring requirements. Establish procedures for using the results of monitoring to adjust the process and maintain control.
5. Establish corrective actions to be taken when monitoring indicates that there is

adeviation from an established critical limit.

6. Establish effective record-keeping procedures that document the HACCP system.

7. Establish procedures for verification that the HACCP system is working correctly.

HACCP systems have to be accompanied by other preventive measures. Food safety during transportation, storage, retail sale and preparation are the next important links in the farm-to-table chain (Stevenson, 1990). Safe handling beyond the plant becomes the responsibility of distributors, employees in retail stores and restaurants, and consumers.

The FDA has worked very hard to develop standards governing the safety of foods during transportation and storage and to support local and state authorities in their efforts to improve food safety practices at the retail level (Tisler, 1991). As progress is made in reducing contamination that occurs in the farm-to-retail process, increased awareness of proper storage, handling and preparation practices in the home and in foodservice facilities becomes even more critical in maintaining the safety of fresh produce. In addition to their strong support for the government inspection service's transition to a more science-based inspection program, fresh produce industries have invested millions of dollars U.S. to develop HACCP plans and new technologies to reduce the risk of bacterial contamination. For instance, in recent years, millions of dollars U.S. have been invested in research designed to reduce the risk from *E. coli* O157:H7 (Rajkowski, 1995; Tisler, 1991).

Food Quality and Food Quality Assessment

The consumer expects a wide range of competitively priced food products of consistently high quality. As the technology of agriculture and food preparation have advanced and as populations have increased, the analytical problems concerning food have become very complex. Problems faced include, among others, discerning of food products of different quality, frauds, food preservation and development of novel food (Boelkel, 1996; Jay, 1996; Sims and Rodrick, 1995). Moreover, production processes for preservation of nutritious food have become very important because of the need to store food for long periods and to prevent chemical and microbiological deterioration, insect infestation and bacterial contamination. Fierce competition in a saturated market has forced food producers to introduce increasingly more new products to the market. Modern food technology can lead to the production of components in food which possess new significant biological effects and result in the formation of unknown chemical compounds, and microbiological development (Bianco, 1984; Diaz et al., 1997; Charalambous, 1986; Murphy, 1983; Thorner and Manning, 1983; Troller, 1986). One of the most common methods to assess quality of food is by sensory evaluation (SE). SE uses selected consumers and/or trained panelists to assess the characteristics of product under controlled conditions and using appropriate techniques (Cichy, 1983; Heintz and Kader, 1983). SE provides feedback on eating quality, gives an estimation of shelf life, allows analysis of product failure, indicates product development direction, provides a quality assurance check and ingredient substitution (Chambers et al., 1981; Harries and Smith, 1991). The major drawback of sensory analysis is the subjectivity of the results

and high variability. SE is different from other type of analysis that may be more difficult to perform, but could offer greater precision and reproducibility (Gunasekaran and Ding, 1994; Martin, 1971; McIntire, 1983; Resurreccion, 1988; Tarrant, 1980).

Volatile and semi-volatile organic compounds are largely responsible for the flavor qualities of the foods we eat (Kader et al, 1985) . Flavor is always considered one of the most important factors of any of the quality categories when comparing various horticultural products. Fresh produce, particularly fruits provide a great sense of eating pleasure for their characteristic flavor and taste as well as have an important role in a well balanced diet.

Benady et al. (1994) developed an electronic sniffer to measures fruit ripeness rapidly and nondestructively by measuring the aromatic volatiles that are naturally emitted by ripening fruit. In this study, the potential of using a 'sniffer' in the quality sorting of blueberries was evaluated. Blueberries were first visually classified into four distinct ripeness classes: unripe; half-ripe; ripe; and over-ripe and quantitatively measured for color, firmness, TSS, and sugar acid ratio. Ripeness classification accuracy with the sniffer matched or exceeded that of all other ripeness indices. The sniffer differentiated unripe, ripe and over-ripe berries within one second, but could not distinguish between the unripe and half-ripe class. Detection of 1 to 2 damaged or 1 to 2 soft fruit spiked within a large container of 24 to 37 high quality ripe fruit was also achieved, but required a response time of 10 seconds. Electronic sensing of aromatic volatiles may be a useful new technique in the grading and sorting. Simon et al. (1980) reported a study on carrot volatiles collected on porous polymer traps. In this study, an accurate and precise porous

polymer trapping method was adapted for the gas-liquid chromatography analysis of volatiles from raw carrots. It was found that more volatiles are synthesized in the crown of roots than in midsection or tip sections and that terpinolene and caryophyllene levels were higher in the phloem than in the xylem of roots. Fragrance and odor characteristics are critically important to many commercial products and applications (Gray and Robert, 1970; Lindsay, 1984; Kokini et al., 1977). Volatile analysis of fruits and vegetables has been gaining importance as a tool to assess the quality of produce. Consumers rely a great deal on their perception of aroma of the food they buy to assess quality. This is particularly true for produce.

Chromatography as a method of instrumental analysis is capable of producing information which may describe the qualitative and quantitative composition of mixtures of compounds (Schomburg, 1990). The two important parts of the chromatographic system are the column, in which the separation takes place, and the detector. The carrier gas supply unit, the main component of which is a controller for pressures and flows connected to a gas cylinder (for H_2, He, N_2, Ar) or to a liquid source (for N_2 or Ar) generates an optimized gas flow through the column (Schamburg, 1990).

Analytical mass spectrometry (MS) was introduced commercially in 1941. For two decades its main application was quantitative analysis of light hydrocarbon mixtures and similar samples, often with accuracies of plus and minus 1% absolute (McLafferty and Turecek, 1993; Turro, 1980). Gas chromatography (GC) is the most common combination of chromatography interfaced directly to mass spectrometry (Anon., 1995). For all GC/MS combinations, the data consist of a series of mass spectra that are acquired

sequentially. Instrument systems combining GC and MS have proven to be of high value for analysis of complex mixtures, such as those from human biological fluids, plant extracts, pollutants, industrial processes, and samples for forensic study (Anon., 1995; McLafferty and Turecek, 1993). A variety of detectors and column types can be used in gas chromatography to assess the chemistry of food flavor. The combination of gas chromatography/mass spectrometry (GC/MS) analysis is becoming increasingly prevalent due to the advantages it offers in terms of spectral information for peak identification along with high resolution and sensitivity (Hartman et al., 1990; Manura et al., 1990 ; Schomburg, 1995; Yeung et al., 1995). The specific instrument configuration will depend on the requirements of the analysis, and different options can be used even for a particular method, such as that used by the Environmental Protection Agency (EPA) low-level volatiles in drinking water.

GC-MS technology has been utilized for the detection and identification of volatile organic, aromas and flavor components in milk over time and how these differences in volatile organic composition relate to the quality of milk (Careri et al., 1990). This analytical technique can be utilized for the quality control during the production of milk as well as a technique for the detection of potential contamination of milk. GC/MS systems have successfully been used to characterize the aroma and flavor of fruits and vegetables (Song et al., 1997).

Volatile metabolites produced by microorganisms can be measured directly in growth media by membrane inlet mass spectrometry (Lauritsen and Lloyd, 1992; Schomburg, 1990). Membrane inlet mass spectrometry has become a popular technique

for the direct measurement of gases and volatile organic compounds in aqueous solutions. The technique has been used especially for on-line measurements of dissolved gases in bioreactors, kinetic studies of chemical and biological reactions, identification of volatiles produced by microorganisms and environmental monitoring (Lauritsen and Loyd, 1992).

SPME Sampling System

Solid phase extraction (SPE) has become a widely used technique in the analysis of food. Since its introduction to the analytical community in the 1970's (Hurst, 1991), it has gained a wide acceptance. A relatively new SPE technique has been commercialized and is referred to as solid phase micro extraction or SPME. SPME is designed to be used with a gas chromatograph. A 1998 on-line search performed using the Yahoo and Alta Vista web search engines to determine the number of citations on the use of SPME in food analysis since 1991 garnered almost 300 citations.

Sample preparation techniques have been developed using SPME preconcentrate trace compounds and/or separate analytes from sample matrix (Anon 1996; Arthur and Pawliszyn, 1990; Arthur et al., 1990; Arthur et al., 1992; and Zhang et al., 1993). A SPME unit consists of a length of fused silica fiber coated with a polyacrylate or polydimethylsiloxane phase (Anon., 1996). The fiber is attached to a stainless steel plunger in a protective holder. When the SPME fiber is immersed in an aqueous sample, a partitioning of the compounds in the sample between the aqueous phase and the fiber surface occurs (Yang, 1994; Matich et al., 1996). SPME requires no solvents or complicated apparati. It can be used to concentrate volatile and nonvolatile compounds in

both liquid and gaseous samples (Yang and Peppard, 1994).

Yang and Peppard (1994) conducted a study using SPME, headspace and liquid sampling to test 25 common flavor components in dilute aqueous solutions. They found out that the addition of salt generally enhances SPME absorption and that larger sample volume and a smaller volume of headspace over the liquid sample also increase the sensitivity of SPME-based analysis. Matich et al. (1996) investigated SPME for use in the quantification of aroma volatile production by 'Granny Smith' apples during cool storage. Matich et al. (1996) suggests that collection of high molecular weight volatiles by SPME is hindered by the slow transport of analytes into the gaseous phase, which results in long equilibration times and headspace depletion of analytes during sampling, and by adsorptive losses onto walls of containers. Steffen and Pawliszyn (1996) investigated the flavor compounds of orange juice by using headspace SPME. They found that the addition of salt to the matrix significantly enhanced the amount of analytes extracted into the fiber coating and that the partial removal of suspended solids from the juice was found to enable a standard addition quantitation of the target analytes.

Song et al. (1997) examined SPME and time-of-flight mass spectrometry for their suitability and compatibility for rapid sampling, separation and detection of apple flavor volatiles. This study suggests that flavor-contributing volatiles compounds are of relatively high partition coefficient on a 100 μm-thick SPME coating of polydimethylsiloxane (PDMS). The time required to saturate the PDMS coating was dependent on the absorbed material, varying from less than 2 minutes to greater than 30 minutes. Theyn also determined that GC response was linear across several orders of

magnitude. SPME has been used to compare various packaging materials to assess their suitability for storing and shipping analytical materials (Penton, 1997) as well as in the characterization of volatiles in wines (Penton, 1997; Yang and Peppard, 1994). In order to make effective use of SPME, there needs to be an additional concentration step prior to separation by chromatography. Generally, this is accomplished by cooling the GC inlet or a portion of the GC column in a proces known as cryofocusing.

Cryo-cooling Technology

A cryofocusing trap consists of a small chamber cooled with liquid nitrogen through which the volatiles pass. Cryofocusing permits the concentration of analytes in a narrow band in the column due to the decrease of the kinetic energy of the gas exerted by the abrupt decrease in temperature (Hartman et al., 1991; Manura et al., 1990). Concentrating the analyte then rapidly heating improves instrument response, achieving a higher resolution. Cryofocusing units are usually placed inside the GC oven just under the GC injection port. Some cooling devise are directly installed into the GC oven. Alternatively, the GC entire oven is cryofocussed using liquid CO_2.

Thesis Research

We are presenting the results of a research project in which we have used a combination of SPME sampling method and time-of-flight mass spectrometry for volatile analysis of whole strawberry (*Fragaria ananasa* Duch.), minimally processed carrot (*Carota daucus* L.), and broccoli (*Brassica oleracea* L.) to assess the physiology and

microbiology of these products. We have evaluated the changes of volatiles produced by strawberry during storage and studied the relationship of those volatiles with the process of senescence (Chapter II). Chapter III deals with the monitoring of senescence-related volatiles of carrot and broccoli. In Chapter IV we present the results of the project dealing with the identification of fingerprint volatiles produced by *E. coli* and *S. typhimurium* grown in various substrates including fruits and vegetables. We have also investigated the impact of modified atmosphere packaging on aroma production by strawberry (Chapter V) and by carrot and broccoli (Chapter VI).

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CHAPTER II

SENESCENCE-RELATED VOLATILE PROFILES OF STRAWBERRIES (*Fragaria ananassa* Duch.)

SENESCENCE-RELATED VOLATILE PROFILES OF STRAWBERRIES (Fragaria ananassa Duch.)

Additional index words. methyl acetate, aroma, volatiles, ripening, fruit aging

Abstract. Volatiles of strawberry stored under refrigerated air (2°C) changed as fruit senesced. Esters were the dominant volatile class. Of ester types, those with a methyl group on their alcohol portion comprised proportionally 74.64% of total , ethyl esters comprised 11.48%, propyl esters comprised 8.28%, and hexyl esters made up 5.60%. All ester classes decreased during storage, except methyl esters, which increased slightly over time. The production of methyl acetate was found to negatively correlate with loss in firmness, suggesting that the production of this volatile may be related to the cellular breakdown of the fruit. The ratios of methyl acetate production to the product of other methyl esters (methyl butanoate, methyl hexanoate, ethyl butanoate and ethyl hexanoate) increased continuously as the fruit senesced. Methyl acetate production was also found to have a good correlation with extractable lipoxygenase and peroxidase activities suggesting thereby that methyl acetate may be linked to senescence-related enzymatic changes of strawberry during storage. Methyl acetate is a volatile compound that appears to have potential for use in assessing the physiological status of stored strawberry.

Aroma is an essential component of food flavor, particularly in fruit, and constitutes one of the key elements in food quality. The flavor quality of food is a combination of the sensory impressions of taste detected by the tongue and aroma sensed by the nose (Manning, 1993). To assess quality, consumers rely a great deal on their perception of the aroma of the food they buy. Understanding of the biochemistry of naturally-occurring aroma of fruit is of great importance to the food industry for determining optimal harvesting dates, storage conditions and shelf life. The relative amount of particular volatiles and type of volatiles are like "fingerprints" for different fruit species. Very little has been done to evaluate the volatile profile of fruit, including strawberry, directly from the field versus fruit that have been stored for any length of time. Therefore, little is known about changes of volatile profiles as the fruit senesces. Nevertheless, volatiles of strawberry have been thoroughly identified and characterized (Douillard and Guichard, 1990; Pyysalo et al., 1979). The aroma biosynthetic process yet to be fully understood. Esters, alcohols and carbonyls are important for strawberry flavor (Dirinck et al., 1981). It seems unreasonable to expect a different enzyme to catalyze the formation of each compound as this would require a considerable expenditure of energy for protein synthesis (Manning, 1993). At the same time, it seems logical to think that more than one enzyme is responsible for aroma production in strawberry.

New advances in the field of gas chromatography, mass spectrometry and headspace sampling have made possible detection of volatiles with sufficient rapidity to permit real-time monitoring (Arthur et al., 1992; Schomburg, 1990; Song et al., 1997). Work is under way in the development of sensors for volatiles or classes of volatiles at the

part-per-billion level (Hetzroni et al., 1995). Volatile sensors for industrial process control systems are already in use. In addition, the field of biosensor applications continues to expand. With regard to volatiles emanations from plant products, there already exists a biosensor for alcohols (Chematics, Inc., North Webster, Inc.). Thus, volatile sensing in the headspace of packaged products has the potential to be performed as an in-package biosensor or via automated, rapid instrumentation

In this work, we have evaluated the changes of stored-strawberry volatiles over time to identify those volatiles that could potentially be used as senescence indices. Physic and biochemical analyses were performed to assess the physiological status of the fruit and to thereby establish the relationship between the senescence state and the production of particular volatiles. Physicochemical parameters include changes in ascorbic acid, citric acid and pH. Biochemical parameters included changes in the senescence-related enzymes lipoxygenase and peroxidase. We have also examined the pattern of volatile production by strawberry during storage and tried to understand the biochemical nature of volatile production by the fruit while it senesces.

Material and Methods

Plant material. Strawberry (*Fragaria ananasa* Duch. cv. Kent) were obtained as fresh as possible from the Detroit Produce Terminal. Fruit were sorted for visible defects and further sanitized in a solution of 0.5 $g \cdot L^{-1}$ Benlate (Du Pont de Nemours Co., Agricultural Product Department, Wilmington, Del.) and 0.5 $g \cdot L^{-1}$ Rovral (Rhone-poulenc Ag. Company, Research Triangle Park, N.C.) and then rinsed with distilled water

and allowed to air-dry. Approximately 400 g of the fruit were placed in ventilated glass jars. A stream of air was passed through each jar at a flow rate of 10 to 15 $mL \cdot min^{-1}$. Three jars were used for continuous gas measurement (CO_2 and O_2) and volatiles. One jar containing about 20 berries was used for firmness measurements. Fruits used for firmness were taken out every day for measurement. Firminess was achieved by using the FirmTech1 Instrument (BioWork Co., Stillwater, Okla). FirmTech1 Instrument measures firmness by individual compression tests of each fruit, i.e., each fruit is squeezed much in the same manner as a consumer might do with their fingers. As the fruit is squeezed in a step manner, the force increases. The rate at which the force increases ($g \cdot mm^{-1}$) is defined as firmness. Berries in six other jars were used for destructive physicochemical and biochemical analysis. These analysis included soluble solid, pH, ascorbic acid, citric acid and enzymatic evaluations. Four or five berries were taken out every day for this purpose.

Analysis of volatiles. Volatile collection was accomplished using solid-phase micro-extraction (SPME) device (Supelco, Inc., Bellefonte, Pa.). A fiber with a 100 μm-thick, non-bonded, polydimethylsiloxane-based stationary phase was used. The absorption time was 5 min. After absorption, volatiles were then thermally desorbed from the fiber coating by directly inserting the fiber into the injection port (200°C) of a gas chromatograph (HP-6890, Hewlett Packard Co., Wilmington, Del.) for 90 s. Gases were cryofocused on-column using liquid N_2 and separated using a gas chromatograph fitted with a 10-meter, 0.1 mm I.D., DB-5 capillary column (Hewlett Packard Co., Boise, Idaho). The detector was a time-of-flight (TOF) mass spectrometer (Pegasus II, LECO Co., St. Joseph, Mich.). The TOF mass spectrometer permitted the resolution and

quantification of compounds not separated chromatographically. Identification of volatile material was done by comparison of the spectra and GC retention times with authenticated standard compounds. High purity (>95%) authenticated standards obtained from Aldrich (Aldrich, Co., Milwaukee, WI). For the standard compounds, a mix of the targeted compounds was prepared in a 4.85-L jar (standard jar), equipped with a self-sealing rubber sampling port. The mix was prepared by first placing 50 µL of each compound into a 2.5-mL vial to generate a stock mix. Then, 10 µL were taken from the stock mix and placed into the 4.85-L jar to be used as standard. Standard preparation was done at 5 °C and the standard jar was kept at the temperature of the experiment. Calculation of the concentration of standard compounds was expressed in ppm. As sampling from the packages was done, so was from the standard to estimate the actual production of the targeted compounds regardless of the sensitivity of the detection system. Data were reported in $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

Lipoxygenase activity. A modification of the method of Lester (1990) was used as extraction protocol for lipoxygenase. Fresh strawberry (10 g) were frozen by dipping fruit slices into liquid N_2. Fruit were then kept at -80 °C until use. For extraction, samples were grounded using liquid nitrogen in a mortar and by using a pestle. The powder was placed in a beaker containing 40 mL 0.05 M potassium phosphate buffer adjusted to pH 7 containing 61.5 mL 0.5 M monoacid potassium phosphate (K_2HPO4) and 38.5 mL of 0.5 M biacid potassium phosphate (M KH_2PO_4). After shaking and blending for 30 s, the homogenate was filtered through miracloth and further centrifuged (RC5C, Sorvall Instrument Co, Duont, Colo.) at 4000x g for 15 min. The supernatant was used for the

enzyme assay. For the substrate preparation, 0.5 mL Tween 20 was dissolved in 10 mL 0.05 M potassium phosphate (pH 7.0) and 0.5 mL linoleic acid was added, as described by Surrey (1964). The linoleic acid was added drop by drop to facilitate solubility. The contents were throughly mixed so as to dispose the acid into a fine emulsion. Following this, 1.3 mL of 1 N NaOH was added and the mixture was again agitated until a clear solution was obtained. To this solution, 90 mL of the potassium buffer was added and the final volume was brought to 200 mL with distilled water. For the assay cocktail, 150 μL of the enzyme extract was added to 2.85 mL of the linoleic acid substrate solution in a quartz cuvette and mixed. The lipoxygenase activity was measured from the change in absorbance at 234 nm over time using a spectrophotometer (Model 300 Hitachi Instruments, Inc., Naperville, Ill.). The assay temperature was 25°C. One unit of enzyme activity was defined as the amount of protein that produced a change of 1 unit in absorbance. Enzyme activity was expressed in $\mu mol^{-1} \bullet min \bullet g^{-1}$ in a fresh weight basis.

Peroxidase activity. A combination of the Prestamos and Manzano (1993) and Rodriguez-Saona et al., (1995) methods was used for the peroxidases (s) enzyme extraction from strawberry. Fresh strawberry (10 g) were frozen by dipping fruit slices into liquid N_2. Frozen tissue was then kept at -80 °C until use. For extraction, samples were grounded using liquid nitrogen, mortar and a pestle. Ten grams of strawberry powder was placed into a beaker containing 40 mL 0.1 M phosphate buffer at pH 7.8. The mixture was blended by using a Waring blender for 30 s and the homogenate was filtered through miracloth and further centrifuged (RC5C, Sorvall Instrument, Duont Co.) at 17000x g for 30 min at a temperature of 4 °C. The supernatant was used as enzyme

extract. For the peroxidases substrate preparation, a modification of Kar and Mishra (1975) method was used. Catechol was used as substrate. 3.3 g of 99% catechol was dissolved into 40 mL of distilled water to yield a 0.3 M catechol solution. One mL of 30% H_2O_2 was diluted into 30 mL of distilled water to create a 0.1 M solution of H_2O_2 which served as the electron donor. The assay cocktail for the spectrophotometric determination of peroxidase activity consisted of 2 mL of 0.07 M phosphate buffer (pH 5.9), 0.4 mL of catechol solution, 0.2 mL of the 1% H_2O_2 solution and 0.2 mL of freshly prepared strawberry extract. The reference cuvette consisted of 2.5 mL of the buffer. Measurements were made at 420 nm and activity was calculated based on the increase in absorbance during the first 30 s of the reaction. A unit of peroxidase activity is the increase of absorbance of 0.001 per min. Results were expressed in $\mu mol \bullet .min^{-1} \bullet g^{-1}$ in a fresh weight basis calculated by considering an extinction coefficient of 25.5 $cm^2 \bullet \mu mol^{-1}$ at wavelength of 420 nm and at a temperature of 25 °C.

Physicochemical Analysis. For pH measurements, 50 g mesocarp tissues from strawberry fruit was homogenized in a blender for 2 min. The pH of the resulting puree was measured with a pH meter (Accumet, Fisher Scientific Co., Liberty Lane, NH). For the determination of citric acid, a modification of the Lee and Lee (1992) method was used. Strawberries were blended for 2 min and strained through 4 layers of cheesecloth. Ten mL of juice was diluted with 90 mL of distilled water and titrated with 0.1 N NaOH to pH 7. Citric acid was expressed in % on w/w basis. Ascorbic acid was determined by the method described by Ruck (1963). A 30 g portion of homogenised sample was blended with about 100 mL of 0.5% oxalic acid for 2 min in a Waring blender. The

blended misture was made to 500 mL with 0.4% oxalic acid and was filtered; 20 mL of the filtrate were titrated with standard 2,6-dichorophenol inophenol. Results were expressed as mg per 100 g fresh weight.

Results

Strawberry volatile profile. Eighteen volatiles of strawberry stored under refrigerated air (2 °C) could be detected (Fig. 1). Some strawberry volatiles increased over time while others decreased (Table 1, Fig. 2). Volatiles that steadily increased as the fruit senesced included methyl acetate, hexyl acetate, and 3-buten-2-one. Volatiles that decreased included methyl butanoate, methyl pentanoate, methyl hexanoate, methyl octanoate, ethyl butanoate, hexyl hexanoate and 2,5-dimethyl-4-hydroxy-3(2H) furanone. Esters were the dominant volatile class. Of ester types, those with a methyl group on their alcohol portion comprised proportionally 74.64%, ethyl esters 11.48%, propyl esters 8.28%, and hexyl esters 5.60% when averaged over the duration of the study. The total amount of esters declined as the fruit senesced (Fig. 2A). The proportion of methyl esters slightly increased over time relative to the other classes of esters (Fig. 2B). However, the only methyl ester that increased as the fruit senesced was methyl acetate (Fig. 3). Both methyl butanoate and methyl hexanoate decreased while methyl propanoate and methyl octanoate were unchanged throughout storage. The ratios of methyl acetate to methyl butanoate, methyl hexanoate, ethyl hexanoate and ethyl butanoate were evaluated. The four ratios showed a continuous increasing trend during storage (Fig. 4). The ratios of methyl acetate over most of the other strawberry esters showed an increasing trend

throughout storage (data not shown).

Methyl acetate production as related to changes in firmness and some chemical changes. The production of methyl acetate by strawberry during storage correlated (r^2 =0.86) with the rate of softening of the fruit (Fig. 5). Methyl acetate production was negatively correlated with citric acid (r^2 = 0.8), ascorbic acid (r^2 = 0.96) and positively correlated to soluble solid (r^2 = 0.8) and pH (r^2 = 0.92) (Fig. 6).

Volatiles and Senescence-related enzymes. Extractable lipoxygenase and peroxidase activity increased as the fruit senesced (Fig. 7). Methyl acetate correlated with both lipoxygenase (0.9) and peroxidases activities (0.9).

Discussion

The increase of some aroma compounds of strawberry while others decrease as the fruit senesces gives way to several thoughts. First, the data indicate that there is not a single enzymatic system or a single metabolic pathway responsible for the aroma production in strawberry as suggested by Hamilton-Kemp (1996). Second, the data also suggest that the activities of the enzymes that catalyzes the biosynthesis of volatiles in strawberry are not uniformly affected by the senescence process, resulting in the suppression of some volatiles and in the enhancement of others. This finding is consistent with results published by Yamashita et al. (1977). Third, the data may be taken to mean that the degradation products of some volatiles are necessary for the formation of other volatiles as reported by Yamashita et al. (1976) and as shown in our results when evaluating the ratios of methyl acetate to methyl butanoate, methyl hexanoate, ethyl

hexanoate and ethyl butanoate. As some substrates such as butanoate become limiting, the fruit necessarely favors the use of others substrates such as acetyl-CoA. Fourth, perhaps, the genes responsible of expressing the enzyme (s) which catalyze the biosynthesis of aroma in strawberry, are differentially activated by the phenomenon of senescence, resulting in the increase of the expression of particular enzymes and a decrease in the expression of others.

The increase in lipoxygenase may result in a higher peroxidative modification of polyunsaturated fatty acid and ultimately in the formation of various secondary lipid oxidation products. The principal substrates for LOX in higher plants are linoleic (18 carbons long and two double bonds with one of the double bond being 6 carbons in from the last methyl) or omega (*w*) end C18:2, *w*6 and alpha linolenic (C18:3, *w*3) (Hildebrand, 1989; Schewe, et al., 1986). The initial event in the lipoxygenase pathway is thought to be the release of free fatty acids from glycerolipids such as phospholipids. Lipoxygenase has been implicated as playing a role in senescence (Hildebrand, 1989). Lipoxygenase and its hydroperoxide products might directly participate in senescence by inactivating protein synthesis, inhibition of photochemical activity in chloroplast and deterioration of cellular membranes. Oxidation of polyunsaturated fatty acids in the membranes could lead to increased membrane permeability which could then result in increase Ca^{2+} levels in cells, stimulation of phospholipase and release of free fatty acids. The free fatty acids might then be peroxidized by lipoxygenase, accelerating the cycle and increasing the deterioration of membranes and metabolic function.

Peroxidase activity also increased during storage of strawberry. An increase in

peroxidase activity suggests a possible role of this enzyme in degrading unsaturated fatty acids and activating cell membrane and cell wall breakdown, yielding volatiles such as methyl acetate thus linking them directly to quality deterioration of strawberry. Some of the direct products of peroxidase activity in fruits include the production of *cis*-3-hexenol, *trans*-2-hexenal, and other aldehydes and alcohols typical of some fruits and vegetables. One ester of unsaturated aldehydes was detected in the volatile profile of senescing strawberry. Peroxidase catalyzed reactions may be envisioned as an integral aspect of the biochemistry of aging cells since sub-cellular organelles undergo degeneration with subsequent loss of compartmentation of enzymes and substrates (Dilley, 1970). Peroxidase is considered to be a membrane bound or soluble enzyme.

Source of methyl acetate in senescent strawberry. Methyl acetate is formed by the condensation of methanol and acetyl CoA in a reaction castalyzed by alcohol acyl-CoA transferase (AAT). Methanol has been reported to be produced from the degradation of pectins (Siragusa et al., 1988). Pectins are a group of heterogenous polysaccharides with a high molecular weight. They can be found in the intercellular regions and cell walls of most fruits and vegetables (Talmadge et al., 1973; Siragusa et al., 1988; Ahmed et al., 1980). Pectins are composed of D-anhydrogalacturonic acid units linked through alpha (1-> 4) glycocidic bonds, forming a polygalacturonic acid with some of the carboxyl groups esterified with MeOH (Siragusa, 1988). When pectins are degraded, the methoxyl groups are released as MeOH. MeOH has been reported as the major volatile produced during seed growth and maturation in soybean (Obendorf et al., 1990) and peanut (Pattee et al., 1970). Obendorf et al. (1990) reported that pectin methyl esters are the most likely

source of methanol released in plant tissue. As a result, methanol has been used to quantify pectin ester content and pectin methylesterase activity (Wood and Siddiqui,1971). The source of the acetate moiety of methyl acetate may be membrane lipids. Following cellular breakdown as a result of the programmed physiological event of senescence, both unsaturated and saturated free fatty acids are released by cellular membranes as reported by previous researchers (Hildebrand, 1989; Vick and Zimmerman, 1976; Zimmerman, 1976; Brader, 1980). In order for most free fatty acids to be oxidized, they must be primed or activated for reaction in an ATP-dependent acylation reaction to form fatty acyl-CoA (Voet and Voet, 1990). The products of this reaction are are CoA derivatives of fatty acids including acetyl-CoA. Acetyl-CoA, in the presence of methanol and acetyl-CoA transferase would form methyl acetate. Nurten (1970) reported that co-enzyme A is involved in the formation of esters and that therefore, it is associated with the fundamental metabolism of the cell.

Given the high correlation between methyl acetate and an increase in softening of strawberry during storage and considenring that methanol has been linked to cell wall degradation, it may indicate that the cell wall degradation, membrane breakdown and the resulting fatty acid release activate the mechanism of methyl acetate formation. It can also be suggested that methyl acetate is the result of the esterification of MeOH and acetic acid (Brader, 1980; Hildebrand, 1989; Nurten , 1970; Vick and Zimmerman, 1976).

In conclusion, our results indicate that, in strawberry, ester volatiles as a whole decrease as the fruit senesces and thus a decrease of esters may be used as a general indicator of the physiological age of the fruit during storage. Among esters, methyl

acetate seems to have unique-type relationships with other physiological parameters, which may link this volatile with the senescence process of strawberry. The increase of methyl acetate produced by strawberry during storage and the positive correlation of methyl acetate with softening and changes of activity of the senescence-related enzymes lipoxygenase and peroxidase and may give indication of a casual link between methyl acetate production and senescence. Therefore, methyl acetate has the potential to be used as a fingerprint-type volatile to assess senescence in strawberry.

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Table 1. Selected volatiles produced by strawberry over time. Esters are classified according to the type of substrates that lead to their formation. Amount of the compound is given in pmol/kg.s, fresh weight basis

Volatiles									
Alcohol Portion	Acid Portion	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Methanol	acetate	19	20	22	24	28	30	31	33	34
	propanoate	12	12	11	11	9	10	11	11	11
	butanoate	67	65	24	29	28	27	34	17	16
	hexanoate	36	34	24	22	20	17	18	15	13
	octanoate	1	.9	.9	.6	.5	.5	.4	.2	.2
Ethanol	butanoate	29	26	15	14	7	4	4	4	4
	hexanoate	4	3	8	6	4	4	4	1	1.5
Propanol	acetate	4	8	6	7	7	6	8	7	7
	butanoate	6	6	3	3	3	3	2	2	3
Hexanol	acetate	.9	.9	.9	1	1.1	1.1	1.2	1.2	1.4
	hexanoate	31	17	9	4	1.1	1.1	1	.9	.9
Other Volatiles										
3-buten-2-one		11	16	10	11	20	20	20	34	31
1-butanal 3-methyl Acetate		7	8	5	4	3	3	1.9	2.1	2.5
3-hexen-1-ol, acetate		36	29	6	6	3	4	3	7	6
1-pentanol, 2-methyl, 4-methyl		8	9	6	4	3	4	2	2	2
nonanal		1	2	1	1	.9	2	1	2	2
2,5-dimethyl-4-hydroxy-3(2H) furanone		6	2	3	1	1	1	1	1.1	1.8

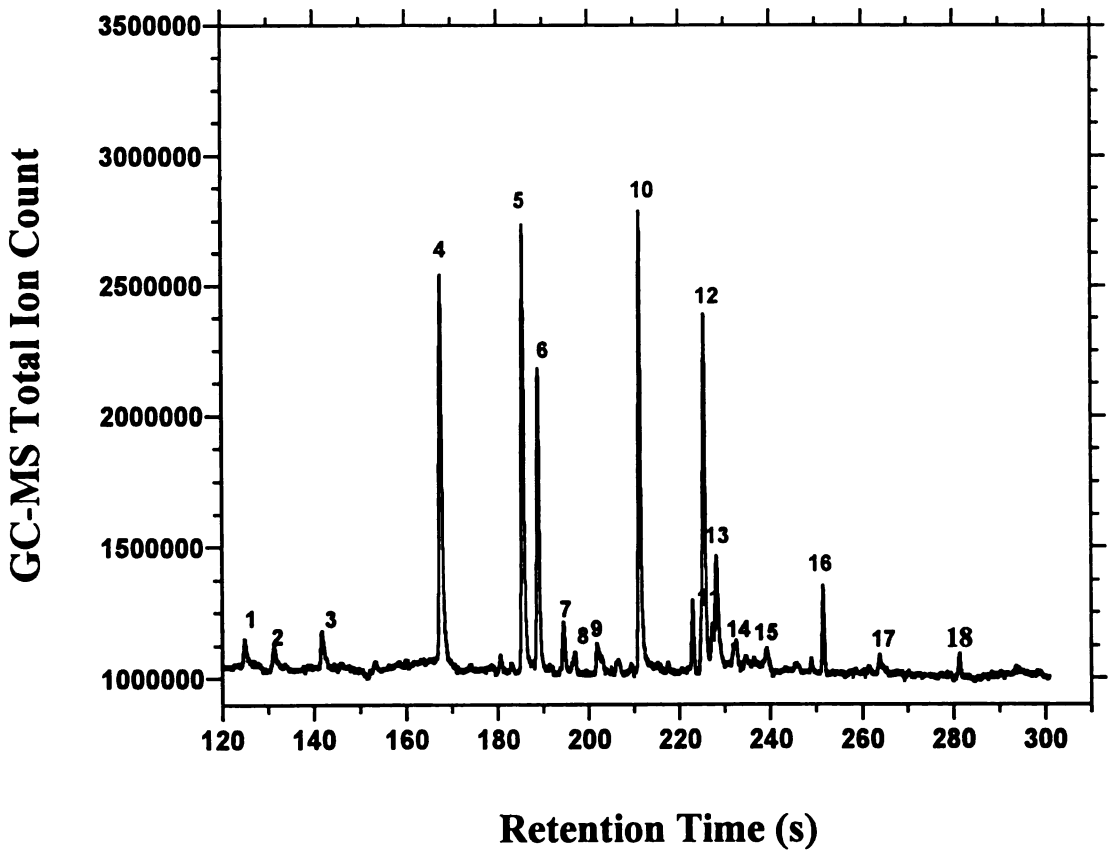

Figure 1. Strawberry volatiles produced on day 1 of study in a flow-through system: (1) carbon dioxide, (2) methyl acetate, (3) methyl propanoate, (4) methyl butanoate, (5) propyl acetate, (6) ethyl butanoate, (7) 1-pentanol, 2,3-dimethyl, (8) 3-methyl butyl acetate, (9) methyl hexanoate, (10) propyl butanoate, (11) hexyl acetate, (12) 3-hexen-1-ol, acetate, (13) ethy,2-methyl, 2-heptene, (14) 2,5-dimethyl-4-hydroxy-3(2) Furanone, (15) ethyl hexanoate, (16) methyl octanoate, (17) hexyl hexanoate, (18) octyl butanoate. Qauntitation of identified peaks is presented in Table 1.

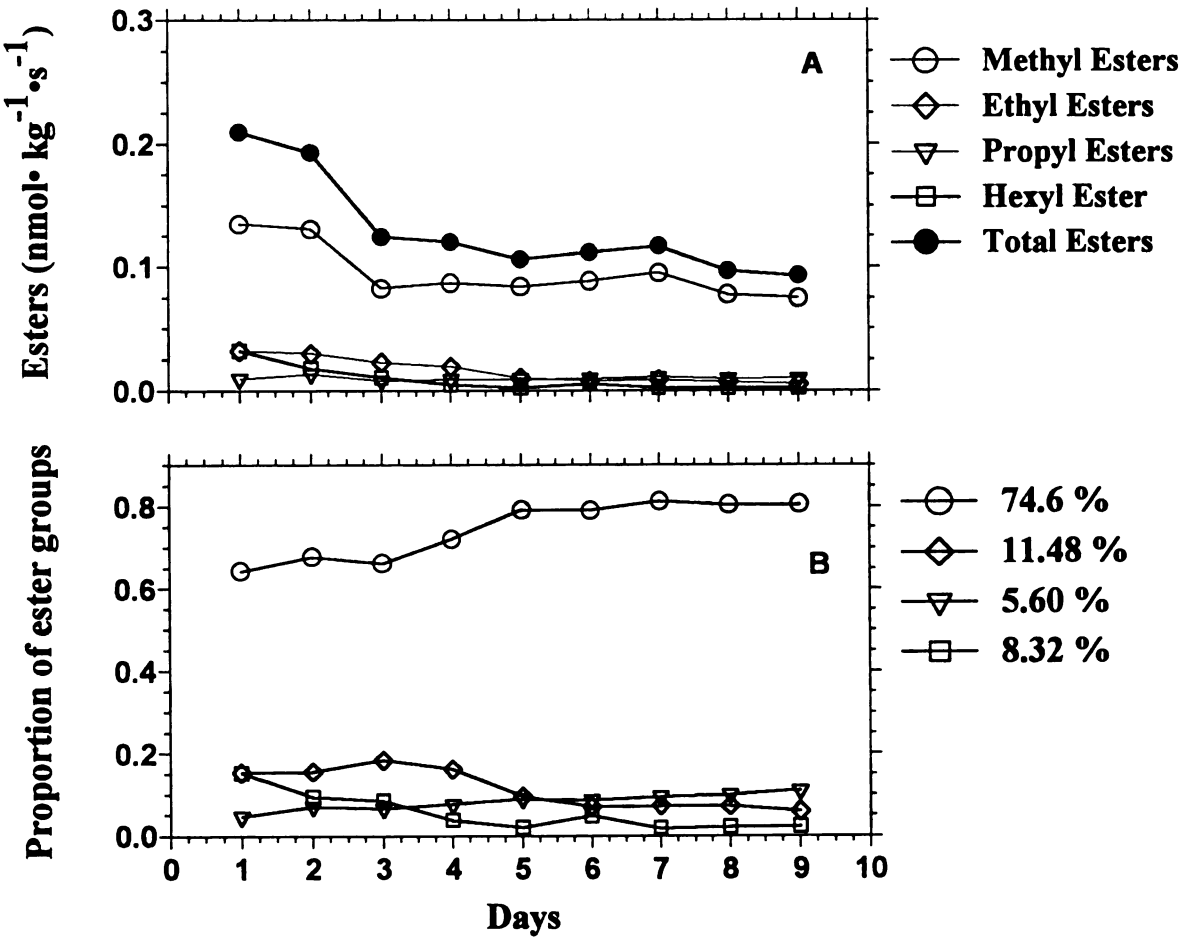

Figure 2. Ester production by strawberry during storage classified by group (A) and the proportion of each class of esters (B). Solid line represents the changes of total esters during storage time. In B, open circles represent methyl esters, squares are hexyl esters, diamonds are ethyl esters, and triangles are propyl esters.

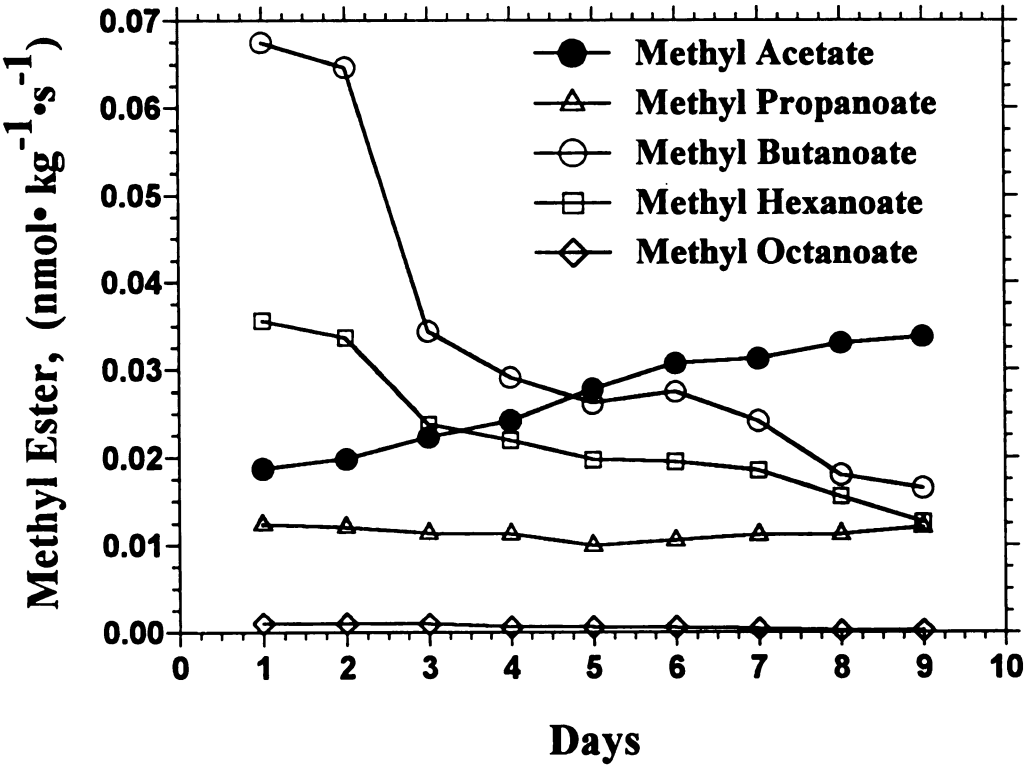

Figure 3. Changes of methyl esters produced by strawberry during storage. Closed circles represent methyl acetate, open circles methyl butanoate, squares, methyl hexanoate, and diamond methyl octanoate.

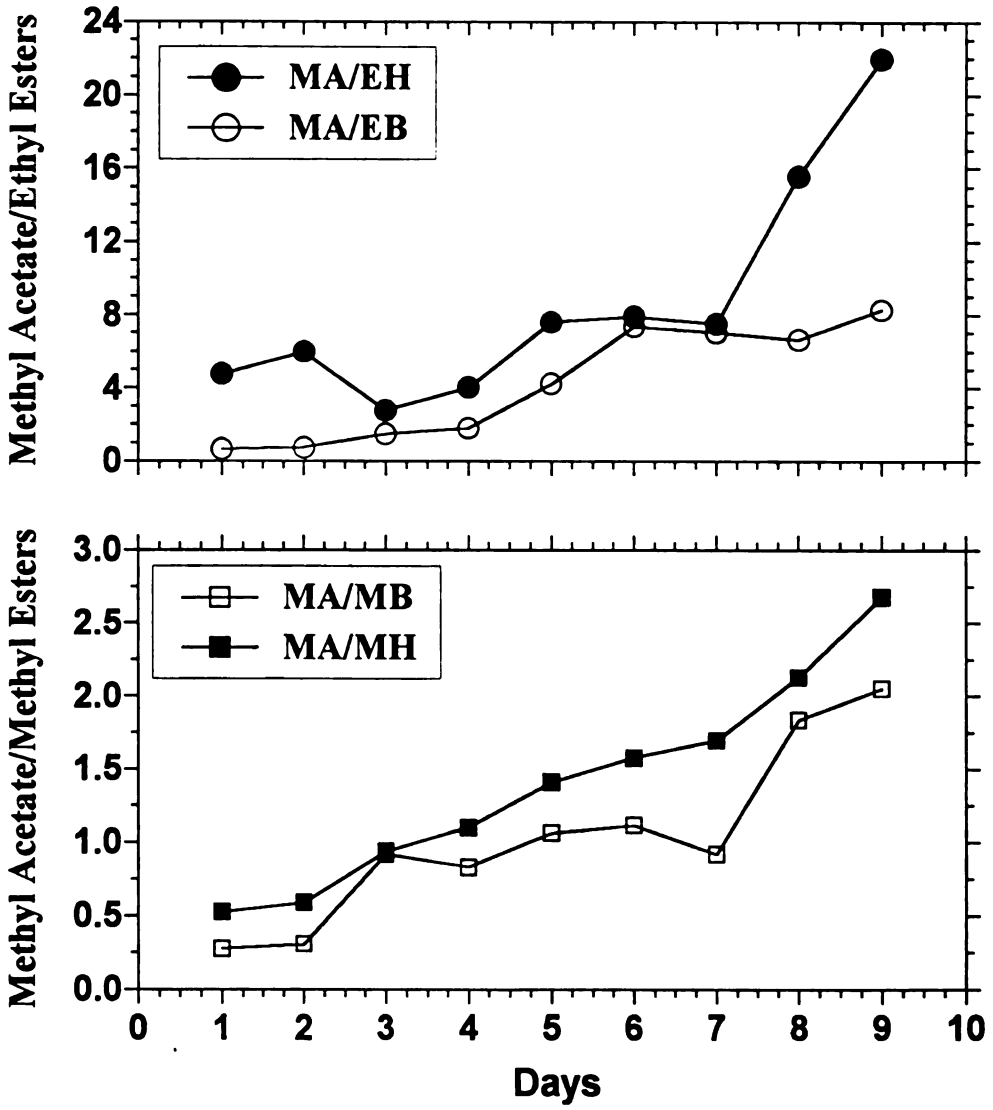

Figure 4. Ratios of methyl acetate to methyl hexanoate (closed squares), methyl acetate to methyl butanoate (open squares), methyl acetate to ethyl hexanoate (closed circles), and methyl acetate to ethyl butanoate.

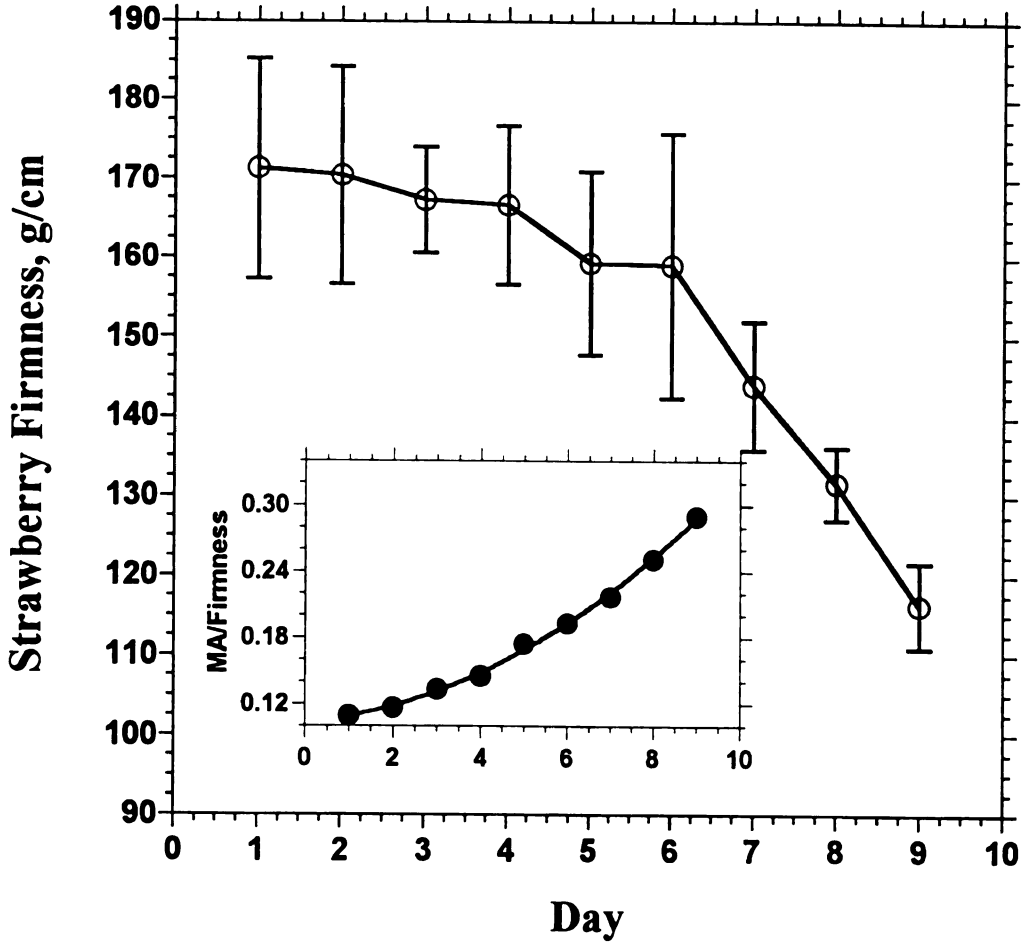

Figure 5. Softening of strawberry during storage. Insert figures represents changes of the ratio MA to firmness over time.

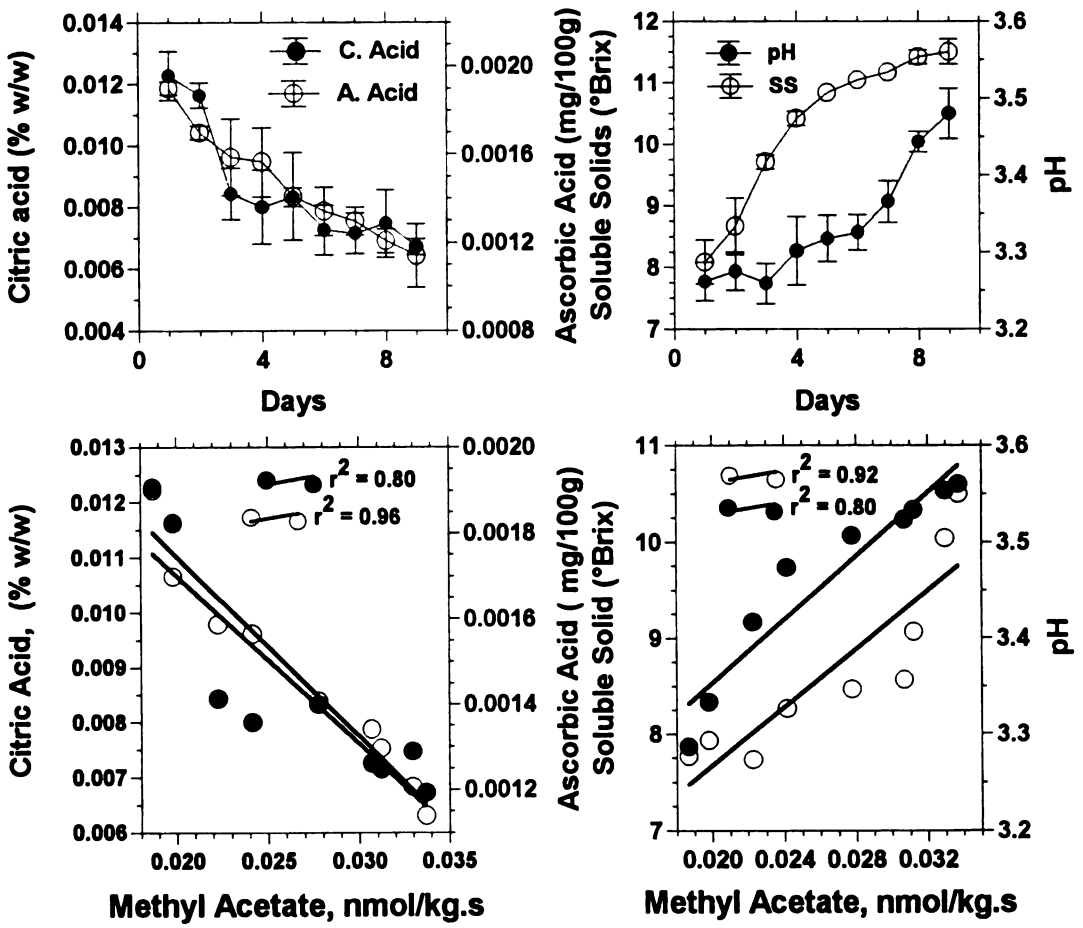

Figure 6. Changes in strawberry physicochemical parameters (top) and correlation of methyl acetate produced by strawberry and changes in physicochemical parameters (bottom).

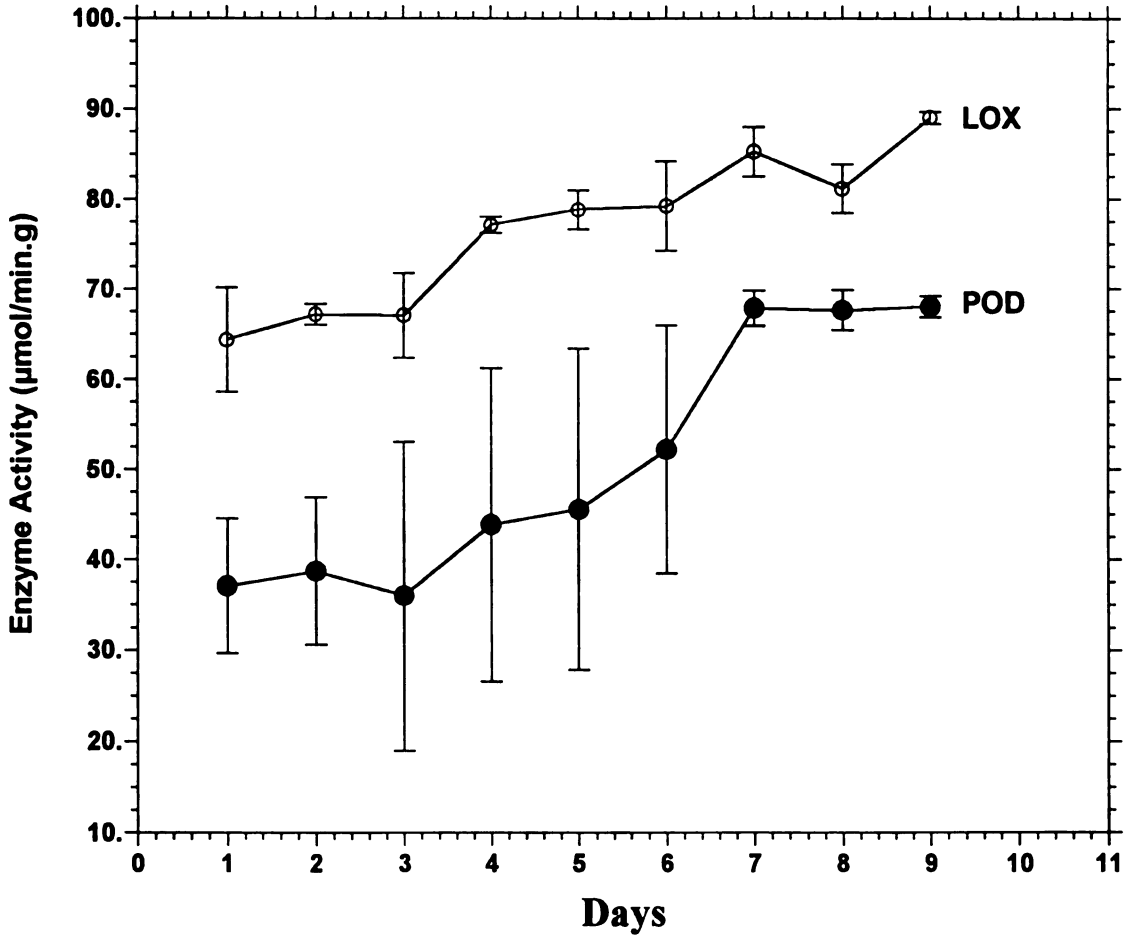

Figure 7. Activity of lipoxygenase (open circles, bottom) and peroxidases (closed circles, bottom). Calculation were made in a fresh weigth basis.

CHAPTER III

VOLATILE PROFILES OF BROCCOLI (*Brassica oleracea* L.) AND CARROT (*Daucus carota* L.).

VOLATILE PROFILES OF BROCCOLI (Brassica oleracea L.) AND CARROT (Daucus carota L.) DURING STORAGE

Additional index words. terpenes, sulfide compounds, volatile ratios

Abstract. Carrots were cut into sticks and broccoli into florets for the monitoring of volatile production during 9 days in a flow through system. The most abundant class of carrot volatiles were terpenes. Of the terpenes measured all but alpha phellandrene declined as the commodity aged. This decline may give an indication of the physiological status of carrot during storage and may provide information regarding shelf-life. For broccoli there was no single class of volatiles that predominated. In broccoli, the types of volatiles detected included terpenes (limonene and O-cymol), aldehydes (heptanal), fatty acid derivatives (pentene-3-methyl, hexene-2,5-dimethyl, and 1-heptene-4-methyl), sulfides (dimethyl disulfide), ketones (acetone), alcohol (linalool) and benzenoid (ocimene). Dimethyl disulfide production increased as broccoli senesced. Dimethyl disulfide, which has before been reported as anaerobic metabolism product is likely also a senescence-related volatile compound. Dimethyl disulfide therefore has the potential to serve as a senescence-type volatile in the designing of shelf-life prediction models.

To date, to our knowledge, research to assess the volatile organics in fresh produce, such as broccoli and carrot as the commodity senesces has yet to be fully explored (King and Morris, 1994). As tissues senesce they produce enzymes necessary to recycle metabolically expensive materials and reroute the subunits to areas for use by active systems elsewhere. Some of these materials are likely to be specific to the process of senescence (King and Morris, 1994). Volatile production of produce during storage could be a valuable asset to indirectly and non-destructively assess the physiology of the produce and serve as a tool to make prediction regarding storage or shelf-life.

Broccoli is a compact, rapidly developing floral vegetable which is usually harvested when the flowering heads are immature and growing. When harvested, the floral apices are separated from nutrients, hormones and energy supplied by the roots and leaves. Consequently, broccoli florets senesce rapidly as is typical of commodities harvested before physical growth has ceased (Huber, 1987). The most obvious feature of broccoli postharvest senescence is sepal degreening due to degradation of chlorophyll (King and Morris, 1994). To find favor with consumers, however, broccoli must also be mild in flavor (Werner and Harris, 1974).

Tollsten and Bergstrom (1988) reported that terpenes were the major compounds found in plants og the genus *Brassica*. Among terpenes, the most abundant compound was the monoterpene trans-ß-ocimene. Other prominent monoterpenes found were ß-pinene, sabinene, myrcene, limonene, and ß-phellandrene. Buttery et al. (1976) reported some other groups of importance. These include sulfides (dimethyl disulfide, dimethyl sulfide, dimethyl trisulfide), isothiocyanate, cyanides, aliphatic aldehydes, aliphatic

alcohols and aromatic compounds. Pentina et al. (1995) reported a possible enzymatic pathway for the production of some of the sulfur-containing compounds. They suggested that for a cell-free system of fresh broccoli, sulfur containing compounds are formed from L-methionine and S-methionine. Terpene and benzenoid compounds are flower-fragrance components, being mainly released from undamaged inflorescences. They also reported that alcohols and aldehydes are mainly degradation products from lipids while nitrogen-containing compounds are formed through enzymatic degradation of different glucosinolates (Tollsten and Bergstrom, 1988).

Carrot volatiles are mostly terpenes (Wagner and Warthesen, 1995). These include alpha pinene, beta pinene, myrcene, alpha phellandrene, alpha terpinene, limonene, gamma terpinene, terpinolene, terpinen-4-ol, methyl acetate, caryophyllene and gamma bisabolene (Simon et al., 1980; Kopas-Lane and Warthesen, 1995). Terpenoid biosynthesis begins with the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This reaction is catalyzed by the enzyme HMG-CoA synthase (Dewick, 1997). Studies of plant-derived HMG-CoA have suggested that in radish, both HMG-CoA synthase activity and that responsible for acetoacetyl-CoA synthase (Acetoacetyl-CoA thiolase) are localized on a single polypeptide. Thus, the soluble monomeric protein from radish membranes catalyzes the two-step conversion of acetyl-CoA into HMG-CoA. Because of the critical role of HMG-CoA on the terpenoid biosynthesis pathaway, this enzeme is considered the most important control point in terpenoid biosynthesis. Whereas the mevalonate pathway to terpenoids has been studied in considerable detail (Dewick, 1997; Grayson, 1997;

Cullmann and Becker, 1998), it has been observed on several occasions that angiosperms incorporate little, if any, ^{14}C-mevalonate into ß-carotene and the phytol side of chlorophyll as well as into other terpenoids (Arigoni et al., 1997). It has been suggested that different pathways of terpenoid biosynthesis might be operative even in different compartments of plant cells and carbohydrate (Arigoni et al., 1997). The conversion of geranyl diphosphate (GPP) into simple cyclic monoterpenes involves initial isomerization to (3R)-(-) or (3*S*)-(+)-linalyl diphosphate (LDP) (Dewick, 1997). GPP is typically bound to the enzyme as a complex with a divalent metal ion, and undergoes ionization to the allylic linalyl cation, which allows formation of LPP, and the opportunity for cyclization *via* the stereochemically favorable cation. A series of cation-diphosphate ion pairs participates in the sequence, and both the isomerization and cyclization reactions are catalyzed by a single enzyme. Interestingly, the biosynthesis of linalool in the flowers of Clarkia breweri does not proceed as far as LPP, but involves quenching of the linalyl cation with water. The enzyme limonene synthase catalyzes the isomerization-cyclization of GPP to limonene, the chirality in the product being determined by the enzyme and its source. Thus, limonene synthase from spearmint (*Mentha spicata*) yields (4S)-(-)-limonene (Dewick, 1997).

The Biosynthesis of (+)-3carene, a major component of turpentine, has been studied by feeding experiments with labeled mavalonic acid precursors. Accordingly, the formation of (+)-3-carene has been reinvestigated using cell-free extracts of Douglas fir (*Psedotsuga menziesii*) and partially purified carene synthase preparation from lodgepole pine (*Pinus contora*). It has been established that label from (1-H)GPP was retained at C-

5 in carene, in keeping with the intermediacy of terpinyl cation, then cyclopropane ring formation by loss of proton C-5 of the cation (Dewick, 1997)

There is a concern in the produce industry as to what effect time of storage has on the quality and flavor of produce to the consumer. In this project, we examined the changes of volatile profiles of both broccoli and carrot during storage, with the objective of identifying trends of volatile biosynthesis by these two commodities as they senesced. We have also attempted to identify those particular senescence signature-type volatiles with which we would be able to elaborate on the designing of prediction models to assess shelf-life.

Material and Methods

Plant material. Broccoli and carrot were obtained as fresh as possible from the Detroit Produce Terminal. Broccoli was cut into florets and carrot was cut into sticks ranging from 2 to 5 inch length. The lightly processed products were then sanitized in a smixture of 0.5 $g \cdot L^{-1}$ of Benlate (Du Pont de Nemours Co., Agricultural Product Department, Wilmington, Del.) and 0.5 $g \cdot L^{-1}$ Rovral (Rhone-poulenc Ag. Company, Research Triangle Park, N.C.) To prevent decay and then rinsed with distilled water and allowed to air-dry. Approximately 450 g of carrot and 350 g of broccoli were placed in glass jars for use in experiments. The storage temperature was 15 °C.

Flow through sampling system. The jars were fitted with T-shaped glass gas sampling ports at the inlet and outlet. Connectors and gas lines were constructed of glass and teflon, respectively, to minimize sorption and loss of volatiles from the system.

Temperature of the system was regulated by placing the apparatus in a controlled temperature room. The jars were ventilated with air at flow rates that ranged from 15-19 $mL \cdot min^{-1}$.

Headspace Analysis and Volatile Identification. Volatile collection was accomplished using solid-phase micro-extraction (SPME) device (Supelco, Inc., Bellefonte, Pa.). A fiber with a 100 μm-thick, non-bonded, polydimethylsiloxane-based stationary phase was used. The absorption time was 5 min. After absorption, volatiles were then thermally desorbed from the fiber coating by directly inserting the fiber into the injection port (200°C) of a gas chromatograph (HP-6890 Hewlett Packard Co., Wilmington, Del.) for 90 s. Gases were cryofocused on-column using liquid N_2 and separated using a gas chromatograph fitted with a 10-meter, 0.1 mm I.D., DB-5 capillary column (Hewlett Packard Co., Boise, Idaho). The detector was a time-of-flight (TOF) mass spectrometer (Pegasus II, LECO Co., St. Joseph, Mich.). The TOF mass spectrometer permitted the resolution and quantification of compounds not separated chromatographically. Identification of volatile material was done by comparison of the spectra and GC retention times with authenticated standard compounds. High purity (>95%) authenticated standards obtained from Aldrich (Aldrich, Co., Milwaukee, Wis.). For the standard compounds, a mix of the targeted compounds was prepared in a 4.85-L jar (standard jar), equipped with a self-sealing rubber sampling port. The mix was prepared by first placing 50 μL of each compound into a 2.5-mL vial to generate a stock mix. Then, 10 μL were taken from the stock mix and placed into the 4.85-L jar to be used as standard. Standard preparation was done at 5 °C and the standard jar was kept at the

temperature of the experiment. Calculation of the concentration of standard compounds was expressed in ppm. As sampling from the packages was done, so was from the standard to estimate the actual production of the targeted compounds regardless of the sensitivity of the detection system. Data were reported in $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

Results

Trend of carrot and broccoli volatile profile. The most abundant chemical class among carrot volatiles were the terpenoids (Table 1, Fig. 1). Fourteen terpenoids were evaluated during the storage time and among these, only alpha phellandrene showed an increase over time (Fig.2). Other terpenoids were found to decrease as the product aged (Figs. 2 and 3). Most terpenoids decreased markedly during the first 1-3 d of evaluation and declined more slowly thereafter.

Ratios among carrot terpenoids were generated to assess the type of relationship between the production of different carrot volatiles during storage (Fig. 4). Ratios included camphene to alpha pinene, ocimene to camphene, sabinene to ocimene, alpha phellandrene to cymene, myrcene to alpha phellandrene, and alpha pinene to myrcene. Ratios of ocimene to camphene, myrcene to alpha phellandrene and alpha pinene to myrcene decreased over time. The ratios of camphene to alpha pinene and alpha phellandrene to cymene increased as the product aged.

In broccoli no single group of volatiles predominated. Volatiles detected included terpenes (limonene and O-cymol), aldehydes (heptanal), fatty acid derivatives (pentene-3-methyl, hexene-2,5-dimethyl, and 1-heptene-4-methyl), sulfides (dimethyl disulfide),

ketones (acetone), alcohol (linalool) and a benzenoid (ocimene) (Table 2, Fig. 5). Among these groups, terpenes and fatty acid derivatives were the most abundant. Broccoli volatiles which increased over time included heptanal and dimethyl disulfide. Volatiles that decreased included acetone 3-methyl pentene, 4-methyl-1-heptene-, limonene, and linalool. 2,5-dimethyl hexene, ocimene, and O-cymol stayed approximately at the same levels throughout storage (Fig. 6). We have also provided a series of possible modes of formation of particular terpenoid volatiles produced by carrot and broccoli that exist in the literature (Dewick, 1997). (Figs. 7, 8, 9, 10 and 11).

Discussion

The carrot volatiles identified in this work including sabinene, ocimene, camphene, alpha pinene, myrcene, alpha phellandrene, cymene, limonene, 3-carene, terpinolene, 1,2-dimethyl-4-vinyl benzene, caryophyllene and bisabolene were consistent with previous reports (Heatherbell et al., 1971a; Heatherbell et al., 1971b; Heatherbell and Wrolstad, 1971; Simon et al., 1980). Simon et al. (1980) reported the volatile profiles of eight carrot lines either blended, grated or sliced. They found that the volatile profiles for the eight carrot lines were the same and that the difference was only in concentration. Heatherbell and Wrolstad (1971b) reported the volatile composition of carrot as influenced by variety, maturity and storage. They reported that differences were quantitative rather than qualitative and that variation in concentration of individual terpenes appeared to be consistent with descriptions of flavor characteristics of different varieties.

The formation of cyclic monoterpene such as alpha phellandrene in carrot is usually *via* the intermediacy of alpha terpinyl cation which may be produced in either 4R or 4S configurations depending on whether the LPP produced from GPP has 3R or 3S configuration respectively (Dewick, 1997). Biosynthesis of many other monoterpenes then requires migration of the positive charge from the side-chain into the ring, and this may be achieved by hydride shift. The precise nature of such shift in the formation of alpha terpinene, for instance and alpha phellandrene has been investigated using cell-free enzymes systems and specifically-labelled GPP substrates (Dewick, 1997). It has been established that the a 1,2-hydride shift from C-4 to C-8 of the alpha terpinyl cation thus generating the terpinen-4-yl cation is occurring during alpha terpinene formation (Dewick, 1997).

The decrease of terpene production by carrot as the product aged observed in our experiment may help to understand the biochemical nature of the volatile biosynthesis system of the product. These data indicate there may be a step in the terpenoid pathway that is increasingly inhibited as the crop senesces. Most terpenes detected in carrot are cyclic including sabinene, camphene, alpha pinene, alpha phellandrene, cymene, limonene, 3-carene, terpinolene, 2,2-dimethyl 1,4-vinyl benzene, caryophyllene and bisabolene. George-Nascimento and Cori (1969) reported that the biosynthesis of cyclic monoterpenes involves the transformation of mevalonic acid into 10-carbon phosphorylated intermediates, and subsequent elimination of the pyrophosphate moiety. The cyclization process leading to the formation of mono- or bicyclic-monoterpenes such as limonene or the pinene apparently requires an adequate stereochemistry of the 10-

carbon precursor. The *cis* isomer neryl pyrophosphate has been postulated as the more likely precursor of cyclic monoterpenes (George-Nascimento and Cori, 1969). The process of senescence in carrot may have an impact in both the necessary stereochemistry of the 10-carbon cyclic terpene precursor. However, the two straight-chain terpenes ocimene and myrcene also behaved the same way as cyclic terpenes as the product aged, implying that probably the effect of aging on terpene biosynthesis is somewhere in the pathway before the formation of mevalonic acid. Ova et al. (1970) reported that both the acetate and mevalonate used in terpene biosynthesis are produced through the formation of pyruvate and acetyl-CoA in mitochondria.

A particular carrot volatile that increased over time while the product aged was alpha phellandrene. Dewick (1997) reported that beta phellandrene is a derivative product of geranyl pyrophosphate (GPP) (Fig. 10). It is then likely that isomerization occurs and alpha phellandrene is produced. Biosynthesis of beta phellandrene requires migration of the positive charge from the side-chain of the GPP molecule into a ring, and this may be achieved by hydride shifts. The precise nature of such shift in the formation of beta phellandrene in (*Pinus cortorta*) has been investigated using cell-free enzyme system and specifically labeled GPP substrate (Fig. 10) (Dewick, 1997). A 1,3-hydride shift from C-3 to C-8 of the alpha terpinyl cation giving the terpin-3-yl cation and the intermediacy of (3S)-LPP. It has been established that a 1,2-hydride shift from C-4 to C-8 of the alpha terpinyl cation thus generating the terpinen-4-yl cation was occurring during landa-terpinene formation. Alpha terpinene formation also involves the same 1,2-hydride shift, and a similar proton loss, this time from C-3 of the terpinen-4-yl cation. An increase of

alpha phellandrene in carrot as the product ages as observed in our study, may indicate that the 1,3-hydride shift, which results in the formation of (4S)-(+)-alpha terpinyl cation into terpinen-3-yl cation, is favored. This results in an increase in the final product beta and alpha phellandrene and a decrease in the production of landa and alpha terpinene. Our results are consistent with this theory.

Broccoli volatiles identified in this study are typical of the genera *Brassica* as reported by Tollsten and Bergstrom (1988). Both heptanal and dimethyl disulfide increased as broccoli senesced and it may be worthwhile to evaluate their potential as senescence-fingerprint compounds. The diversity of types of volatiles during storage may indicate that volatiles in broccoli come from different sources. Thus, the impact of senescence on volatile production of broccoli may not be as easily targeted as in carrot. Pentina et al. (1995) and Forney et al. (1991) have both reported sulfur compounds as off-odor indicators in experiments conducted under anaerobic conditions. Dimethyl disulfide was included in both studies. In our study, conducted under aerobic conditions, none of the other sulfur containing compounds but dimethyl disulfide was detected. It may indicate that dimethyl disulfide is rather a senescence-related volatile and not necessarily a low-oxygen synthesized compound. It also suggests that dimethyl disulfide is the responsible for the unpleasant odor of decaying broccoli under aerobic conditions. Dimethyl disulfide may have the potential to serve as a senescence-type volatile.

The production of volatiles during storage merits further evaluation. Monitoring of terpenes may provide a method to assess the physiological status of the product during storage and should be judged for its value in prediction of shelf-life. Besides the general

trend of terpenes over time, ratios among terpene compounds may have potential to serve as markers of aging and may have an application in the design of models to assess the stage of development of the product as well. As for broccoli, dimethyl disulfide seemed to be related to senescence and may have potential to be used as a senescence indication.

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Table 1. Selected volatiles produced by carrot over time. Concentration of volatiles is given in $\mu g \cdot kg^{-1} \cdot h^{-1}$, in fresh weight basis.

Compounds	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
1. Sabinene	.05	.0138	.0083	.0027	.00047	.0004	.0009	.0004	.00018
2. Ocimene	.21	.0304	.021	.024	.006	.0036	.0025	.0025	.001
3. Camphene	.029	.0015	.0013	.0013	.001	.0009	.0009	.0007	.0007
4. Alpha pinene	.683	.158	.028	.020	.01	.0059	.0046	.0036	.0019
5. Myrcene	.124	.088	.058	.048	.009	.007	.0032	.003	.0012
6. Alpha phellandrene	.0015	.0016	.0034	.0093	.0101	.0284	.0483	.0932	.0222
7. Cymene	.214	.125	.096	.085	.021	.0142	.0053	.0064	.0056
8. Limonene	.204	.152	.0414	.0235	.0094	.0055	.0067	.0010	.00134
9. 3-carene	.174	.105	.055	.0271	.0171	.0101	.0036	.0026	.0007
10. Terpinolene	1.363	.2265	.1697	.0612	.0714	.0375	0.0134	.0078	.0019
11. 1,2-dimethyl-4-vinyl benzene	.5186	.453	.415	.3503	.349	.205	.0947	.0299	.0196
12. Caryophyllene	.615	.501	.366	.0721	.0692	.033	.020	.0183	.0035
13. Bisabolene	.156	.0523	.0382	.0113	.0061	.0053	.0019	.0008	.0007

Table 2. Selected volatiles produced by broccoli over time. Concentration of volatiles is given in $\mu g \cdot kg^{-1} \cdot h^{-1}$, in fresh weight basis.

Compounds	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
1. Acetone	.283	.1268	.0871	.0252	.0159	.0112	.0094	.0069	.0059
2. Heptanal	.0027	.0046	.0083	.0131	.018	.152	.174	.2024	.2233
3. Pentene-3-methyl	.142	.088	.0453	.0100	.0098	.005.0	.0021	.0019	.0016
4. Hexene, 2-5-dimethyl	.0174	.0153	.0181	.0174	.0174	1731	.0174	.0165	.0176
5. 1-heptene-4-methyl	.0245	.0237	.0189	.0182	.0183	.0169	.0134	.0093	.0018
6. Dimethyl Disulfide	.0085	.0309	.0694	.0719	.1215	.132	.224	.241	.298
7. Ocimene	.0166	.0172	.0299	.025	.026	.0187	.0279	.0286	.0182
8. Limonene	.1560	.0389	.0186	.01865	.0169	.0149	.0132	.0098	.0041
9. O-cymol	.0407	.0348	.0192	.0157	.0122	.0111	.0101	.0039	.0035
10. Linalool	.0462	.0357	.0382	.0314	.0259	.0197	.0165	.0131	.0068

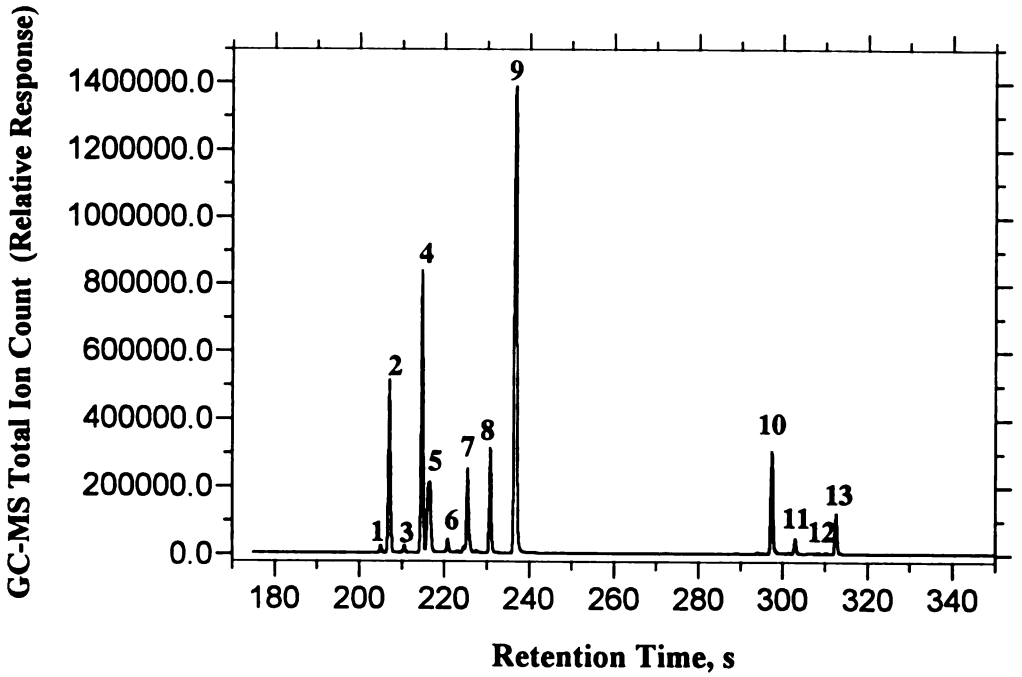

Figure 1. Volatiles profile of carrot using time-of-flight mass spectrometry: 1) sabinene, 2) ocimene, 3) camphene, 4) alpha pinene, 5) myrcene, 6) alpha phellandrene, 7) cymene, 8) limonene, 9) 3-carene, 10) terpinolene, 11) 1,2-dimethy-4-vinyl benzene, 12) caryophyllene, 13) bisabolene.

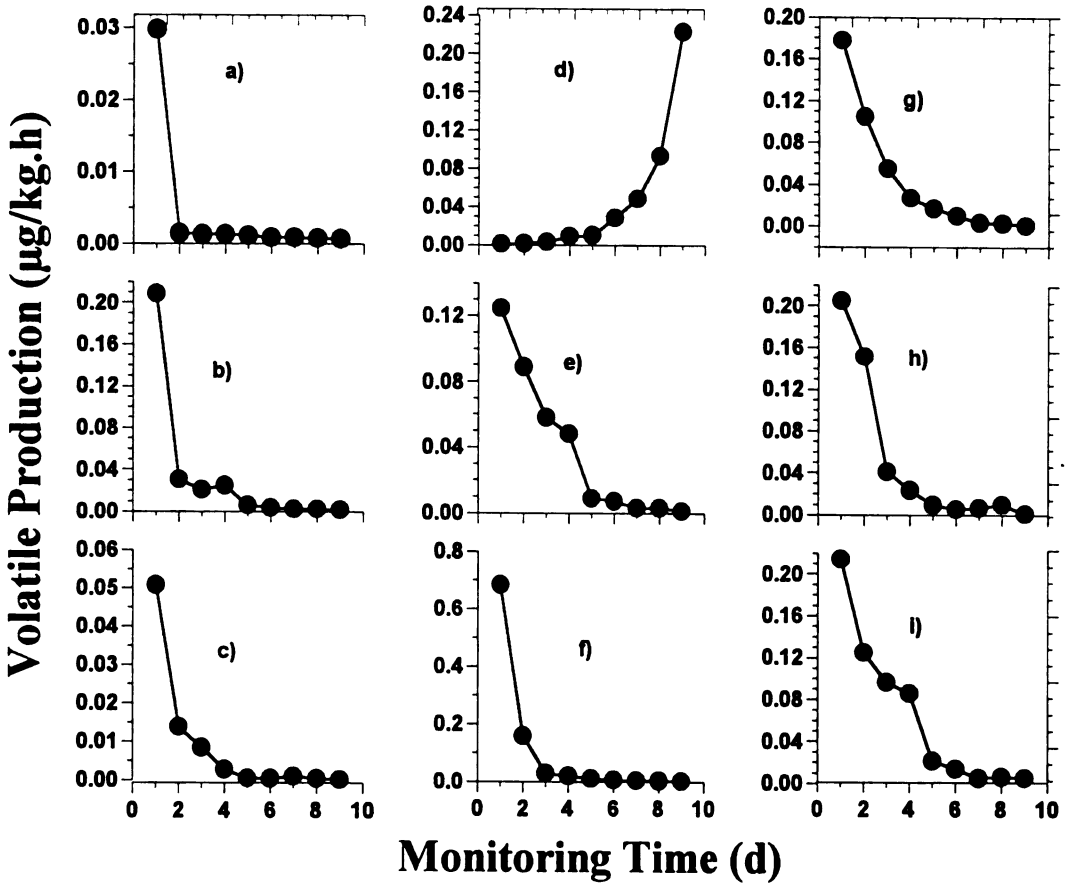

Figure 2. Production over time of selected carrot volatiles. a) camphene b) ocimene, c) sabinene, d) alpha phellandrene, e) myrcene, f) alpha pinene, g) 3-carene, h) limonene, i) cymene.

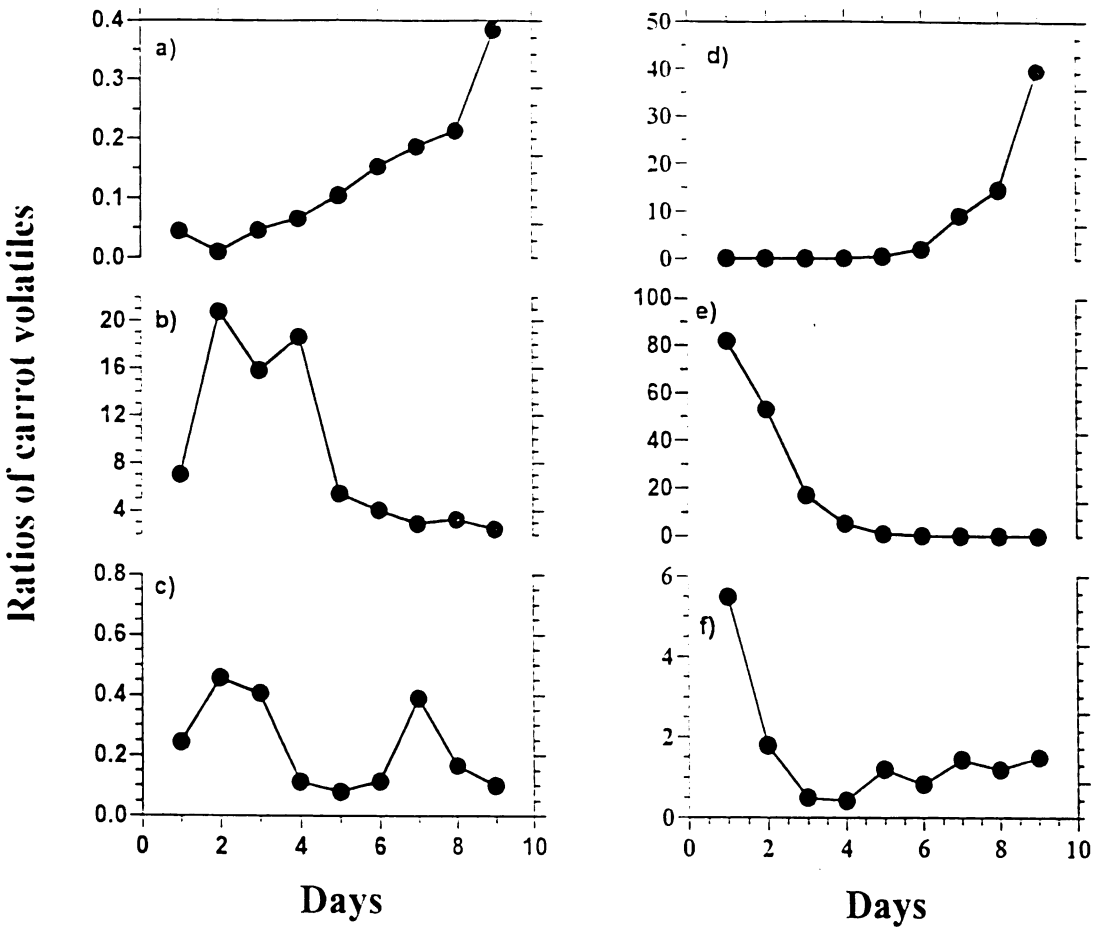

Figure 3. Ratios of selected carrot volatiles over time: a) camphene to alpha pinene, b) ocimene to camphene, c) sabinene to ocimene, d) alpha phellandrene to cymene, e) myrcene to alpha phellandrene, f) alpha pinene to myrcene.

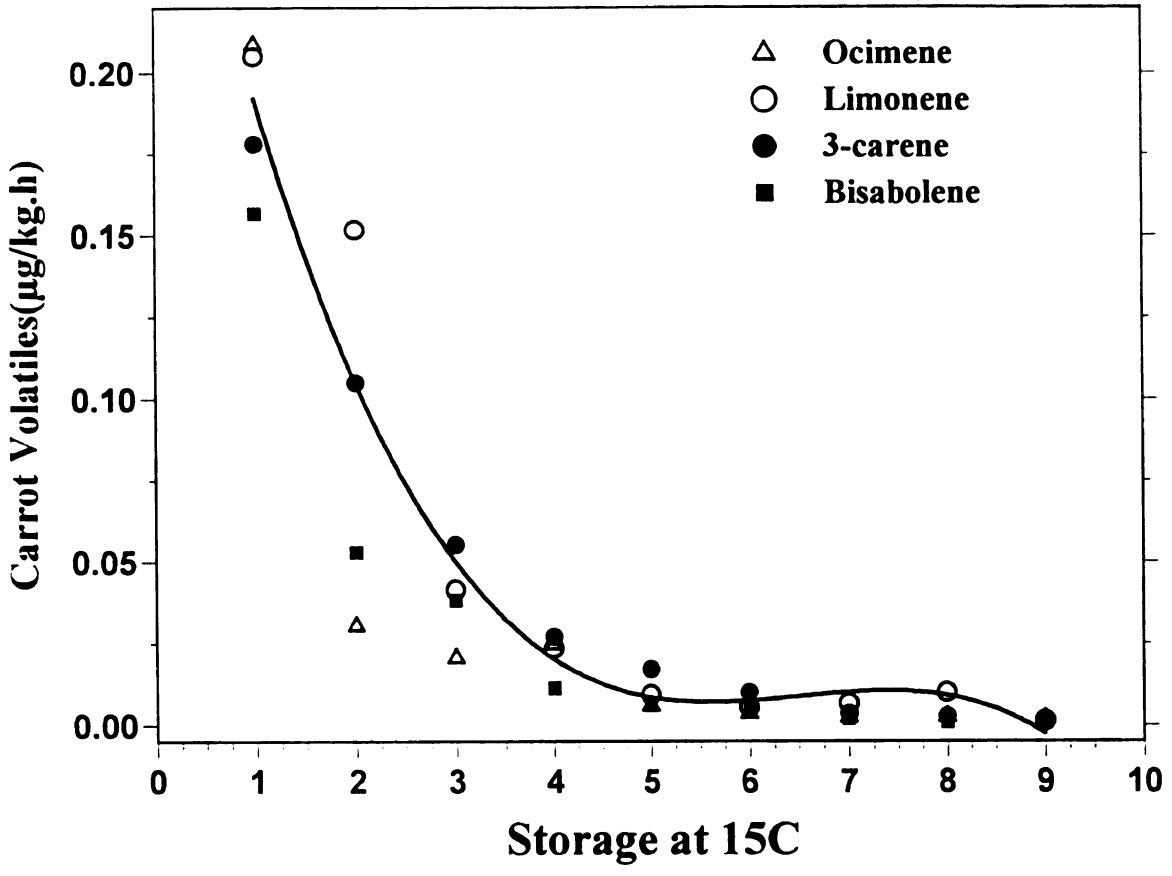

Figure 4. Effect of storage duration at 15 °C on the production of selected carrot volatiles over time. ocimene (open triangles), limonene (open circles), 3-carene (closed circles), bisabolene (closed squares), alpha mycerene (open circles).

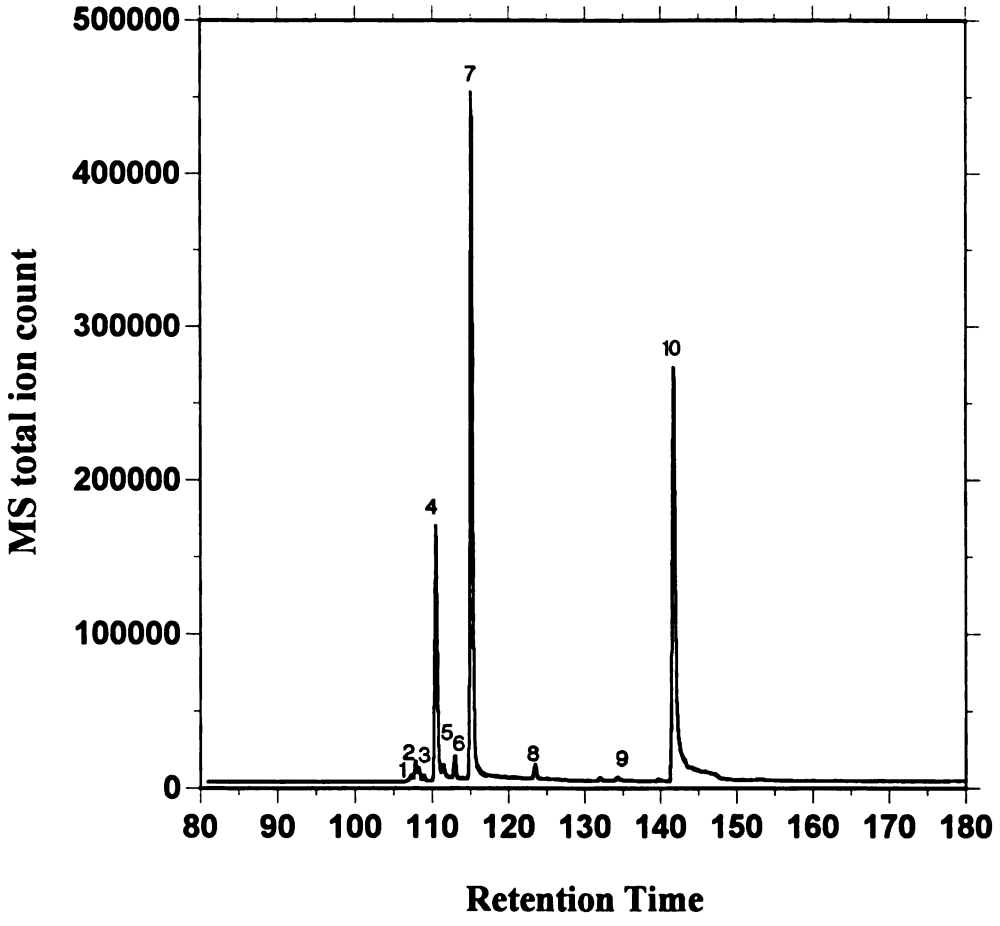

Figure 5. Volatiles profile of broccoli using time-of-flight mass spectrometry: 1) acetone, 2) heptanal, 3) pentene-3-methyl, 4) hexene, 2-5dimethyl, 5) 1-heptene-4-methyl, 6) dimethyl disulfide, 7) ocimene, 8) limonene, 9) o-cymol, 10) linalool.

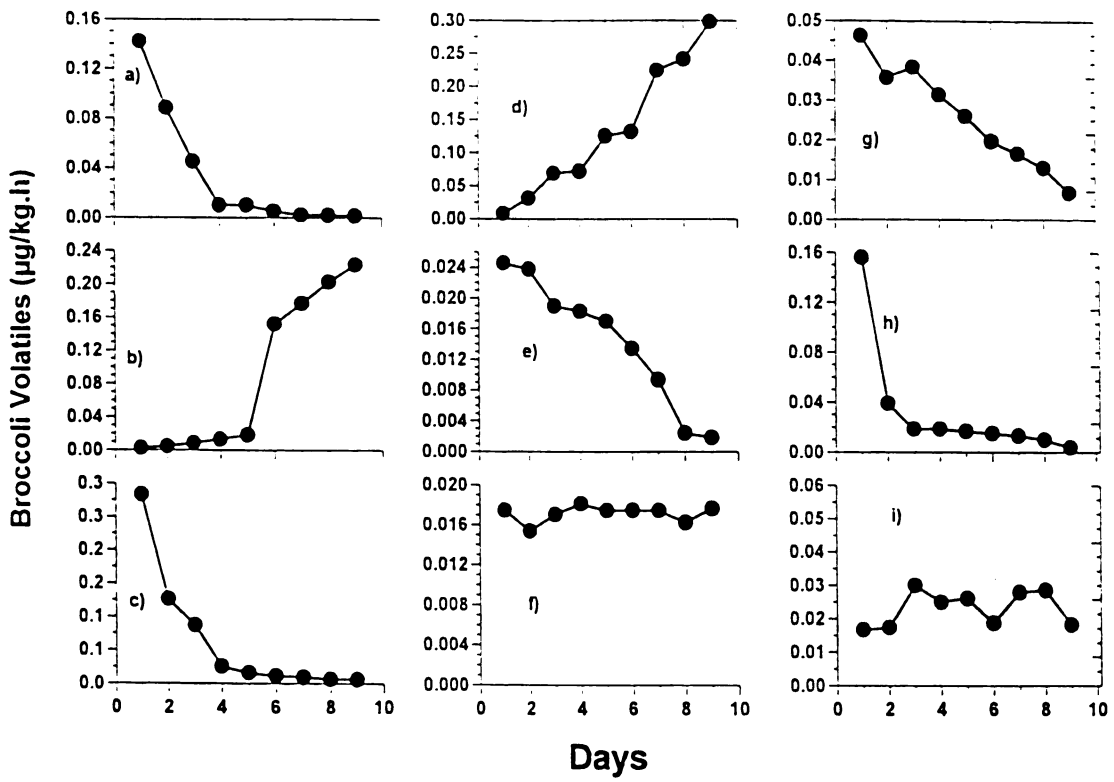

Figure 6. Production over time of selected broccoli volatiles. a) pentane-3-methyl, b) heptanal, c) acetone, d)dimethyl disulfide, e) 1-heptene-4-methyl, f) hexene-2,5-dimethyl, g) linalool, h) limonene, i) ocimene.

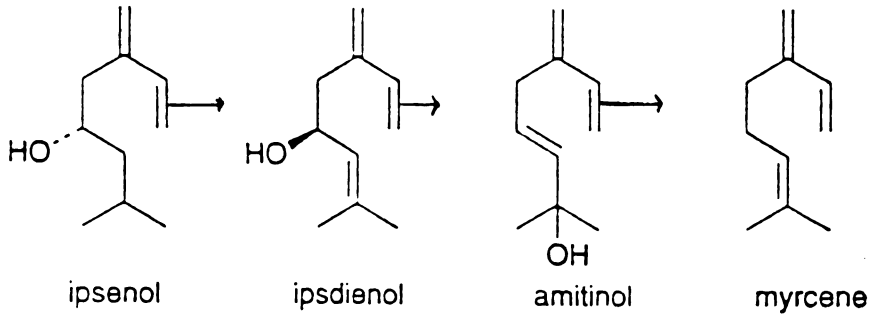

Figure 7. Mechanism of formation of the monoterpene myrcene. From: Dewick (1997).

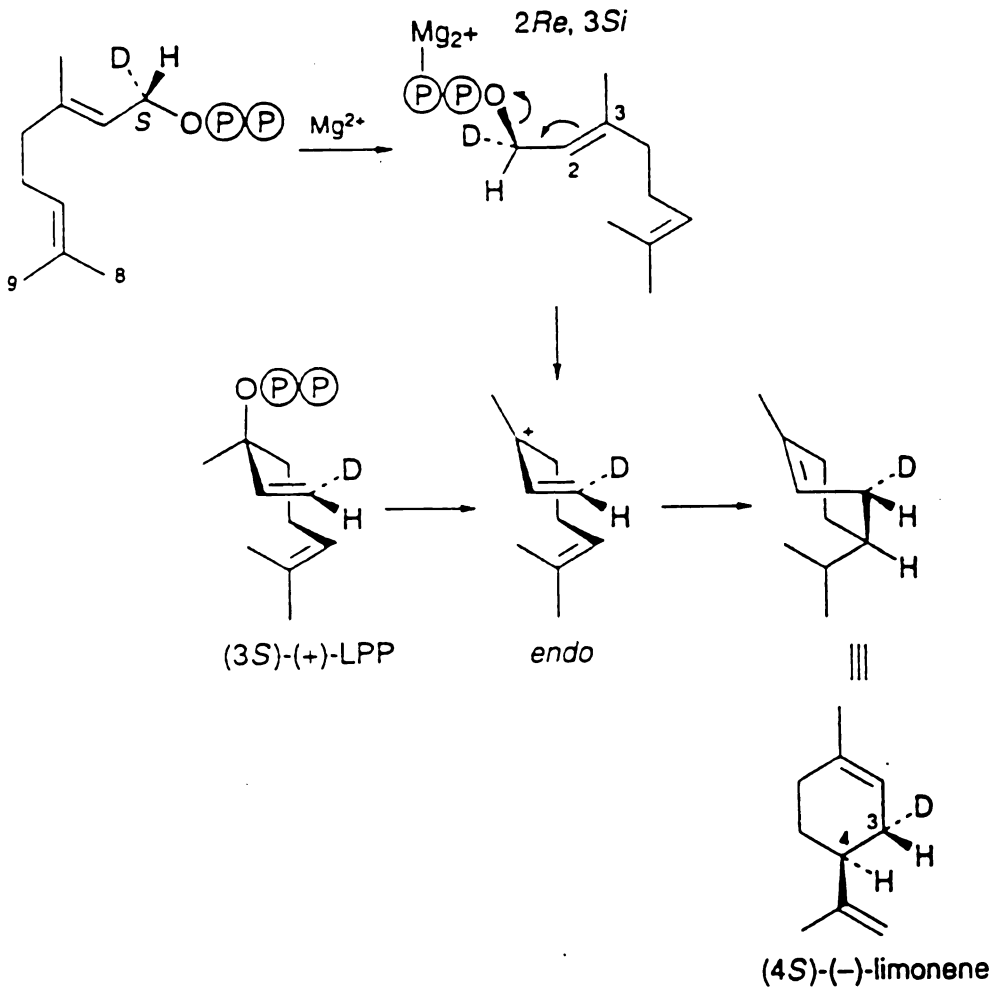

Figure 8. Mechanism of formation of the monoterpene limonene. From: Dewick (1997)

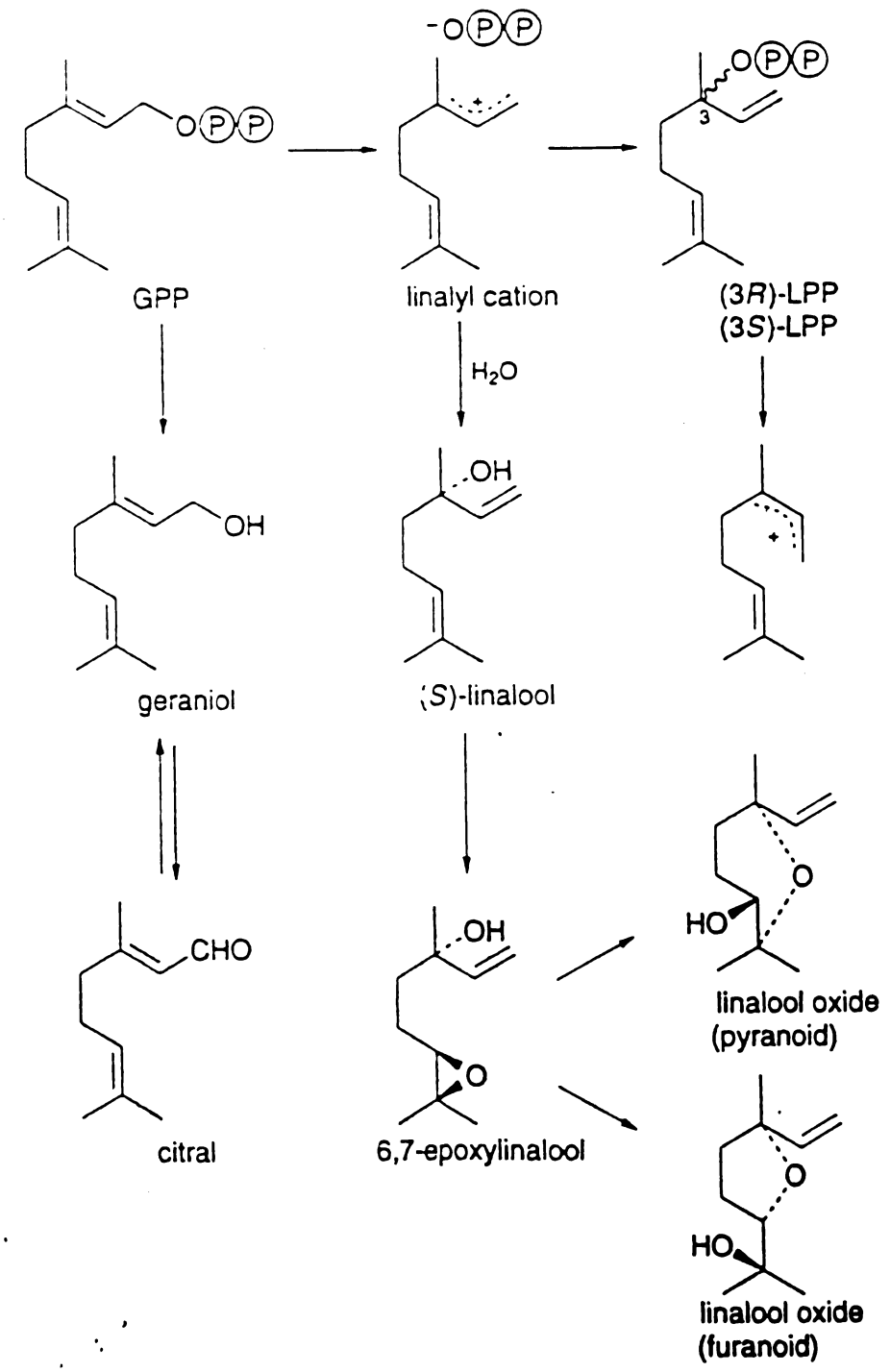

Figure 9. Mechanism of formation of the monoterpene linalool. From: Dewick (1997)

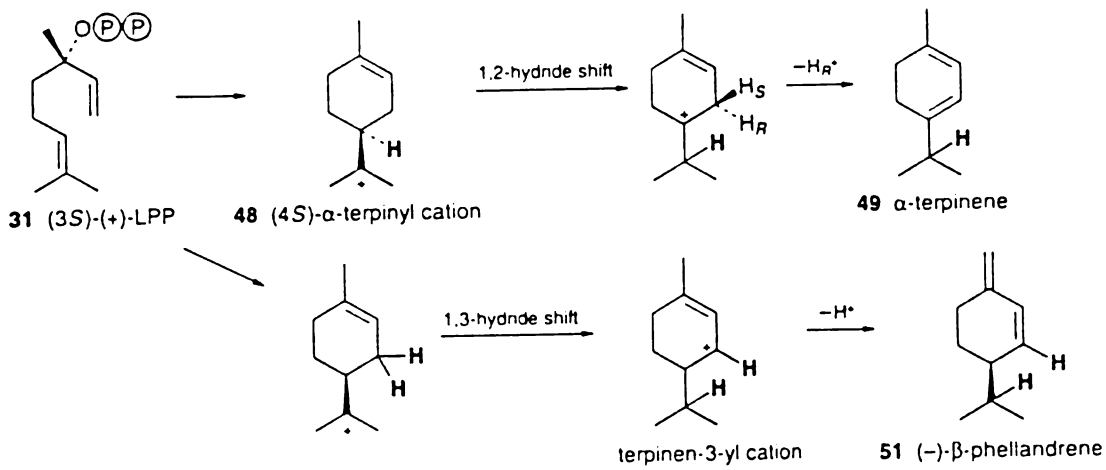

Figure 10. Mechanism of formation of the monoterpenes b-phellandrene and alpha terpinolene. From: Dewick (1997)

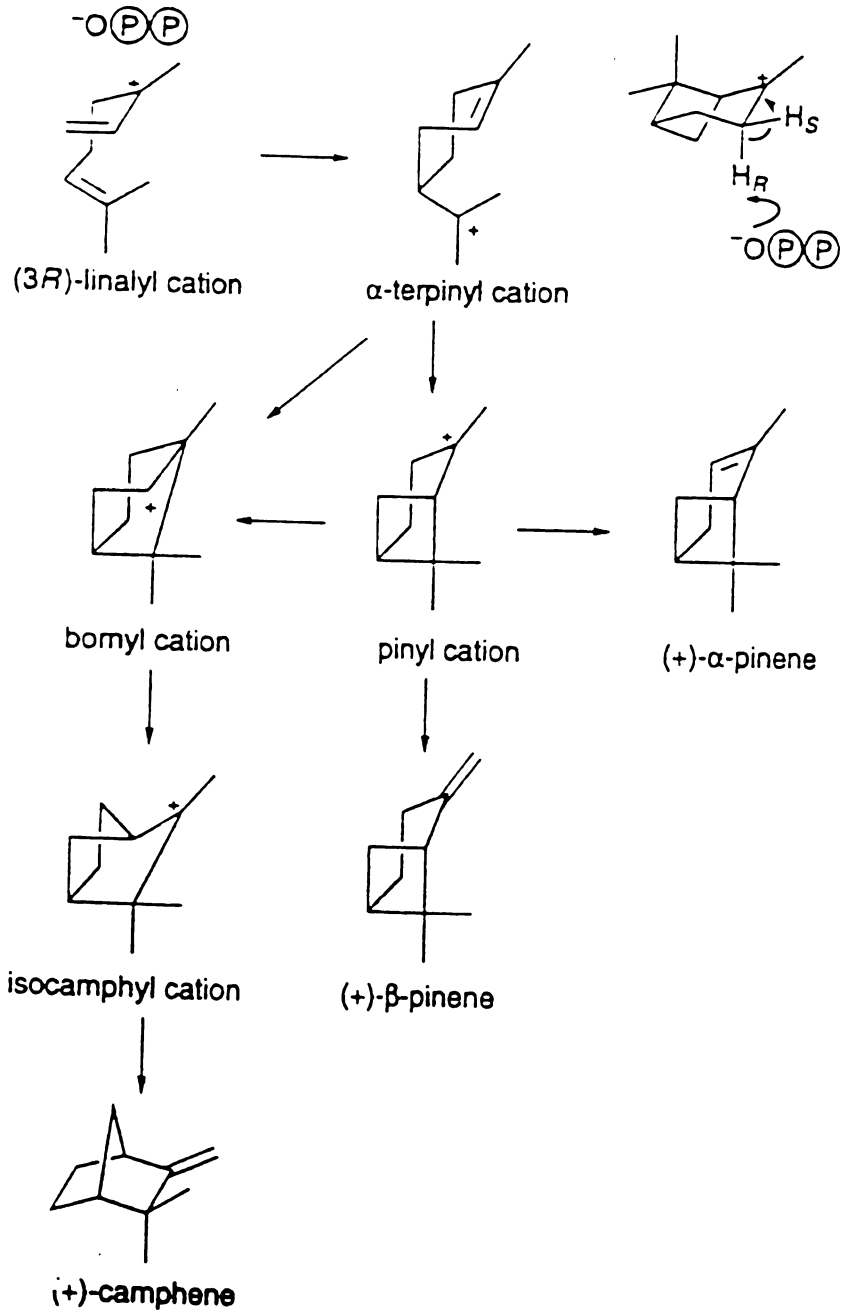

Figure 11. Mechanism of formation of the monoterpenes alpha pinene, beta pinene and camphene. From: Dewick (1997)

CHAPTER IV:

VOLATILE PROFILES OF MICROORGANISMS GROWN IN LABORATORY MEDIA AND FOOD PRODUCTS

VOLATILE PROFILES OF MICROORGANISMS GROWN IN LABORATORY MEDIA AND FOOD PRODUCTS

Additional index words. *Escherichia coli, S. typhimurium*, indole, propanol, vegetables, carrot, broccoli

Abstract. The volatile profile of *E. coli* and *S. typhimurium* grown on both laboratory media and selected horticultural products was studied to determine whether fingerprint volatiles could be identify for nondestructive assessment of human pathogen presence in food. Mass spectrometrically-detectable volatile compounds produced by *E. coli ATCC-25922* in standard laboratory media in a closed system included carbon dioxide, ethanol, 1-propanol, cyclobutanol, 1-butanol, 1-pentanol, disulfide dimethyl, cyclopropane-pentyl, octanol and indole. Detectable volatiles produced by *S. typhimurium* grown under the same conditions as *E. coli* included carbon dioxide, cyclobutanol, 1-butanol, 2-methyl cyclopentanol, 3-ethyl cyclopentanol, 2-hepten-1-ol, octane-3,3-dimethyl, 3-decanol, 2,2,3-trimethyl decane, 3,5-dimethyl dodecane, 3- methyl tridecane, 5,8-dimethyl dodecane, pentadecane, 2,4,6-trimethyl dodecane, 2-heptadecanol, octadecanol, nonadecanol, 2,5,6-trimethyl heptadecane. As a general assessment, it appears that alcohols and some other compounds such as indole result from amino acid breakdown and are typical volatile compounds of *E. coli ATCC-25922* grown in standard laboratory media. *S. typhimurium* produced a wider range of detectable alcohols. When *E. coli* was present on broccoli, carrot and lettuce packages, the only reliably detected volatile was 1-

propanol. Indole, which is a compound produced in abundance by *E. coli ATCC-25922* in standard laboratory media was not detected in produce packages. Indole production appeared to be inhibited by other sources of carbon as suggested by previous workers. 1-propanol may have the potential to be used as a fingerprint compound for *E. coli* in both different growing media and in different produce packages. Heavier alcohols such dodecanol, octadecanol and nonadecanol seemed to be characteristic for *Salmonella*, even though we did not performe extensive monitoring nor investigate whether these compounds are detectable in packages using the SPME/GC/MS system.

While the U.S. food supply is among the safest in the world, there are still millions of U.S. residents stricken by illness every year from microbial pathogen contamination of food they consume, and some 9,000 people die each year as a result (Jay, 1996). The number of food-related human pathogens is large and their routes for ingestion varied (Eley, 1996; Hobbs et al., 1993; Mott and Foster, 1995). *E. coli* O157:H7 has been detected in a wide array of food including meat, apple juice and lettuce (Griffin and Tauxe, 1991; Moyer, 1996; Tarr et al., 1997). *Salmonella* has been found in eggs, vegetables and poultry (Eley, 1996; McNab, 1997). Suzuki, 1994). *Cyclospora* has been encountered on fruit, *Cryptosporidium* has infected people via drinking water and hepatitis A virus was detected in frozen strawberries (Anonymous, 1997). As of May of 1997, it was estimated that as many as 6.5 to 33 million illnesses in the United States each year are food-related (Anonymous, 1997). The United State Department of Agriculture (USDA) estimates that medical costs and productivity losses for 7 specific pathogens range between $6.5 billion and $34.9 billion annually (Anonymous, 1997). Total costs for all foodborne illnesses are

likely to be much higher. Those estimates do not include the total burden placed on society by the chronic illness caused by some foodborne pathogens. In comparison, the total annual value of fresh produce grown in the U. S. is over $50 to 60 billion.

The foodborne outbreak paradigm has shifted. In the past, an outbreak affected a small local population, had a high attack rate, and involved locally prepared food products with limited distribution (Eley, 1996). Nowadays, outbreaks involve larger populations and may be multistate and/or international. In many cases, the pathogenic organism has a low infective dose and it is unlikely that can be isolated from the food product. Delay in identifying the causative agent can allow the outbreak to spread, increasing the number of cases with time. Most pathogens cannot be easily detected in foods (Jay, 1996). Among the needs for improved diagnostics include greater sensitivity, accuracy and speed for detection of human pathogens in food (Briggs, 1987; Giese, 1993; Schaertel and firstenberg-Eden, 1988).

At present, most of the microbial detection methods are time consuming and very sophisticated, in many cases involving DNA amplification. This does not allow real-time monitoring (Jay, 196: Watanabe, 1987). The lack of technology for real-time pathogen evaluation in food has encouraged the study of alternative detection technologies. Such technologies may include volatile analysis for the detection of figerprint volatiles produced by pathogenic organisms.

The development of new volatile analysis technologies in the last decade and the increasing interest of designing automated quality control sensors in the food industry make it worthwhile to explore the potential of possible fingerprint or signature-type

volatiles emitted by microorganisms while present in food. The most prolific methods of detecting and classifying bacteria have been focused on the detection of specific fatty acids for particular group of microorganisms (Phillips et al., 1976; Van den Bogaard et al., 1986). Analytical instrumental methods have been used to identify and classify these microorganisms (Fukuda et al., 1971). Identification has been based on the determination of the chemical composition of certain constituents in the microorganism such as profiling of lipids, or the detection of a biomarker that signifies a particular microorganism. A variety of analytical techniques have been used in these analyses including gas chromatography (GC), gas chromatography/mass spectrometry (GC-MS), pyrolysis mass spectrometry (Py-MS), and pyrolysis gas chromatography/mass spectrometry (Py-GC/MS). The most common approach in the classification of bacteria through profiling is the analysis of their fatty acid methyl esters.

Volatile emanations from human pathogenic microorganisms associated with fresh or lightly-processed produce are, in many cases, unknown. However, volatile emanations from some microorganisms have been measured. Many fungi are known to synthesize ethylene (Fujii et al., 1985; Fukuda et al., 1971; Sembdner and Parthier, 1973). Additionally, bacteria are known to emit C2 to C7 fatty acids which can be somewhat volatile with enough specificity to permit identification of the organism in clinical settings (Phillips et al., 1976; Van den Bogaard et al., 1986). The potential to identify volatiles associated with the presence of human pathogens has not received the same level of scrutiny as, say, volatiles associated with aroma development by fruit during ripening. Interesting or novel metabolites may lead to new avenues of investigation. Similarly,

volatile emanations from bacteria in relation to atmospheric composition and temperature has not been previously described and may form the basis of future inquiries in the area of microbial ecology.

In this work we have investigated the volatile profiles of *E. coli ATCC-25922* and *S. typhimurium* grown in or on various substrates including fresh produce. The major aim was to identify those volatiles which could serve as fingerprints for the identification of the microorganisms in food.

Material and Methods

Bacterial cultures and stocks. The medium for the growth of both *E. coli* and *S. typhimurium* was LB (Luria-Bertani) broth (Maniatis et al., 1982). The broth was prepared with 10 g of Bacto-tryptone, 5 g of Bacto-yeast, and 5 g of NaCl in a liter of distilled water (Maniatis et al., 1982). The broth was autoclaved for 15 min. *E. coli ATCC-25922* was added to the broth and incubated at 37 °C in a gyratory shaker at a speed of 100 rpm (G-25 New Brunswick Scientific Corporation, New Brunswick, N.J.). Growth was monitored at 595nm using a spectrometric densitometer (model 601-G, Milton Ray Co., Rochester, N.Y.). The target concentration was 3.75×10^8 cells/mL (OD=0.375). Measurements with the densitometer were made every half hour. A growth curve was constructed.

When the target population was achieved, starting cultures were prepared. One liter of solution containing *E. coli ATCC-25922* was dispensed to 6-125mL rotor-GSA centrifuge bottles with screw caps. The four bottles were then centrifuged (model Rc 5c

Superspeed Centrifuge, Sorvall Instruments, Dupon Co., Haffman Estate, Ill.) at 1600x g for 10 min and the supernatant decanted. Cells were re-suspended in 3 mL of 20% glycerol solution. The bacterial population was rechecked using spectrometry at a wave length of 595 nm. Cultures were then diluted to $2.6x10^{10}$ cells/mL. One mL aliquots were placed in 1.5-mL microcentrifuge tubes. Tubes were quickly frozen using ethanol and dry ice (CO_2) and stored at -80 °C.

Viability Analysis. The number of viable cells per mL of solution was determined by serial dilutions of the starter culture on agar plates. Agar-based medium was composed of 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 5 g of NaCl, and 12 g of agar. Distilled water was added to the mix and brought to a volume of 1 L. Agar was added after all other components were dissolved. Once the medium was prepared and autoclaved for 30 min, 1 mL of starter culture was diluted serially and the solution was dispersed evenly over the surface of the agar. Plates were incubated overnight at 37°C and colony count was performed.

E. coli and S. typhimurium in standard laboratory media in a closed system. E. coli and S. typhimurium microorganisms were prepared in five different growing media to monitor their volatile profiles over time. Media included NZY broth (5 g NaCl, 2 g MgSO4.7H2O, 5 g yeast extract, 10 g NZ amine or casein hydrolysate and adjusted to pH 7.5 with NaOH), 2X YT broth (10 g NaCl, 10 g yeast extract, and 16 g bacto-tryptone), SM Buffer (5.8 g NaCl, 2 g MgSO4.7H2O, 50ml 1M Tris-Cl, pH 7.5 and 5 mL of 2% bacterial gelatin), LB broth (10 g NaCl, 10 g bacto-tryptone and 5 g yeast extract) and Super Broth (5 g NaCl, 20 g bacto-yeast extract, 35 g tryptone and 1 mL 5 M NaOH).

About 50 mL of each media were placed in 120-mL glass jars equipped with sampling ports. Media then were inoculated with 1 mL of overnight-enriched stock bacterial sample. Jars were sealed and sampling of the headspace using SPME was initiated. Alternatively, tryptophan or threonine was added to the Super Broth medium to evaluate the capacity of *E. coli* to breakdown both amino acids and yield indole and 1-propanol, respectively. At this point we should mention that only the Super Broth medium was used for time course monitoring and the other media were employed only in the preliminary and exploratory stage. In Super Broth medium, the two compounds (indole and 1-propanol) that were evaluated more extensively were produced in copious amount relative to the other media.

Headspace sampling of volatile. Volatile collection was accomplished using a solid-phase micro-extraction (SPME) device (Supelco, Inc., Bellefonte, Pa.) as described by Song et al. (1997). Using SPME, analytes are absorbed into a coating on a fused silica fiber exposed to the jar headspace for 5 min. For this study, a fiber was coated with a 100 μm-thick polydimethylsiloxane-based non-bonded stationary phase. After absorption, volatiles were then thermally desorbed from the fiber coating by directly inserting the fiber into the injection port of a gas chromatograph (HP-6890, Hewlett-Packard Co., Wilmington, Del.). For most fruit and vegetable volatiles, 5 min was sufficient time to achieve equilibrium, between the volatiles in the headspace and the fiber coating. However, as much as 25 minutes were required for some volatiles. As long as absorption time is held constant, however, detector response is linear with volatile partial pressure (Song et al., 1997).

MS volatile analysis. Volatile analysis was conducted by using gas chromatography/time-of-flight mass spectrometry. Gases were cryofocused on-column using liquid N_2 and separated in the GC (HP-6890, Hewlett-Packard Co., Wilmington, Del.) Using a 10-meter, 0.1 mm I.D., DB-5 column (Hewlett-Packard Co., Wilmington, Del.). The detector was a benchtop time-of-flight (TOF) mass spectrometer (Pegassus II, LECO Co., St. Joseph, Mich.). This mass spectrometer permitted the resolution and quantification of compounds not separated chromatographically. Identification of volatile material was done by comparison of the mass spectra with high purity authenticated standard compounds (Aldrich, Co., Milwaukee, Wis.). For the standard compounds, a mix of the targeted compounds was prepared in a 4.85-L jar (standard jar), equipped with a self-sealing rubber sampling port. The mix was prepared by first placing 50 µL of each compound into a 2.5-mL vial to generate a stock mix. Then, 10 µL were taken from the stock mix and placed into the 4.85-L jar to be used as standard. Standard preparation was done at 5 °C and the standard jar was kept at the temperature of the experiment. Calculation of the concentration of standard compounds was expressed in ppm. As sampling from the packages was done, so was from the standard to estimate the actual production of the targeted compounds regardless of the sensitivity of the detection system. Data were reported in $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

Monitoring E. coli ATCC-25922 volatiles in produce packages. Produce (brocoli, carrot and lettuce) were obtained as fresh as possible from a local shipper (Steve Produce Company, Lansing, Mich.). Produce were sorted out so that defect-free material would be used in experiments. Broccoli florets were used, lettuce was shredded and carrot was

cut in sticks. Produce was weighed and inoculated with *E. coli*. The number of cells applied to the produce varied between 8 and $1x10^9$ cells per 500 mL of dipping solution. The inoculation was performed by dipping the product for 20 s in solutions containing *E. coli*. The range of number of *E. coli* cells in packages was achieved by serially diluting 1 mL of stock solution containing *E. coli* in jars with 500 mL of tryptophan-based dipping media. We were also interested in determining the effect of tryptophan availability on indole production. The dipping solution was prepared in a way that different amounts of tryptophan were available for the bacteria to evaluate the impact of substrate on the production of targeted volatiles. Five different concentrations of tryptophan were prepared (5, 4, 3, 2, and 1 g of tryptophan in 250 mL of distilled water). Alternatively, threonine was used as the amino acid to evaluate the potential of *E. coli* to metabolize it to 1-propanol. Produce were then placed in pouches of low density polyethylene (LDPE) (LDPE, Dow chemical company, Midland, Mich.) equipped with a gas-sampling septum, made of Dupont Silicone II tub/tiling glue on a short strip of electrical tape as described by Beaudry et al. (1992). Pouches were heat-sealed and stored at 23 °C for evaluation. The dimensions and thickness of the LDPE flim used to construct the modified atmosphere packaging was determined by using the model of Cameron et al. (1989) so that the produce would generate an in-package O_2 level of about 3-5 kPa.

Results

Volatile profiles of E. coli and S. typhimurium in standard laboratory media. Volatile compounds produced by *E. coli* in LB media tentatively identified as carbon

dioxide, ethanol, 1-propanol, cyclobutanol, 1-butanol, 1-pentanol, dimethyl disulfide, cyclopropane-pentyl, octanol and indole (Fig. 1). Volatiles produced by *S. typhimurium* grown under the same conditions as *E. coli* were tentatively identified as carbon dioxide, cyclobutanol, 1-butanol, cyclopentanol-2-methyl, cyclopentanol-3-ethyl, 2-hepten-1-ol, octane-3,3-dimethyl, 3-decanol, decane-2,2,3-trimethyl, dodecane-3,5-dimethyl, tridecane-3-methyl, dodecane-5,8-dimethyl, pentadecane, dodecane-2,4,6-trimethyl, 2-heptadecanol, octadecanol, nonadecanol, heptadecane-2,5,6-trimethyl (Fig. 2).

Impact of the type of media on the production of indole by E. coli. Indole was the most abundant detectable volatile compound produced by *E. coli*. The highest indole production by *E. coli* was observed in the Super Broth media (Fig. 3).

Indole production as a function of the number of E. coli cells. The biggest difference in the ratios of indole over *E. coli* population was between 10 and 25 h (Fig. 4). The highest ratio of indole/*E. coli* population was achieved by using the medium Super Broth. The ratios of indole to *E. coli* population had the order of Super Broth > 2xYT > NZY. The amount of tryptophan available in the media had the same order. When free tryptophan was added to the Super Broth media, indole production was 90-fold higher than the control (Fig. 5).

Identifying microorganism-fingerprint volatiles in modified atmosphere-packed produce. 1-propanol was detected in packages of broccoli, lettuce and carrot inoculated with *E. coli* in a threonine-containing solution (Figs. 7, 8 and 9). Indole, however, was not detected in the produce packages inoculated with an *E. coli*-containing solution. In produce packages contaminated with *E. coli*, fewer *E. coli*-related alcohols were detected

as suppose in laboratory media.

Discussion

Sources of volatiles produced by bacteria. Carbon dioxide and 1-butanol were the only common compounds between *E. coli* and *Salmonella* volatile profiles, suggesting therefore that each organism has a distinctive way of metabolizing the nutrients available in the media. Microbiologists have long recognized that the enormous diversity of microbial enzymes represent a valuable resource for the biosynthesis of desirable molecules (Harper and Nelson, 1982; Jones and Turner, 1971; Stephenson, 1939; Webb, 1984). The fermentation of sugars to ethanol is one of the earliest applications dating back to earliest recorded history, although its enzymatic basis was only revealed in 1897 using a microbial cell-free system (Buchner, 1897). More recently, molecular biological methods have extended the range of applications for microbial enzymes in biotechnology (Grund, 1995; Weast and Grasselli, 1992). For example, naphthalene dioxygenase genes, cloned and expressed in *E. coli*, provide the last enzymatic step for a fermentation process to make the blue jean dye indigo (Ensley et al., 1983).

In order to assess the mechanism and biochemistry of volatile production by *E. coli* and *S. typhimurium*, we should start by evaluating the growing media and the possible substrates for both organisms. The media used in this experiment for time course analysis was Super Broth. Super Broth is rich in tryptone, a media component rich in tryptophan. *E. coli* has been linked to the generation of indole in media containing the substrate tryptophan (Happold, 1950; Gunsalus et al., 1955; Wood, 1947). *E. coli* synthesizes

tryptophanase which catalyzes the cleavage of tryptophan into indole, ammonia and pyruvate. The indole nucleus of tryptophan undergoes many reactions which can be utilized for analytical purposes. The indole detected in our experiment is likely to be the result of the action of tryptophanase provided by *E. coli* (Gunsalus et al., 1955; Happold, 1950; Wood, 1947). *S. typhimurium*, to our knowledge, has not been reported to have active tryptophanase and therefore does not cleave tryptophan to yield indole, ammonia and pyruvate. Our results are in agreement with the literature since indole was not detected in assays *S. typhimurium*.

Casein, one of the component of the Super Broth medium, has 20 amino acids in addition to tryptophan. Amino acids may be broken down by microorganisms in a number of ways in either anaerobic, semi-anaerobic or aerobic conditions (Stephenson, 1939). These include decarboxylation with the formation of some amines followed by hydrolytic deamination with the formation of hydroxy acids, hydrolytic deamination and decarboxylation with the formation of alcohol, reductive deamination with the formation of saturated acids, reductive deamination and decarboxylation with the formation of hydrocarbons, deamination and desaturation with the formation of unsaturated acid and oxidative breakdown to compounds having fewer carbons. In our experiment, we suggest that the series of alcohols detected in media containing *E. coli* (Fig. 1) and *S. typhimurium* (Fig. 2) are likely to be the result of deamination of amino acids with subsequent hydrolytic decarboxylation. In the case of *E. coli*, ethanol production can be attributed to the action of the bacteria on the serine present in the tryptone of the Super Broth media. Serine is first hydrolytically attacked to remove the carbon dioxide and then deaminated,

thereby producing ethanol. The same mechanism is suggested for the microbial biosynthesis of the other alcohols shown in Fig. 1. The amino acids attacked by *E. coli* to produce other alcohols include threonine for 1-propanol, proline for cyclobutanol, glutamine for 1-butanol, and lysine for 1-pentanol. Octanol may be the result of the condensation and reduction of pentanol and butanol.

Another alternative pathway through which *E. coli* could have biosynthesized ethanol and butanol from Super Broth media is by using the metabolite pyruvate which can be generated by *E. coli* via the action of tryptophanase. Moat and Foster (1995) reported that some bacteria are able to produce at least three different alcohols in the presence of pyruvate as is the case when tryptophan has been broken down into indole, pyruvate and ammonia. Bacteria are able to generate acetyl-CoA from pyruvate by action of the pyruvate:ferrodoxin (Fd) oxidoreductase. Acetyl-CoA can act as a hydrogen acceptor giving rise to ethanol. Two moles of acetyl-CoA condense and form acetoacyl-CoA which further is converted into acetone and butyrate via acetoacetate decarboxylase and beta hydroxybutyryl-CoA dehydrogenase respectively. Acetone is further converted into isopropanol and butyrate into butanol.

The dimethyl disulfide detected in the *E. coli*-containing media may form as a result of the degradation of sulfur-containing amino acids such as methionine and cysteine (Moat and Foster, 1995; Stephenson, 1939). Hydrogen sulfide, methyl mercaptan, dimethyl disulfide, and ethyl sulfide have been reported as products of amino acid degradation by microorganisms (Stutz, 1991). The production of methyl mercaptan, and possibly dimethyl disulfide, depends on the presence of carbohydrate and therefore may

be due to the methylation of hydrogen sulfide. The lack of sulfur-containing volatiles from *S. typhimurium* suggests that perhaps the enzymatic system that catalyzes the production of sulfur-containing compounds from sulfur-containing amino acids is missing in *S. typhimurium*.

In a comparinson of *E. coli* volatiles versus *S. typhimurium* volatiles, it appears that *S. typhimurium* synthesizes higher molecular weight alcohol compounds than *E. coli*. *S. typhimurium* has also been reported to exhibit decarboxylase and deaminase activity for amino acids including aspartate, glutamate, ornithine, lysine, arginine, tyrosine, phenylalanine, cysteic acid, diaminopimelic acid, hydroxyphenyl serine, histidine, tryptophan, 5-hydroxytryptophan, and possibly others (Moat and Foster, 1995). Most of these amino acids were present in the growing media used in this experiment. This may explain the production of various alcohols detected in the media containing *S. typhimurium* (Fig. 2).

Another component of the media used in this experiment was yeast extract. Yeast extracts are concentrates of the soluble components of yeast cells. Fresh yeast contains 6 to 8 % RNA. Special strains may contain 13 % or more (all figures referring to dry solids contents). The yeast RNA is mainly degraded by bacteria into 5 prime nucleotides of guanine (GMP), adenine (AMP), cytosine (CMP) and uracil (UMP) are formed (Stephenson, 1939). The decomposition of nucleotides can occur in three ways: a) by only releasing the phosphate, b) by releasing all the phosphate, the ribose, and the base; and c) by only releasing the base. However, no literature substantiating possible production of alcohols, sulfides, indole, esters, and fatty acids as nucleic acid breakdown

products and by action of *E. coli* or *S. typhimurium was found.*

Potential of 1-propanol and indole as bacterial fingerprint compounds. 1-Propanol offers the best characteristics to be considered as a fingerprint compound for *E. coli* for the types of vegetables in packages (broccoli, lettuce, and carrot) that we used in our experiments (Figs. 7, 8, 9). 1-Propanol increased in packages over time and the rate of increase was dependent on the inoculum concentration (Fig. 8). Presumably the biosynthesis mechanism of 1-propanol by the action of the bacteria on threonine (Fig. 10), is favored by a higher initial population of the bacteria.

Both indole and 1-propanol have the potential to be used as fingerprint compounds for *E. coli* in both standard laboratory media and in different produce packages. 1-propanol and indole are volatiles produced by *E. coli* but not by broccoli, carrot or lettuce and this information may be exploited in a more practical way. Indole was not detected in produce packages, however, due perhaps to the competition of other carbohydrate sources. Nevertheless, indole may be promising in other types of food such as meat, dairy products and egg. Fingerprint volatiles of microorganisms not only offer a new way of identifying microbial development in food but also offer information on the biochemistry and metabolism of the organisms. With the availability of rapid, accurate and more sensitive volatile analysis techniques, it worthwhile to further investigate volatiles produced by microorganisms as a research practical and research applications.

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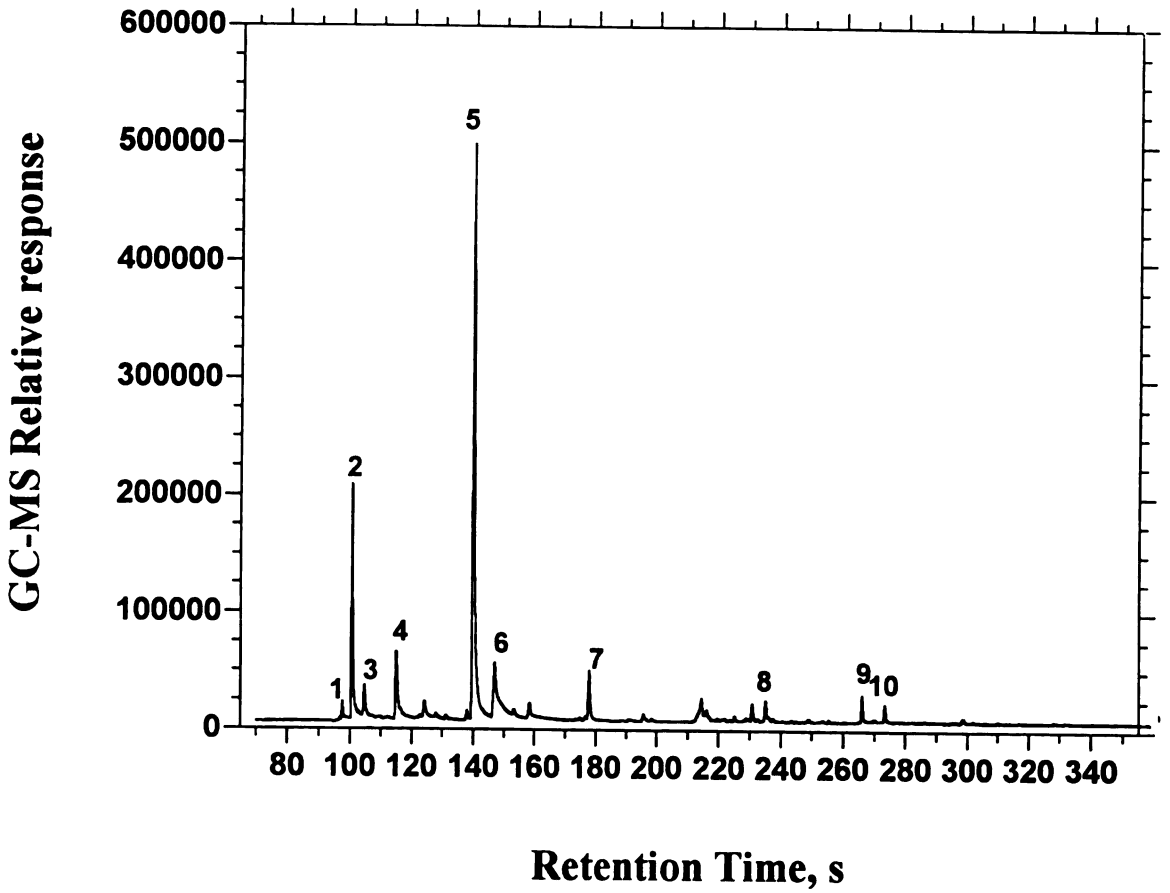

Figure 1. Spectrum of volatiles produced by *E. coli* in Super Broth media. These volatiles were generated in a closed system at room temperature. (1) carbon dioxide, (2) ethanol, (3) 1-propanol, (4) cyclopropanol, (5) butanol, (6) 1-pentanol, (7) disulfide dimethyl, (8) cyclopropane-pentyl, (9) octanol, and (10) indole. Identification of compounds is tentative, based on comparison to spectra in NIST.

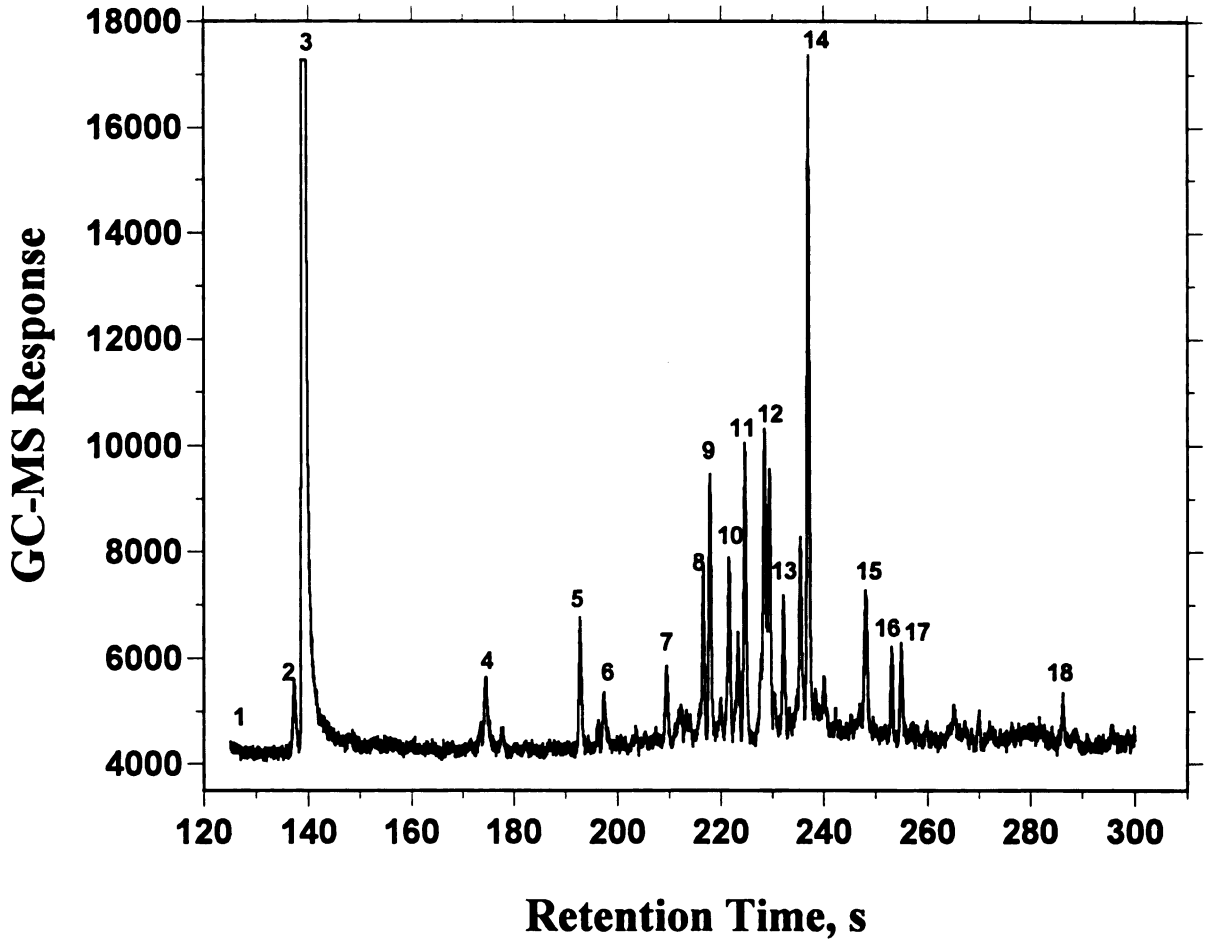

Figure 2. Spectrum of volatiles produced by *S. typhimurium* in Super Broth media. These volatiles were generated in a closed systems at room temperature. (1) carbon dioxide, (2) cyclobutanol, (3) 1-butanol, (4) 2-methyl cyclopentanol, (5) 2-ethyl cyclopentanol, (6) 2-hepten-1-ol, (7) 3,5-dimethyl octane, (8) decanol, (9) 2,2,3-trimethyl decane, (10) 3,5-dimethyl dodecane, (11) 3-methyl tridecane, (12) 5,8-dimethyl dodecane, (13) 2,4,6-trimethyl dodecane, (14) pentadecane, (15) 2-heptadecanol, (16), Octadecanol, (17) nonadecanol, (18) 2,5,6-trimethyl heptadecane. Identification of compounds is tentative, based on comparison to spectra in NIST.

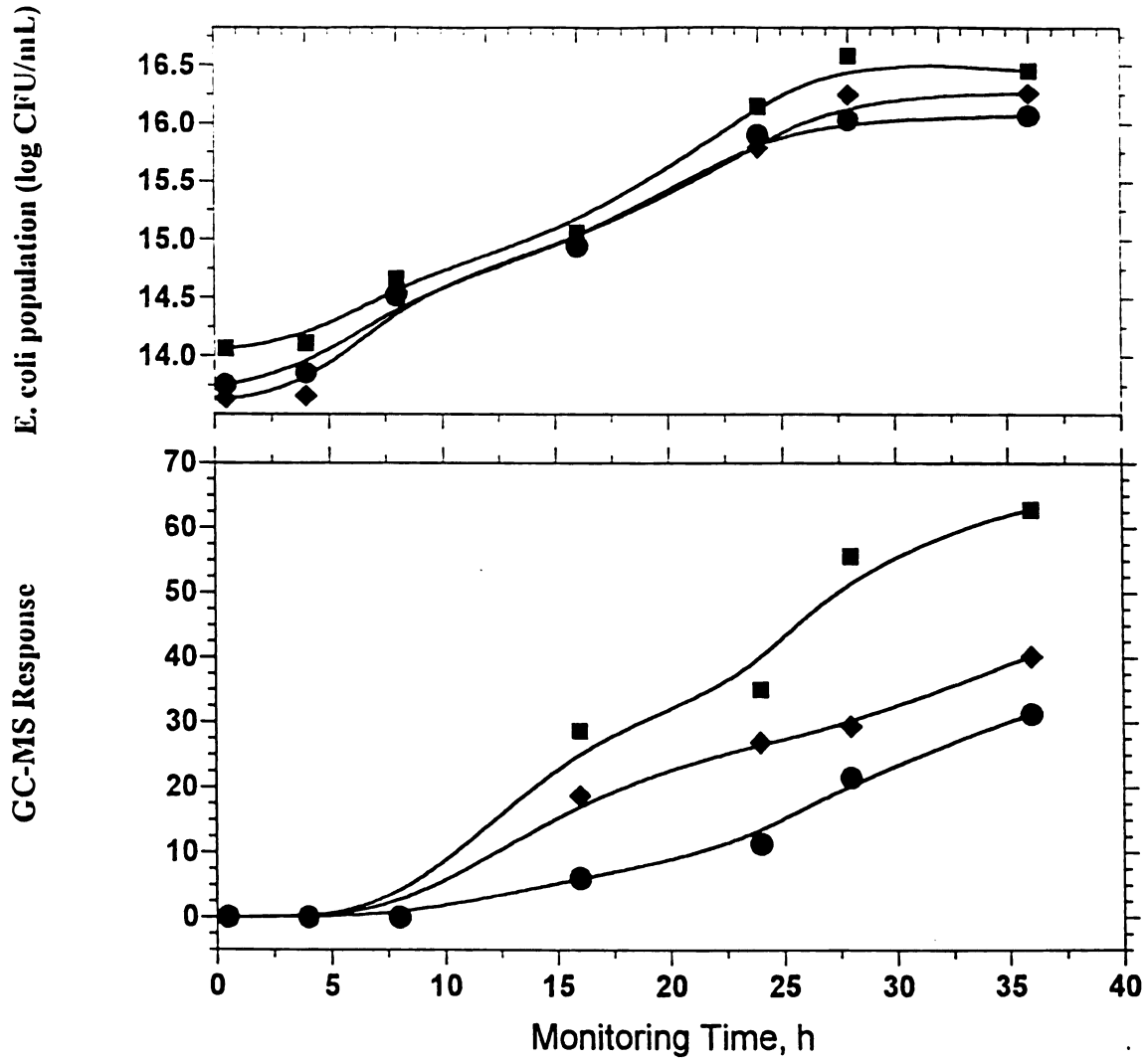

Figure 3. The top graph represents *E. coli* population over time as detected using spectrophotometer measurements at 595 nm. The bottom graph represents indole production by *E. coli* in three different standard laboratory media. Square symbols represent indole in the Super Broth media, diamond symbols are for the 2xYT media and circles are for the NZY media.

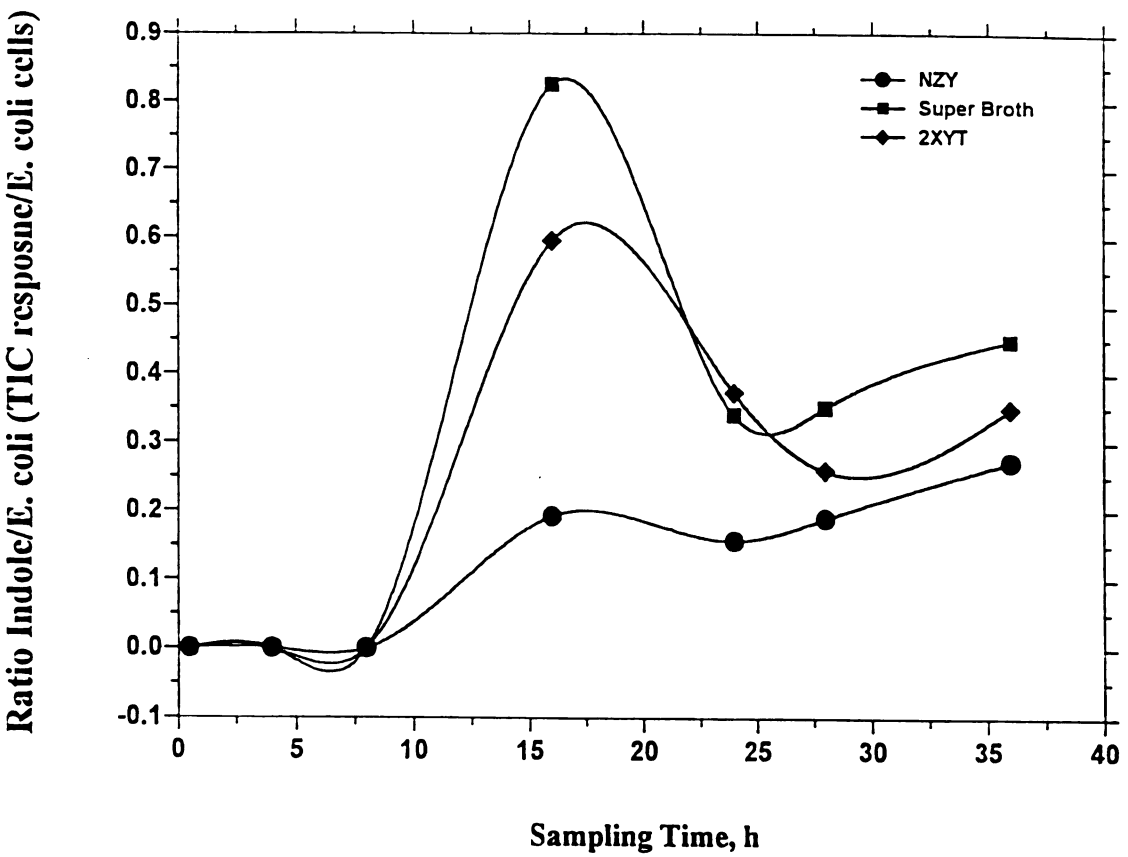

Figure 4. Ratios of indole production by *E. coli* to population of the organism in the media. Square symbols represent indole in the Super Broth media, diamond symbols are indole from the 2xYT media and circles symbols are indole from the NZY media.

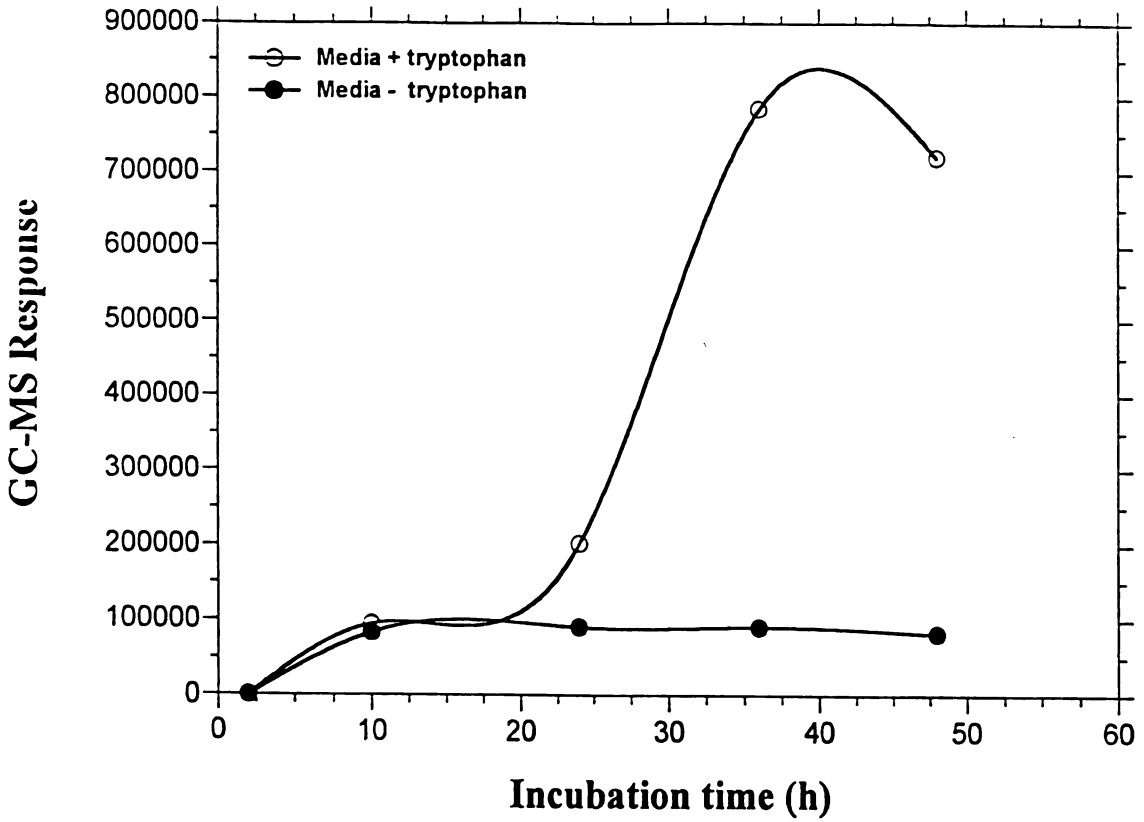

Figure 5. Indole production by *E. coli* in Super Broth media with (open circles) and without (closed circles) tryptophan incubated at RT.

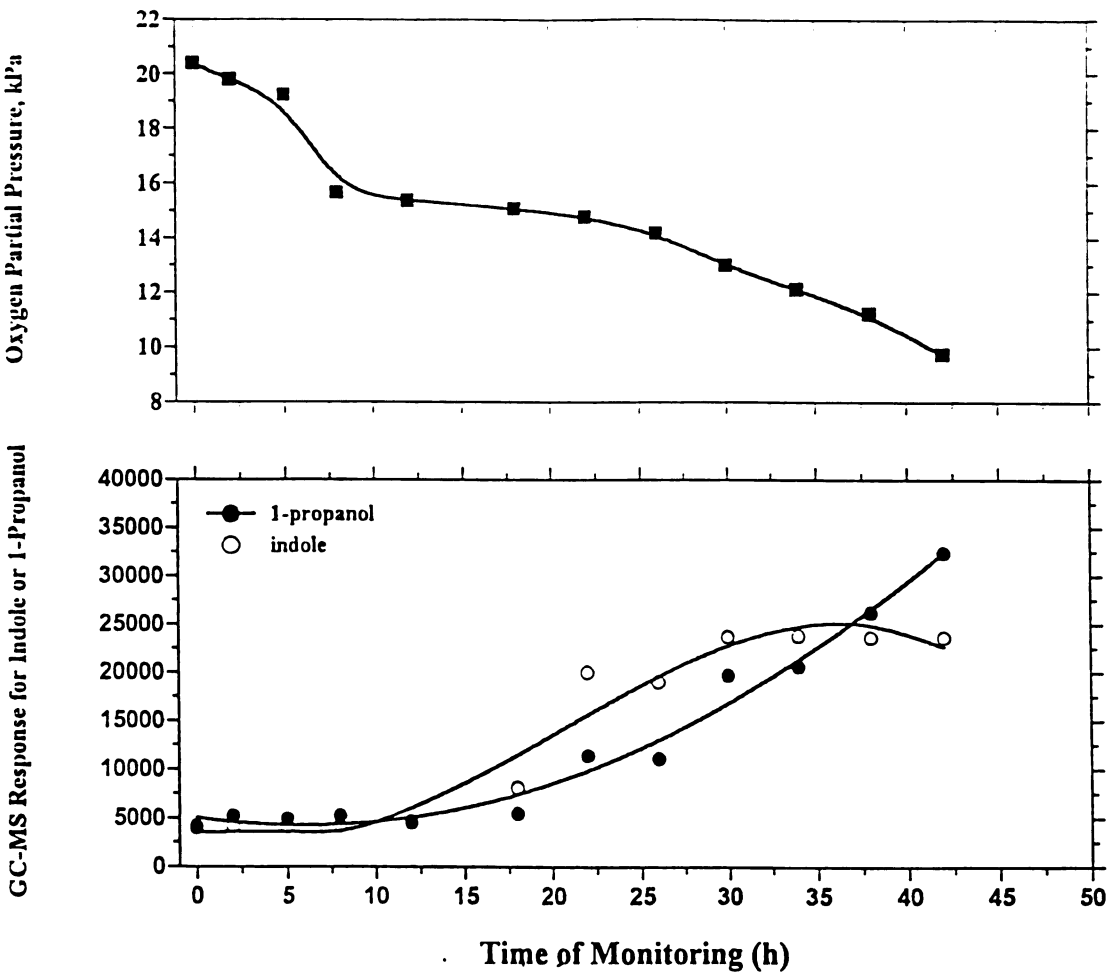

Figure 6. Indole (open symbols) and 1-propanol (closed circles) production. *E. coli* grown in Super Broth medium and a closed system and at RT. Top graph represents oxygen depletion in incubation chamber.

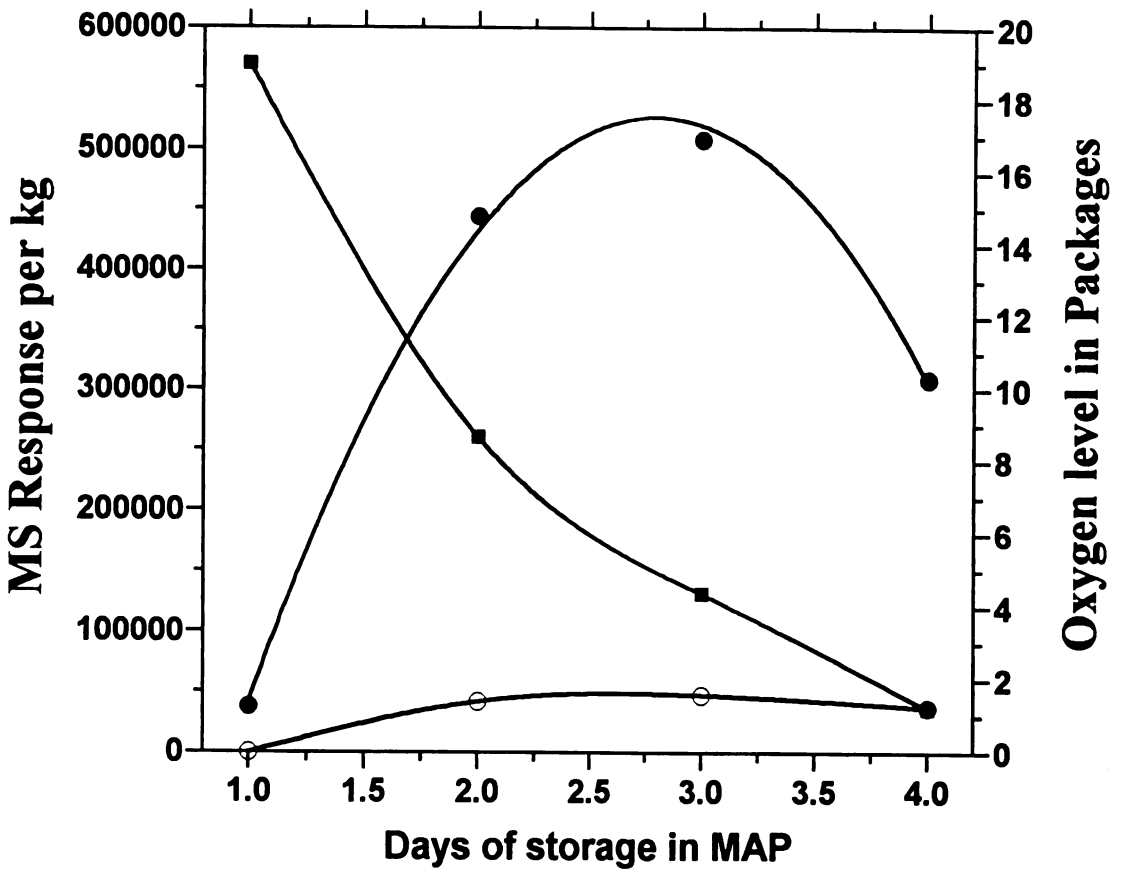

Figure 7. Pproduction of 1-propanol by *E. coli* in broccoli packages at RT. Closed circles represent 1-propanol detected in packages inoculated with *E. coli*, open circles are 1-propanol detected in packages without *E. coli*. Squares symbol represent the depletion of in-package oxygen over time.

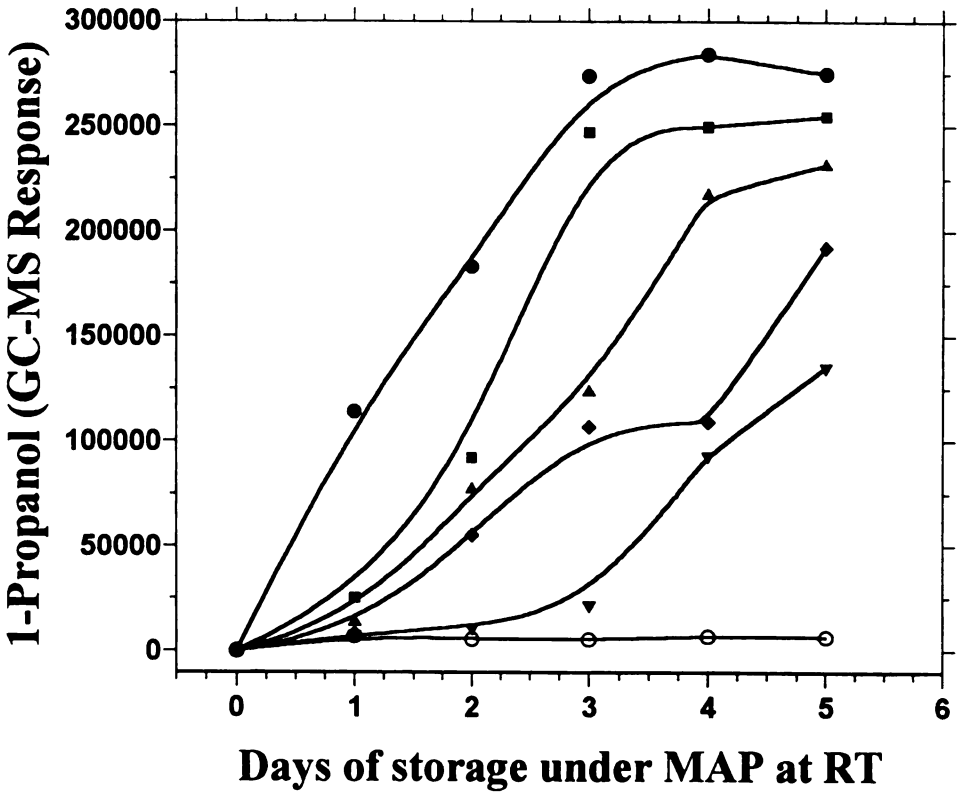

Figure 8. Accumulation of 1-propanol produced by *E. coli* on broccoli in modified atmosphere packages as a function of the initial bacterial population (IP). Circles represent 1x109 cells/mL in the dipping solution, squares represent IP = 2,000,000 cells/mL, triangles represent IP = 4,000 cells/mL, diamonds IP = 8 cells/mL inverted triangles IP = 8 cells/500 mL and open circles IP = 0 cells/mL.

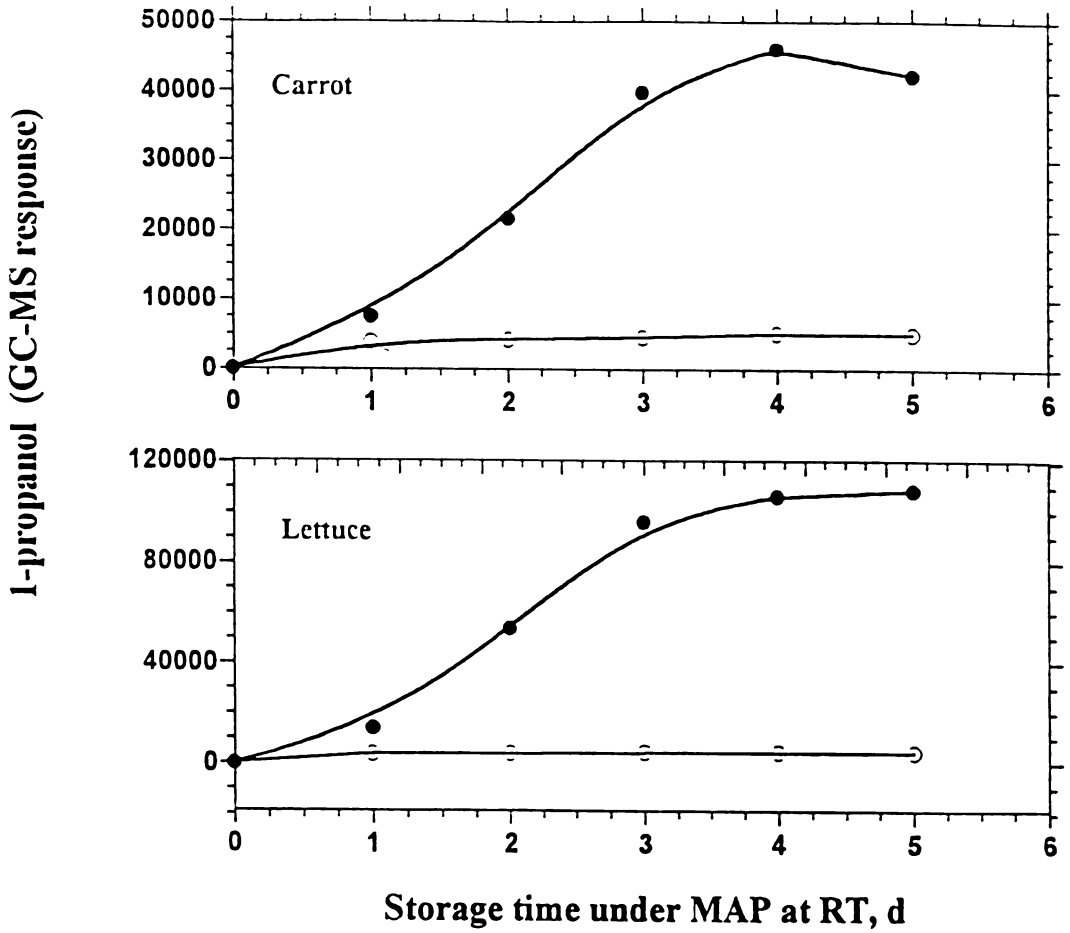

Figure 9. 1-Propanol accumulation in LDPE packages of carrot (top) and lettuce (bottom) incubated at RT inoculated with threonine solution contaminated with (closed circles) and without (open circles) *E. coli*.

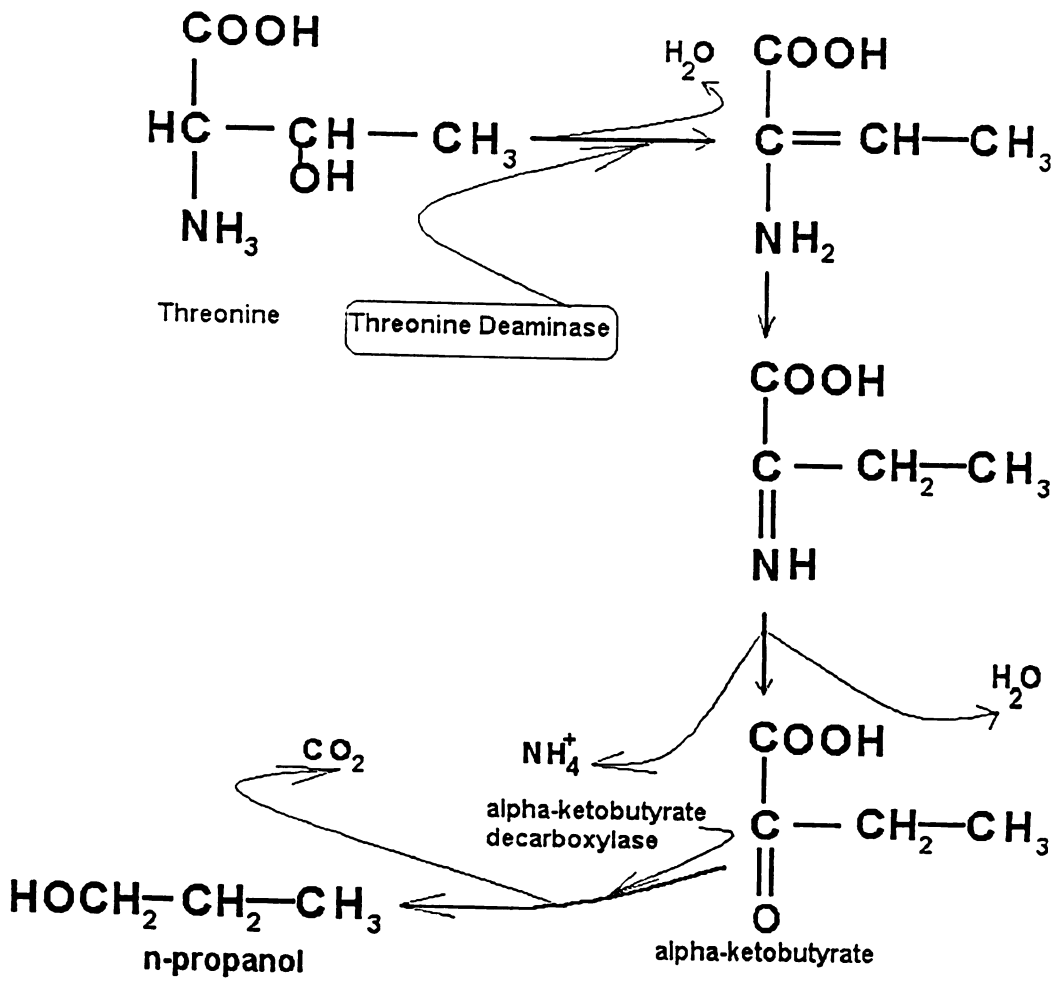

Figure 10. Schematic of the mode of action of E. coli on the breakdown of threonine in packages of broccoli.

CHAPTER V:

STORAGE DURATION, LOW O_2 AND ELEVATED CO_2 ALTER ESTER BIOSYNTHESIS IN STRAWBERRY

STORAGE DURATION, LOW O_2 AND ELEVATED CO_2 ALTER ESTER BIOSYNTHESIS IN STRAWBERRY

Additional index words. modified atmospheres, volatile, mass spectrometry, aroma, *Fragaria ananasa* Dutch.

Abstract. A combination of solid phase micro extraction (SPME) as a sampling method and gas chromatography/time-of-flight mass spectrometry (GC-TOF-MS) for gas analysis were used to investigate the volatiles produced by strawberry *(Fragaria ananasa* Dutch.) stored for eight days in modified atmosphere packages. A range of steady-state atmospheres (2 to 16 kPa O_2, and 2 to 20 kPa CO_2) was developed by enclosing the fruit in LDPE pouches at 0 °C. There was a decrease in volatile production as the concentration of O_2 in the packages decreased and the concentration of CO_2 increased. Volatile esters, known to be very important in the flavor of strawberry, declined with a decrease in package O_2 and a concomitant increase in CO_2. MAP methyl acetate, methyl butanoate, methyl hexanoate and ethyl butanoate. Strawberry fruits were also exposed to a range of O_2 levels in a flow-through system (1.3, 2.4, 3.6, 6.3, 9.5, and 16 kPa). Low O_2 alone reduced the production of methyl butanoate, methyl hexanoate and hexyl acetate but not methyl acetate and ethyl acetate. The data suggest that both low O_2 and high CO_2 alter ester production, each acting in specific ways.

The time devoted to domestic meal preparation has continuously decreased during the last decade and the time spent in restaurants and eating places continue to rise (Hobson and Tucker, 1996). In order to fulfill this consumer-led demand, new storage handling and delivery techniques have had to be developed. One such technique is modified atmosphere packaging (MAP) (Beaudry et al., 1993; Cameron et al., 1994; Cameron et al., 1989; Church and Parsons, 1994). Modified atmosphere packaging could be defined as the enclosure of food products in gas-barrier materials, in which the gaseous environment is manipulated in order to reduce unwanted metabolic events or inhibit spoilage agents and therefore either maintain a higher quality within a perishable food during its natural life (Beaudry et al., 1992; Ben-Arie and Zutkhi, 1992; Cameron et al., 1989; Church and Parsons, 1994; Kader et al., 1989; Vergano and Pertruit, 1993). With modified atmosphere packaging, the atmosphere inside a package generally is low in O_2 and high in CO_2 (Beaudry et al., 1992; Cameron, 1989; Church and Parsons, 1994; Gorris et al., 1996). The composition of the atmosphere at any particular time is a function of the initial gas composition, package volume, gas transmission rate, and the respiratory activity of the produce (Barmore and Rouse, 1976; Gorris et al., 1996; Li and Kader, 1989; Sommer et al., 1973).

The effect of O_2 and CO_2 on aroma compound production has not been well characterized, although elevated CO_2 and or reduced O_2 levels have also been suggested to be the causal factors in off-flavors and off-odors in strawberry stored in MAP. To assess quality, consumers rely a great deal on their perception of the aroma of the food they buy. Therefore, a better understanding of the relationship between the in-package

levels of O_2 and CO_2 in MAP-stored fruit and changes in both aroma-related and off-odor-type volatiles may be of importance in the design of optimized packaging systems. Our objective was to evaluate the impact of modified atmosphere packaging on aroma production by strawberry and more specifically on ester production.

Material and Methods

Plant material. Strawberry (*Fragaria ananasa* Duch.) fruit of the cultivar 'Sweet Darling' not previously treated with CO_2 were shipped overnight under refrigerated air from Florida (BBI Produce, Inc., Dover, Fla.) to the Detroit Produce Terminal in Detroit, MI (Rocky Produce Co., Detroit, Mich.). Fruit were sorted for visible defects and further sanitized in a solution of 0.5 $g \cdot L^{-1}$ Benlate (Du Pont de Nemours Co., Agricultural Product Department, Wilmington, Del.) and 0.5 $g \cdot L^{-1}$ Rovral (Rhone-poulenc Ag. Company, Research Triangle Park, N.C.). Following application of the fungicide, fruits were air-dried at 0 °C for 5 h. Fruit were then placed in pouches of LDPE and stored at 0 °C for evaluation or in flow-through chambers.

Modified atmosphere packaging of strawberry. The design of modified atmosphere packaging was achieved by using the approach of Beaudry et al. (1992) as adapted from Cameron et al. (1989). Fruit were sealed in pouches made of 0.00762 cm (3 mil) thick low-density polyethylene (LDPE; LDF 550, Dow Chemical Company, Midland, Mich.). The initial step in the package design for strawberry was the determination of respiration rates (r_{O2}) of whole strawberries stored at atmospheric O_2 partial pressure (20.7 kPa) at 2 °C. Using the estimated r_{O2}, the needed film area and fruit weight were

calculated to generate 15 kPa O_2. From this high O_2 setting, fruit weight was varied to achieve a range of O_2 levels. Fruit weights used were 15, 41, 120, and 360 g.

Determination of film permeability. The O_2 and CO_2 permeability of the LDPE film (3 mil) was measured on three random film samples at 0, 5, 10, 15, 20 and 25 °C according to the method described by Beaudry et al. (1992). A specially-built stainless steel permeability cell was submerged in a water bath (Lauda RC20; Brinkman Instrument Co., West Bury, N.Y.). Temperature of the permeability cell was measured using thermocouple and the temperature of the bath was verified with a mercury thermometer. The permeability cell contained two circular 25-mL chambers separated by the film sample and sealed in place by an O-ring. The cell chambers were 8 cm in diameter and 0.5 in depth and surface area 50 cm^2. Copper coils tubing were attached to the inlet for the N_2 carrier supply line. Exit lines were composed of stainless steel. The passage of the gas through the coils before entering the cell allowed the system to be isothermic, i.e. the entering gas had the same temperature as the cell. The permeant mixture of O_2 and CO_2 (65 and 35 kPa, respectively) was introduced to one chamber of the cell and N_2 carrier gas was introduced to the other chamber. The rate of O_2 and CO_2 permeation through the film was calculated from the steady-state partial pressure difference between the two streams. The partial pressure of O_2 and CO_2 in the carrier gas stream was determined from concentration measurements obtained using a sequential combination of O_2 and CO_2 analyzers. To measure the O_2 concentration, a calcia-zirconia analyzer (Ametek S3A/II, Ametek Co., Thermox Instrument Div., Pittsburgh, Pa.) Was used. CO_2 was measured with an infrared gas analyzer (ADC 225-MK3, Analytical Development Co.,

Hertfordshire, England). Concentrations were calculated relative to a certified standard gas mixture (106 uL/L O_2 and 100 uL/L CO_2 in N_2 gas). Flow rates were maintained between 110 and 130 mL/min for all gases, and the chamber pressure were equalized and maintained at about 0.4 kPa above atmosphere.

Concentration data were converted to partial pressure using the ideal gas law equation. The partial pressure difference and the measured rate of permeant flux from the receiver cell were used to determine the permeability coefficient using Fick's Law. Values of the permeability as a function of the temperature were fitted by the Arhenius equation:

$$P_i = P_c e^{\left(-\frac{Ep}{RT}\right)}$$

where P_i is permeability coefficient at any temperature in Kelvin. Pc is permeability constant. R is the gas constant (0.0083144kJ•mol^{-1}•K^{-1}). Ep is activation energy in kJ. mol^{-1}. T is temperature in Kelvin. This equation was converted to:

$$\ln(P_i) = \left(\frac{Ep}{RT}\right) + \ln(P_c)$$

Package gas composition. Strawberries were placed into LDPE pouches and the pouches were heat sealed. A gas-sampling septum, made of Dupont Silicone II tub/tiling glue on a short strip of electrical tape was attached to the surface of the package as described by Beaudry et al. (1992). Gas samples were drawn from each package through the self-sealing silicone septum using a 0.5 mL insulin syringe. Three gas samples were

analyzed from each package at each evaluation using an O_2 analyzer (Sevomex Paramagnetic O_2 Transducer, Series 110, Servomex Co., Sussex, England) and CO_2 analyzer (ADC analytical Development Co., Hoddesdon, England) connected in series, with N_2 as the carrier gas (flow rate = 100 mL/min). Average of the three taken samples was used in analysis. The gas composition of individual packages was calculated using a certified standard (1.95% O_2 and 4.85% CO_2). Gas partial pressure were monitored daily for 8 days.

Analysis of volatiles. Volatile collection was accomplished using solid-phase micro-extraction (SPME) device (Supelco, Inc., Bellefonte, Pa.). A fiber with a 100 μm-thick, non-bonded, polydimethylsiloxane-based stationary phase was used. The absorption time was 5 min. After absorption, volatiles were then thermally desorbed from the fiber coating by directly inserting the fiber into the injection port (200°C) of a gas chromatograph (HP-6890 Hewlett Packard Co., Wilmington, Del.) for 90 s. Gases were cryofocused on-column using liquid N_2 and separated using a gas chromatograph fitted with a 10-meter, 0.1 mm I.D., DB-5 capillary column (Hewlett Packard Co., Boise, Idaho). The detector was a time-of-flight (TOF) mass spectrometer (Pegasus II, LECO Co., St. Joseph, Mich.). The TOF mass spectrometer permitted the resolution and quantification of compounds not separated chromatographically. Identification of volatile material was done by comparison of the spectra and GC retention times with authenticated standard compounds. High purity (>95%) authenticated standards were obtained from Aldrich (Aldrich, Co., Milwaukee, Wis.). For the standard compounds, a mix of the targeted compounds was prepared in a 4.85-L jar (standard jar), equipped with a self-

sealing rubber sampling port. The mix was prepared by first placing 50 µL of each compound into a 2.5-mL vial to generate a stock mix. Then, 10 µL were taken from the stock mix and placed into the 4.85-L jar to be used as standard. Standard preparation was done at 5 °C and the standard jar was kept at the temperature of the experiment. Calculation of the concentration of standard compounds was expressed in ppm. As sampling from the packages was done, so was from the standard to estimate the actual production of the targeted compounds regardless of the sensitivity of the detection system. Data were reported in $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

Results

Strawberry volatiles in packages. Strawberry volatiles detected include esters, alcohols, hydrocarbons, and ketones (Fig. 1 and Table 1) although many of the identifications are tentative in that appropriate standards are lacking. This is in agreement with previous reports (Shamila et al., 1992; Shamaila et al., 1992a). Among these groups of volatiles, esters represented the dominant class which is consistent with data reported by Manning (1993). Identified esters included methyl, ethyl, propyl, butyl, pentyl, hexyl, octyl, and nonyl esters. Sulfur compounds, which have been reported to be part of the strawberry volatiles profile (Dirinck et al. 1977; Dirinck et al., 1989; Lieten and Dirinck, 1991), were not detected in study. Furanones detected include, 2-(2-furanylmethyl)-5-methyl furan and, 2,5-dimethyl butyl furan. Furanones have been referred as the character impact compounds of strawberry (Manning, 1993).

Effect of modified atmosphere on strawberry volatiles over time. Aroma

production of most strawberry volatiles declined markedly during the 8 d package sealing, independently of the O_2 and CO_2 concentration in the packages (Fig. 2). Volatiles which declined over time such as methyl butanoate and acetone reached the lowest levels of biosynthesis at d 8 and stayed low thereafter.

The degree of suppression of volatile production as a function of in-package O_2 and CO_2 was evaluated for selected volatiles including methyl hexanoate, acetone, methyl butanoate, ethyl butanoate, methyl acetate and hexyl acetate (Table 2). The rate of suppression for the selected volatiles ranged from 10 fold to 26.2 fold. The rate of suppression was estimated by dividing the GC-MS response in packages with 15 g of fruit over the GC-MS response in packages with 360 g of fruit.

The ratios of methyl acetate to methyl butanoate and hexyl acetate to methyl hexanoate did not seem to be affected by O_2 and CO_2 partial pressure combinations (Fig. 4). The ratio of methyl acetate to methyl butanoate was steady during the first 6 d of storage and increased markedly after d 6. The ratio of methyl acetate to methyl butanoate may be used as indicator of shelf-life of MAP-stored strawberry. The break point was estimated to be day 6. On the other hand, the ratio of hexyl acetate to methyl hexanoate increased continuously over time. This ratio may also have potential to be used as a shelf-life indicator.

Effect of low O_2 alone on the production of strawberry volatiles. In order to determine whether the impact of MA on volatile production by strawberry was a particular effect of the combination of low O_2 and high CO_2, we monitored volatile production by strawberry exposed to low O_2 alone. Low O_2 alone has a different impact on volatile

production by strawberry than a combination of low O_2 and high CO_2. Low O_2, for instance, enhance the production of methyl acetate, acetone and ethyl butanoate (Fig. 5) which appeard to be supressed by the combination of low O_2 and high CO_2 (Fig. 5).

Discussion

In contrast to what has been reported by Yahia et al. (1991) on the differential effect of low O_2 and high CO_2 levels on the production of specific volatiles by 'McIntosh' and 'Cortland apples, in strawberry, there was a severe suppression of volatile production by low O_2 and high CO_2. It may indicate that atmosphere modification in strawberry affected the activity of key enzymes in the ester production system.

Williams and Knee (1977) reported that esterifying enzymes operate at similar rates in fruits stored in air or in 2% O_2. Thus, changes in the suppression of particular volatiles such as methyl butanoate, acetone, and methyl hexanoate (Figs. 2, 3) in steady-state atmospheres are suggested to be due to limited supply of volatile precursors, such as alcohols and CoA fatty acids. Perhaps, at d 8, the production precursors to alcohols such as amino acid derivatives necessary for the production of esters in strawberry stored in MAP was limited. This theory, however, may not be likely in regard to alcohols in strawberry since very few alcohols were identified. Nevertheless, the only alcohol of importance we found, butanol, appeared to be markedly reduced by low O_2 atmospheres (Table. 1). A decrease in the in-package O_2 from 17.5 kPa to 3 kPa induced a decrease in butanol production in by 99.6%.

Isolation of the low-oxygen effect on volatile production by strawberry. The

information regarding ester production formation in modified atmosphere-packaged strawberries with a combination of low O_2 and high CO_2 atmospheres and the response to a range of O_2 partial pressures with no CO_2 permits the isolation of the effect of low O_2 and high CO_2 on ester biosynthesis (Fig. 5). Esters, except those of fermentative metabolism (ethanol, ethyl acetate and ethyl butyrate), have been suggested to be produced *via* ß-oxidation to generate short chain fatty acids and *via* peroxidation to generate a pool of alcohols (Ke et al., 1994). At least three controlling points can be suggested in which low O_2 and or high CO_2 have effect on the production of esters in strawberry (Fig. 5). 1) Low O_2 has a positive direct effect on the activity of both pyruvate decarboxylase and acetaldehyde dehydrogenase. Low O_2 activates these two enzymes and the substrate ethanol is produced in abundance. High carbon dioxide may have less direct effect, as reported by Ke et al. (1994) via induced changes in pH. 2) Low O_2 can also have some effect on one or more of the peroxidases responsible for peroxidation of saturated fatty acids. Equally, high CO_2 may also have some effect on these peroxidases which may not be necessarily the same enzymes affected by low O_2. The effects of either or both low O_2 and or high CO_2 on these peroxidation enzymes would result in changes in the production of alcohols which in turn will affect the total production of esters. This concept is supported by our results which showed that the suppression of ester production in strawberry is a combination of both low O_2 and high CO_2. 3) Low O_2 and or high CO_2 might induce changes in ester production during ß-oxidation of unsaturated fatty acids. Possible enzymes that are affected include: a) the set of fatty acid dehydrogenase enzymes and the ß-ketoacyl-CoA thiolase responsible for the generation of acetyl-CoA out of fatty

acids, 3) The third and last point at which low O_2 and/or CO_2 may exert some effect is during the formation of short chain fatty acids from elongating FA chains via an ACP thioesterase that cleaves the FA from the acyl carrier protein. The synthesis of fatty acids from acetyl-CoA and malonyl-CoA involves seven enzymatic reactions (Voet and Voet, 1990). These reactions include enzymes such as reductases and dehydrases as well as acyl carrier proteins.

In conclusion, the impact of modified atmospheres on the production of esters by strawberry is a combined inhibitory effect of low O_2 and high CO_2 . The mode of action of O_2 and CO_2, however, is not clear yet. It appears to be by interfering the biosynthesis of substrates such as alcohols and acids since the enzyme alcohol acetyl CoA transferase has been reported to have little sensitivity to either low O_2 or high CO_2. However, the results of this study suggest that developing the appropriate package design, as it affects O_2 and CO_2, is of paramount importance to achieve high quality product since the aroma at the time the consumer opens a package is what is perceived and used to judge quality..

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Table 1. Effect of fruit weight in 15 cm x 20 cm LDPE packages on O_2, CO_2 and strawberry volatile production after 3 d at 22 °C.

		MS Response 3 d under MAP for each mass of strawberry in packages									
No.	Mass O_2/CO_2 levels (kPa)	15 g 18.8/3	41 g 15/7.1	120 g 9/13	360 g 3/20	No.	Mass O_2/CO_2 levels (kPa)	15 g 18.8/3	41 g 15/7.1	120 g 9/13	360 g 3/20
1	Ethanol	.23	.28	.3	1.5	22	4-methyl 1-heptane	83.2	9.7	1.82	1.25
2	Butane	6.9	1.98	.29	.23	23	Methyl Hexanoate	5.14	.85	7.16	.11
3	Acetone	4.1	.75	.8	.56	24	3-methyl 1-heptane	12.3	4.14	.47	.018
4	Butanol	28.8	7.3	.31	.12	25	Ethyl hexanoate	12.6	4.06	.19	.29
5	Methyl acetate	10.5	8.5	3.3	2.78	26	Hexyl acetate	11	7.8	1.49	.05
6	Ethyl acetate	0.35	0.60	1.5	1.9	27	2-methyl, 2 heptane Ethyl	64	21.7	.24	4.4
7	Propyl acetate	26	15	2.8	.35	28	2,7-dimethyl octanol	62.24	5.64	10.67	3.7
8	Isopropyl acetate	1.4	1.7	1.6	1.99	29	4,6,8-trimethyl 1-nonene,	4.6	1.4	3.12	.13
9	2,2-dimethyl Hexene	39.44	13	1.6	.04	30	Butyl hexanoate	13.3	4.1	.34	.3
10	Methyl butanoate	116.5	24.3	5.3	4.57	31	5-butyl 4-nonene	.62	.6	.7	.11
11	2,5-dimethyl Hexene	2.72	1.1	8.55	1.57	32	2-(2-furanylmethyl)-5-methyl furan	1.8	.9	.22	.09
12	4-methyl 1-heptyl	26.6	7.16	.35	.095	33	2,5-ditert-butyl furan	6.8	5.27	.33	.02
13	2,3,3-trimethyl pentene	21.4	7.8	2.56	0	34	2,4,6-trimethyl decane	10.30	6.53	.045	.8
14	3-methyl heptane	17.7	3.2	1.38	.71	35	2,5,9-trimethyl decane	.92	.45	5.93	.01
15	Ethyl butanoate	48.7	1.36	1.82	.12	36	Pentyl hexanoate	21.98	.69	.11	.26
16	Butyl acetate	86.7	19.8	.28	.19	37	Nonyl acetate	1.06	.59	.2	.06
17	Propyl butanoate	92.6	34	13	2.78	38	Tridecane	2.34	.41	.054	.08
18	Ethyl pentanoate	23.26	4.46	16.4	.23	39	Octyl butanoate	.72	.39	.21	.04
19	3-methyl octene	2.54	.77	2.51	1.6	40	Octanal dimethyl acetate	2.32	1.06	.23	.03
20	Butyl butanoate	2.39	.78	.76	.22						
21	Tridecene	24	22	.055	.05						

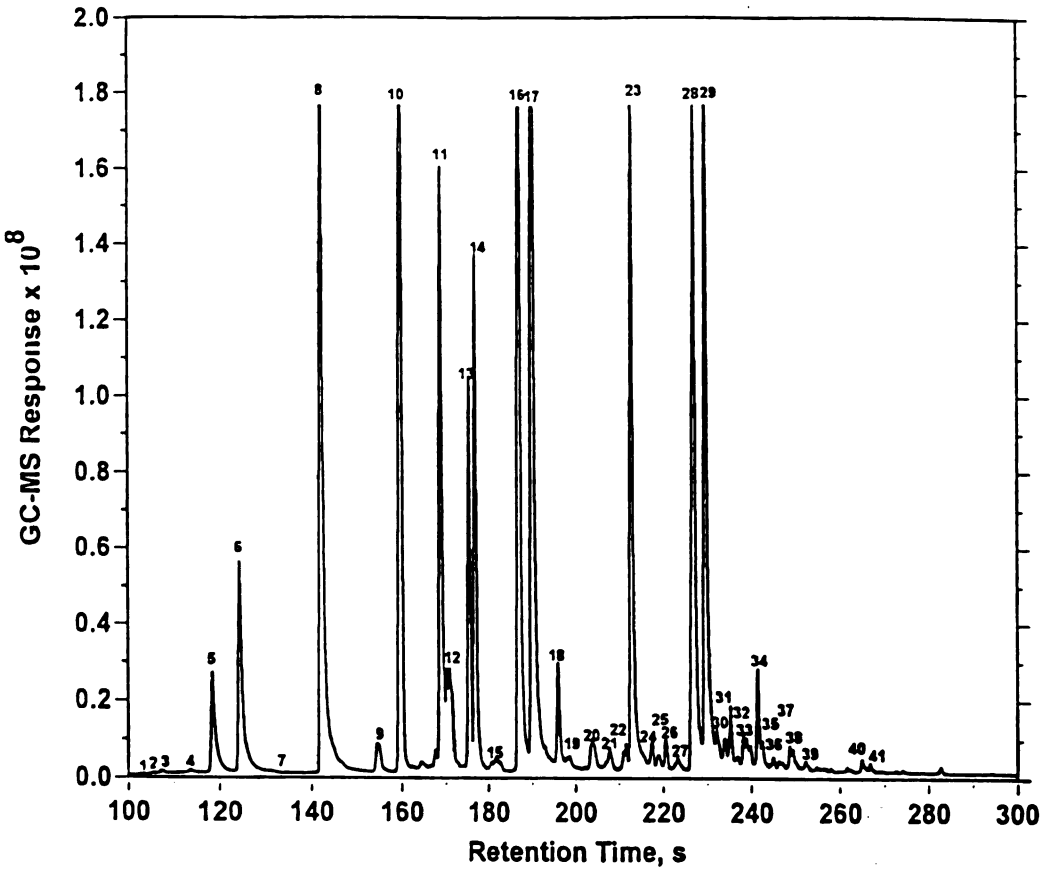

Figure 1. GC-MS Spectrum of the total ion count (TIC) for strawberry enclosed in a sealed jar for 40 min on day 0. Identified volatiles include: 1) carbon dioxide, 2) Ethanol, 3) butane, 4) acetone, 5) butanol, 6) methyl acetate, 7) ethyl acetate, 8) propyl acetate, 9) isopropyl acetate, 10) 2,2-dimethyl hexene, 11) methyl butanoate, 12) 2,5-dimethyl hexene, 13) 4-methyl-1-heptyl, 14) 2,3,3-trimethyl pentene, 15) 3-methyl heptane, 16) ethyl butanoate, 17) butyl acetate, 18) propyl butanoate, 19) ethyl pentanoate, 20) 3-methyl octene, 21) butyl butanoate, 22) tridecene, 23) 4-methyl-1-heptane, 24) ethyl hexanoate, 25) 3-methyl-1-heptane, 26) ethyl hexanoate, 27) hexyl acetate, 28) 2-methyl-2-heptane ethyl, 29) 2,7-dimethyl octanol, 30) 4,6,8-trimethyl-1-nonene, 31) butyl hexanoate, 32) 5-butyl-4-nonene, 33) 2(2)-furanylmethyl)-5-methyl furan, 34) 2,5-dimethyl-butyl furan, 35) 2,4,6-trimethyl decane, 36) 2,5,9-trimethyl decane, 37) pentyl hexanoate, 38) nonyl acetate, 39) tridecane, 40) octyl butanoate, 41) octanal 2,5-dimethyl acetate.

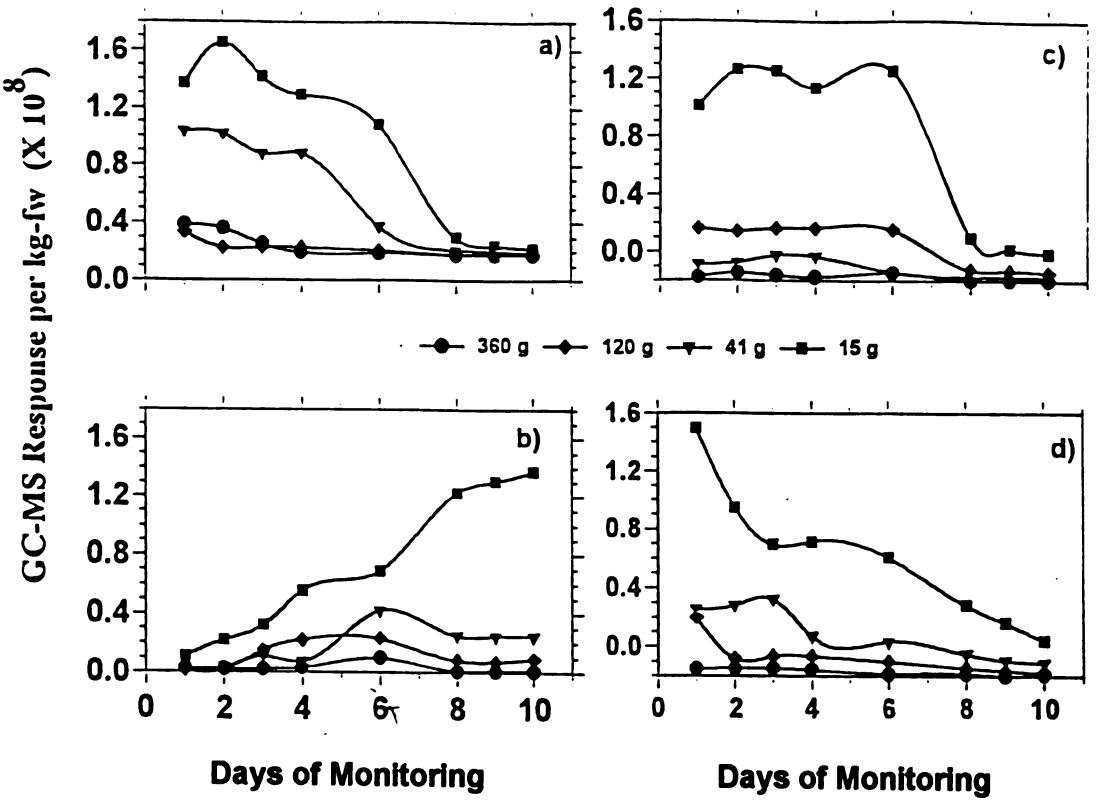

Figure 2. Trend over time of four selected different strawberry volatiles in modified atmosphere packages. Figure (a) represents methyl butanoate, b) Methyl acetate, c) Acetone, d) Methyl hexanoate. Each graph represents four different packages with four different atmospheres as determined by different masses of the fruit in the packages. Each dot is the average of three replicates.

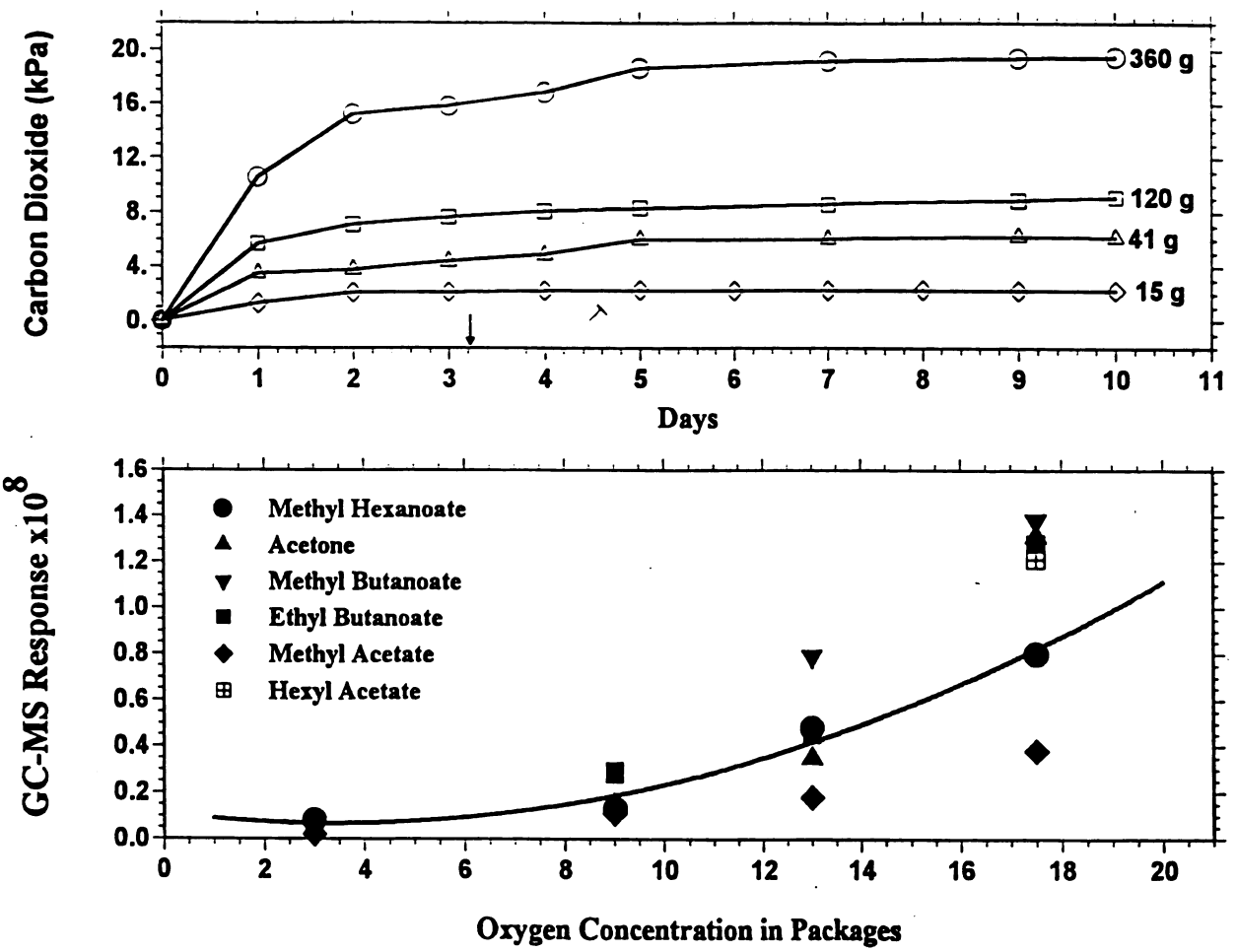

Figure 3. Trend of strawberry volatiles in modified atmosphere packaging and as impacted by in-package O_2 concentration (lower graph). Top graph shows the levels of CO_2 in packages. The arrow shows the day when volatile measurements were made. Amount of product in packages is shown for each level of CO_2 in top graph.

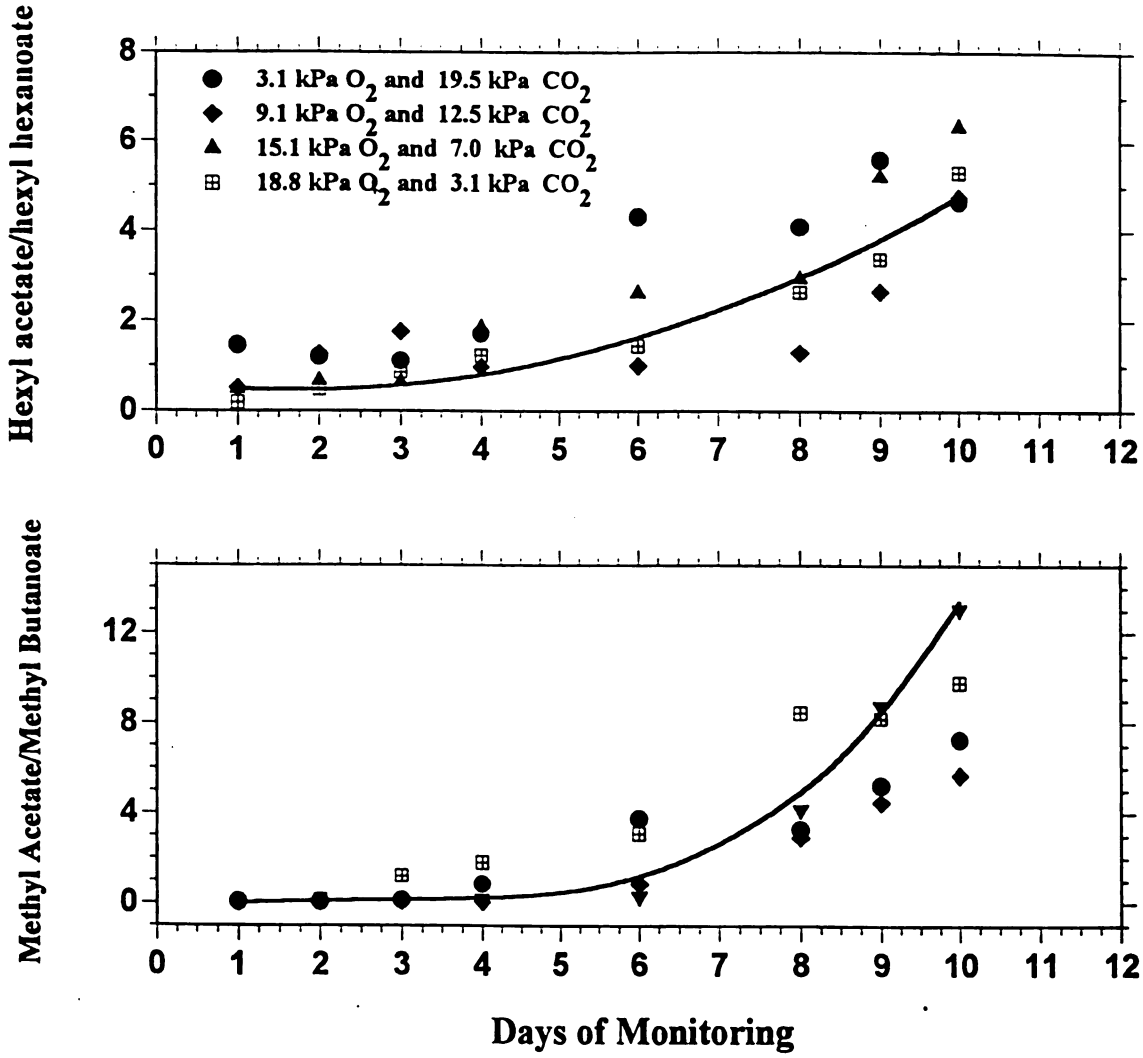

Figure 4. Ratios of methyl acetate/methyl butanoate and hexyl acetate/methyl hexanoate in modified atmosphere-packed strawberry.

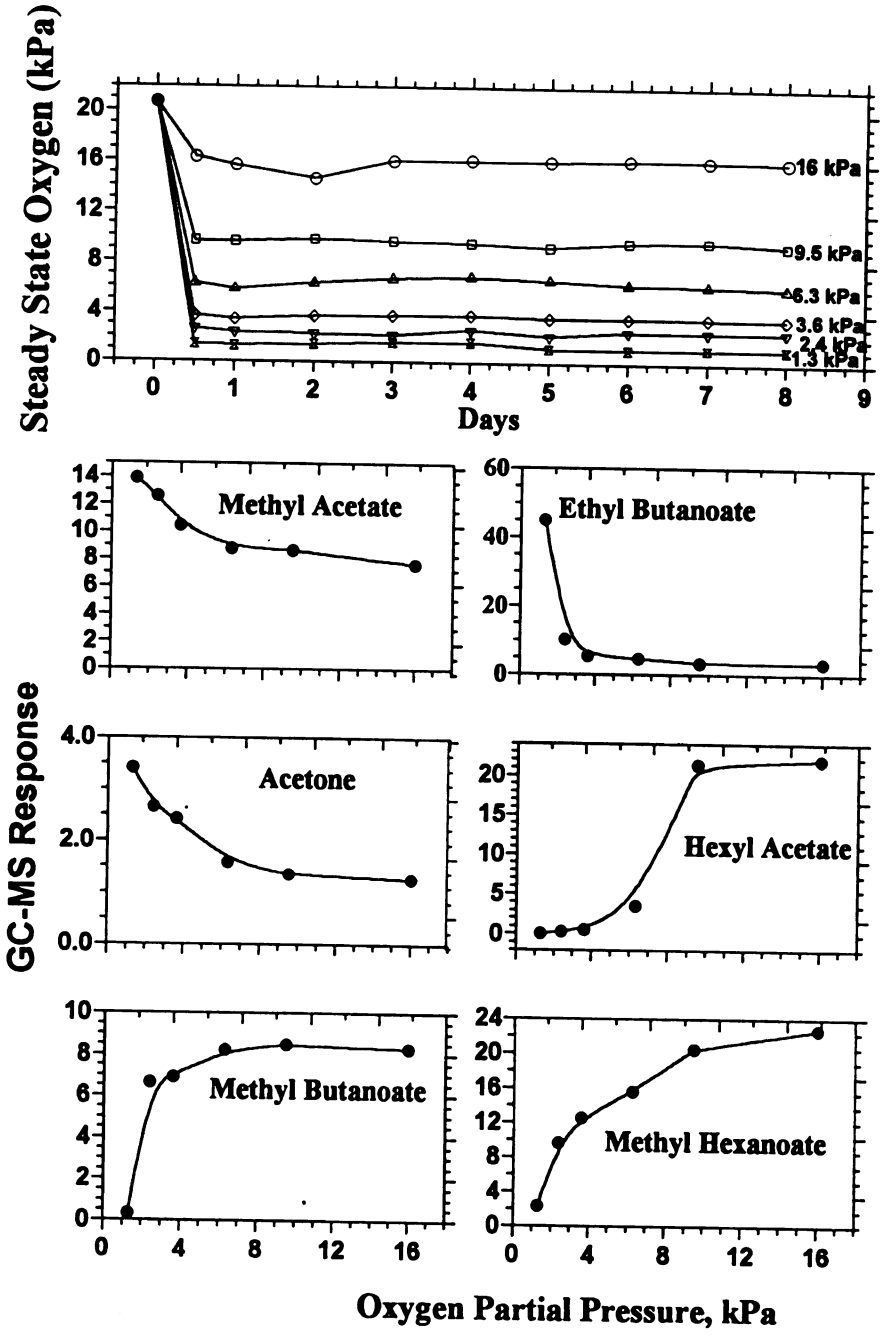

Figure 5. Range of O_2 generated in a flow through system (top) and changes in the production of six different volatile esters at different O_2 concentrations: a) acetone, b) methyl acetate, c) methyl butanoate, d) ethyl butanoate, e) methyl hexanoate, and f) hexyl acetate. Data were generated for day 3.

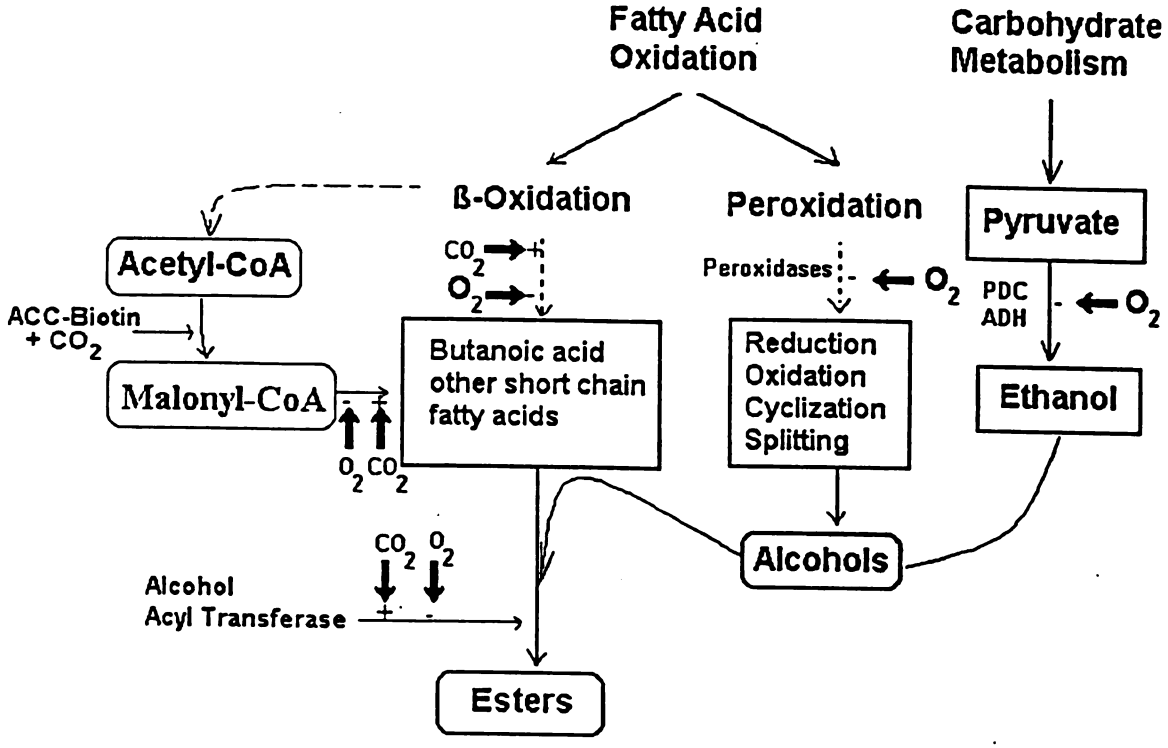

Figure 6. Proposed mode of action of O_2 and CO_2 on the biosynthesis of esters in strawberry packaged under modified atmospheres. The arrows indicate the possible points where low O_2 and high CO_2 may have inhibitory impact on the biosynthesis of strawberry volatiles. The signs (-) or (+) indicate low or high respectively.

CHAPTER VI

AROMA PRODUCTION OF CARROT (Daucus carota L.) AND BROCCOLI (Brassica oleracea L.) AS A FUNCTION OF O_2 PARTIAL PRESSURE IN MAP

AROMA PRODUCTION OF CARROT (Daucus carota L.) AND BROCCOLI (Brassica oleracea L.) AS A FUNCTION OF O_2 PARTIAL PRESSURE IN MAP

Additional index words. modified atmospheres, volatile, mass spectrometry, sulfur compounds, terpenes, quality

Abstract. A combination of solid phase micro extraction (SPME) as a sampling method and gas chromatography/time-of-flight mass spectrometry (GC-TOF-MS) for gas analysis were used to investigate the volatiles produced by carrot sticks and broccoli florets stored for eight days in modified atmosphere packages. Methanethiol, dimethyl sulfide and dimethyl disulfide content in the headspace of packages containing broccoli increased as package O_2 decreased. Our results indicate that packages should be designed to maintain O_2 levels > 5 to 6 pKa to prevent broccoli from producing sulfur volatile compounds should. In carrot, most of the carrot volatiles detected were negatively impacted by MAP when the in-package O_2 levels were below 3-9 kPa. These results provide evidence that the formation of terpenes, major component of the carrot volatile profile, is dependent upon the O_2/CO_2-sensitive carbohydrate metabolism of higher plants. The impact of low O_2 on the production of total terpenes suggests that most carrot volatiles are produced through the same pathway and that thus their O_2-dependent control points, or key enzymatic steps, are common for all of them. Low O_2 may act by restricting the production of important precursors such as acetyl-CoA and mevalonate.

The technology of modified atmosphere packaging has been employed in extending the shelf-life and preserving the fresh-like appearance of broccoli and carrot (Barmore, 1987; Shewfelt, 1987; Riel, 1995). The O_2 levels, however, in modified atmosphere packages of fresh cut vegetables can be depleted to a low level by respiration. When the oxygen drops below the extinction point, aerobic respiration diminishes and anaerobic respiration becomes the dominate pathway. Some produce such as carrot can survive a slight depletion of O_2 below the extinction point (Hisashi and Watada, 1996). The extension of shelf-life of vegetables including carrot and broccoli in modified atmosphere packaging is due to a reduction in the rate of respiration and associated biochemical processes delaying senescence (Ballantyne, 1989). Benefits include delayed ripening, improved color retention and reduction of microbial incidence. However, not much attention has been paid to the impact of modified atmosphere packaging on the production of volatiles by both broccoli and carrot during storage. Aroma is an essential component of food flavor. Flavor constitutes one of the key elements in food quality.

Forney et al. (1991) reported that modified atmosphere-packed broccoli develop strong off-flavors. Forney identified ethanol, methanethiol, hydrogen sulfide, ethyl acetate, dimethyl disulfide, acetaldehyde, methyl acetate, and acetone in in the headspace of packages broccoli stored under an atmosphere of 0.5% O_2. Hensen et al. (1992) reported that ß-ionone also contributes to the objectionable odor of modified atmosphere-packed broccoli.

Heatherbell et al. (1971) reported that the volatiles acetaldehyde, sabinene, myrcene, and terpinolene are considered to be important character-impact compounds in raw carrot aroma. Heatherbell and Wrolstad (1971) conducted a study on the influence of

variety, maturity and storage duration on the type and concentration of volatiles in carrot. Their result showed that the differences were quantitative rather than qualitative and that the variation in concentration of individual terpenes and their summation appeared to be consistent with description of the flavor characteristics of the different varieties. In most research done on modified atmosphere-packed carrot, the effect of MAP on aroma has not been evaluated, even though aroma is on of the key components of minimally processed carrot quality (Bolin and Huxoll, 1991; Bolin, 1992; Brackett, 1987; Bruemmer, 1987; Carlin et al., 1990; Chervin et al., 1994; Heatherbell et al., 1971; Howard et al., 1995). Simon et al. (1980) examined the volatiles of eight carrot lines by using a tenax GC trapping technique. Among the volatiles reported by Simon et al. (1980) include alpha pinine, beta pinene, myrcene, alpha phellandrene, alpha terpinene, limonene, gamma terpinene, terpinolene, terpinen-4-ol, bornyl acetate, caryophellene and gamma bisabolene. Simon et al. also found that slicing of carrot enhanced the production or release caryophellene and alpha bisabolene, which is consistent with results published by Heatherbell et al., (1970).

In this study, our major aim was to evaluate the volatile production of both broccoli and carrot stored under modified atmosphere packaging to gain a better understanding of the relationship between volatiles and levels of O_2 and CO_2 as affected by package design. We wished to identify those volatiles that could serve as fingerprints to assess the physiology of the packed produce and be able to assess shelf-life and storage conditions.

Material and Methods

Plant material. Broccoli and carrot were obtained as fresh as possible from the Detroit Produce Terminal. Broccoli was cut into florets and carrot was cut into sticks ranging from 2 to 5 inch length. The lightly processed products were then sanitized in a smixture of 0.5 $g \cdot L^{-1}$ of Benlate (Du Pont de Nemours Co., Agricultural Product Department, Wilmington, Del.) and 0.5 $g \cdot L^{-1}$ Rovral (Rhone-poulenc Ag. Company, Research Triangle Park, N.C.) To prevent decay and then rinsed with distilled water and allowed to air-dry. The storage temperature was 15 °C.

Modified atmosphere packaging of strawberry. The design of modified atmosphere packaging was achieved by using the approach of Beaudry et al. (1992) as adapted from Cameron et al. (1989). Fruit were sealed in pouches made of 0.00762 cm (3 mil) thick low-density polyethylene (LDPE; LDF 550, Dow Chemical Company, Midland, Mich.). The initial step in the package design for strawberry was the determination of respiration rates (r_{O2}) of whole strawberries stored at atmospheric O_2 partial pressure (20.7 kPa) at 2 °C. Using the estimated r_{O2}, the needed film area and fruit weight were calculated to generate 15 kPa O_2. From this high O_2 setting, fruit weight was varied to achieve a range of O_2 levels.

Headspace Analysis and Volatile Identification. Volatile collection was accomplished using solid-phase micro-extraction (SPME) device (Supelco, Inc., Bellefonte, Pa.). A fiber with a 100 µm-thick, non-bonded, polydimethylsiloxane-based stationary phase was used. The absorption time was 5 min. After absorption, volatiles were then thermally desorbed from the fiber coating by directly inserting the fiber into the injection port (200°C) of a gas chromatograph (HP-6890, Hewlett Packard Co.,

Wilmington, Del.) for 90 s. Gases were cryofocused on-column using liquid N_2 and separated using a gas chromatograph fitted with a 10-meter, 0.1 mm I.D., DB-5 capillary column (Hewlett Packard Co., Boise, Idaho). The detector was a time-of-flight (TOF) mass spectrometer (Pegasus II, LECO Co., St. Joseph, Mich.). The TOF mass spectrometer permitted the resolution and quantification of compounds not separated chromatographically. Identification of volatile material was done by comparison of the spectra and GC retention times with authenticated standard compounds. High purity (>95%) authenticated standards obtained from Aldrich (Aldrich, Co., Milwaukee, Wis.). For the standard compounds, a mix of the targeted compounds was prepared in a 4.85-L jar (standard jar), equipped with a self-sealing rubber sampling port. The mix was prepared by first placing 50 µL of each compound into a 2.5-mL vial to generate a stock mix. Then, 10 µL were taken from the stock mix and placed into the 4.85-L jar to be used as standard. Standard preparation was done at 5 °C and the standard jar was kept at the temperature of the experiment. Calculation of the concentration of standard compounds was expressed in ppm. As sampling from the packages was done, so was from the standard to estimate the actual production of the targeted compounds regardless of the sensitivity of the detection system. Data were reported in $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

Results

Impact of MAP on broccoli volatiles. Typical broccoli volatiles such as limonene appeared to be negatively affect by O_2 levels below 5-6 kPa. Limonene production was markedly reduced at O_2 levels less than 6 kPa (Fig. 1). The same effect is observed for acetone. Methanethiol, dimethyl sulfide and dimethyl disulfide were identified in low O_2 headspace (0.8-5 kPa) in a higher concentration than in headspace with higher levels of O_2

(Fig. 1).

Impact of MAP on carrot volatiles. Most of the carrot terpene volatiles detected using the SPME/GC-MS method were negatively impacted by MAP when the in-package O_2 levels were below 3-9 kPa (Fig. 3). The reduction in headspace content was greater thar 90% for all almost all volatiles as O2 level declined from 17 kPa to 1.3 kPa.

Discussion

A possible enzymatic pathway for the production of methanethiol, dimethyl sulfide and dimethyl disulfide by broccoli was investigated by Pentima et al. (1995). In a cell-free system of fresh broccoli, methanethiol formation occurred in response to L-methionine and S-methylcysteine and dimethyl sulfide is formed from S-methylmethionine. In the same study, Pentima et al. (1995) reported that an increase in free amino acids was observed in broccoli under anaerobic conditions. Our results, are in agreement with Pentima et al. (1995), and suggest that broccoli has the enzymatic capacity to produce volatile sulfur compounds when there are free amino acids available. Modified atmosphere packaging, which usually consists of a range of O_2 of 1.5-6 kPa, enhance the release of the amino acids that serve as precursors for the sulfur volatile compounds detected. Forney et al. (1991) considered the methanethiol primarily responsible for the off-odor produced by broccoli stored under low O_2 atmospheres. Hensen et al. (1992) found that the major compounds contributor to the offensive off-odor are methanethiol, methyl trisulfide, and ß-ionone. In our experiment, we did not detect either methyl trisulfide nor ß-ionone. Perhaps these compounds require lower oxygen levels or maybe the concentrations of these compounds were below the detection limit of our method.

Pentina et al. (1995), reported that the three main factors involved in the formation of volatile sulfur compounds, which are contributors to the objectable odor in broccoli include: (1) the availability of substrates, free sulfur amino acids; (2) the presence of enzyme with specificity to cleave these amino acids; and 3) the accessibility between substrates and enzymes. Our results suggest that one or more of these factors is lacking in modified atmosphere packages where the levels of methanethiol, dimethyl sulfide and dimethyl disulfide are low. Perhaps the enzymatic system responsible for the cleavage of amino acids is more active at low O_2 levels (0.8-6 kPa). Or else, the amount of precursors (amino acids) is higher at low O_2 atmospheres. Our results are also in agreement with Kasmire et al. (1974) who reported the effect of aeration rate on the production of off-flavor by broccoli heads during a simulated transit period. Kasmire et al. (1974) reported that adequate ventilation is essential to prevent accumulation of off-odors .

Makhlouf et al. (1990) reported that the combination of moderately low O_2 and high CO_2 has a protective effect on membrane integrity of broccoli. In the same study they suggested that protection may be achieved indirectly by delay of senescence, or indirectly by the MAP action on the membranes. The later possibility would agree with the hypothesis that states that the very first event during senescence would be membrane lipid degradation (Heemskerk and Witermans, 1987). Direct protection of membrane lipids could be due to the high solubility of CO_2 in hydrophobic environment (Hayduck and Buckley, 1971). Based on our results, we cannot elucidate whether the effect on MAP on sulfur volatile compounds is due to the action of low O_2, high CO_2 or their combination. However, our results suggest that the packaging design that would prevent

broccoli from producing sulfur volatile compounds should not induce O_2 levels below 5-6 kPa in the packages. This information can be important in the formulation of mathematical models for predicting shelf-life of broccoli.

Regarding the impact of modified atmospheres on the production of terpenes, several studies have conducted to investigate the O_2/CO_2-sensitive carbohydrate metabolism of higher plants. Using radiolabeled acetate-2-^{14}C and mevalonate-2-^{14}C, Oba et al. (1970) found that the acetate and mevalonate incorporated into terpenes appear to be produced from carbohydrate metabolism through the formation of pyruvate and acetyl-CoA in mitochondria of sweet potato. Croteau et al. (1972) demonstrated that *in vitro* biosynthesis of acetyl-CoA from sugars yields, concomitantly, ATP and reduced pyridine nucleotides, both of which are required for utilization of acetyl-CoA in terpene biosynthesis in peppermint. Croteau et al. (1972) also study the effect of CO_2 on the assimilation of precursors in the formation of terpenes. This group of researchers found that 5% CO_2 approximately doubled the incorporation of labeled mevalonate into caryophellene.

Based on this work, modified atmosphere-packed carrot, the inhibition of terpene production is likely to be the result of the effect of low O_2 (<5-6 kPa) on the metabolism of carbohydrates. The overall pathway of terpinolene, for instance, include the formation of acetyl-CoA, NADH, ATP and CO_2 from sucrose. The acetyl-CoA generated along with the energy produced as NADH and ATP are then used to produce the corresponding terpenes. George-Nascimento and Cori (1971) conducted a study on terpene biosynthesis from geranyl and neryl pyrophosphates by enzymes from orange flavedo. In this study they reported that the biosynthesis of monoterpenes such as limonene and alpha pinene

involves the transformation of mevalonic acid into 10-carbon pyrophosphorylated intermediates, and subsequent elimination of the phosphate moiety. Terpinolene, limonene, caryophellene and alpha pinene formation requires the supply of acetyl-CoA as well as the generation of energy in the form of NADH obtained from the aerobic pathway of carbohydrate metabolism. These results support our theory and result that the low O_2 levels in MAP-packed carrot is the responsible for the marked reduction of terpene production by carrot (Figs. 3, 4). The impact of low O_2 on the production of total terpenes also gives way to several observations: (1) it appears that most carrot volatiles are produced through the same pathway and that thus their controlling points, or key enzymatic steps, are common for all of them; (2) it seems likely that the low O_2 is the inhibition factor, perhaps by restricting the production of important precursors such as acetyl-CoA and mevalonate; (3) the relationship between O_2 levels in packages and the production of terpene volatiles in MAP-packed carrot allows us to have a better understanding of the biochemistry of the produce during storage and could serve as asset in the designing of automated quality control systems and mathematical models to predict storage time and conditions.

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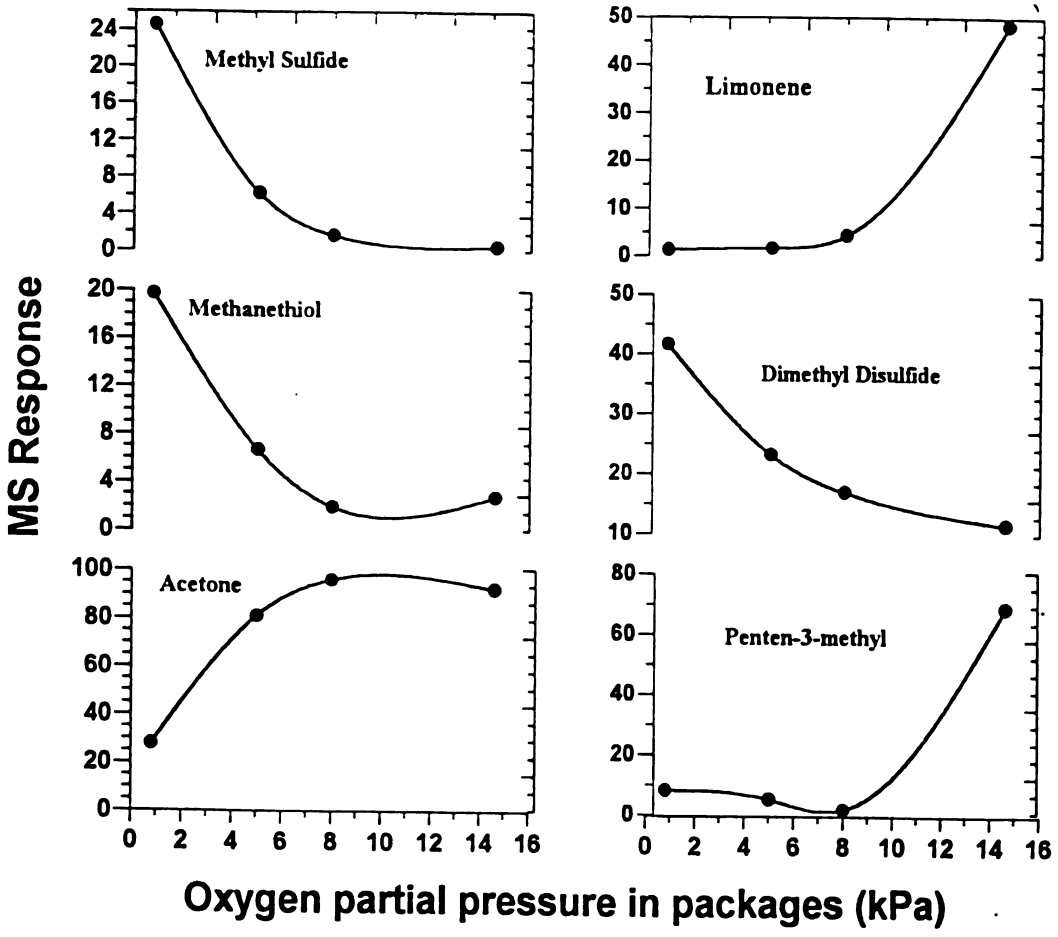

Figure 1. Selected volatile compounds produced by MA-stored broccoli as affected by different levels of in-package O_2 partial pressures.

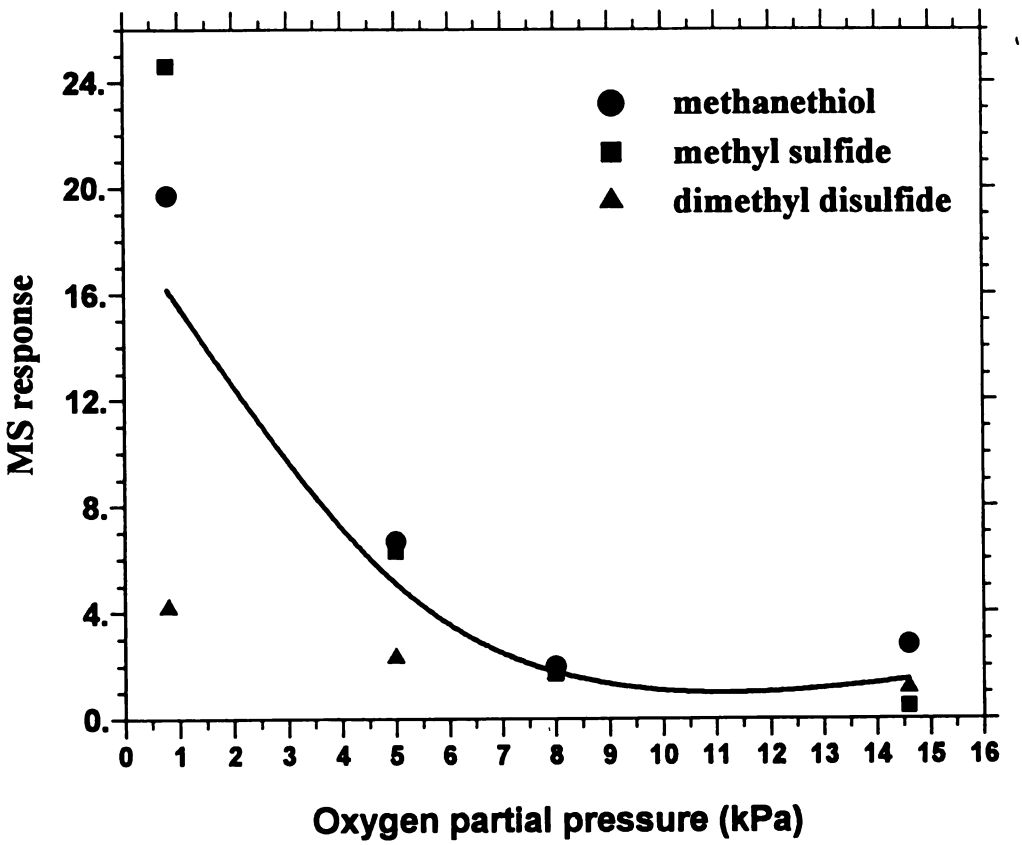

Figure 2. Effect of O_2 on the accumulation of volatile sulfur compounds from broccoli in modified atmosphere packaging on d 3 stored at 15 °C.

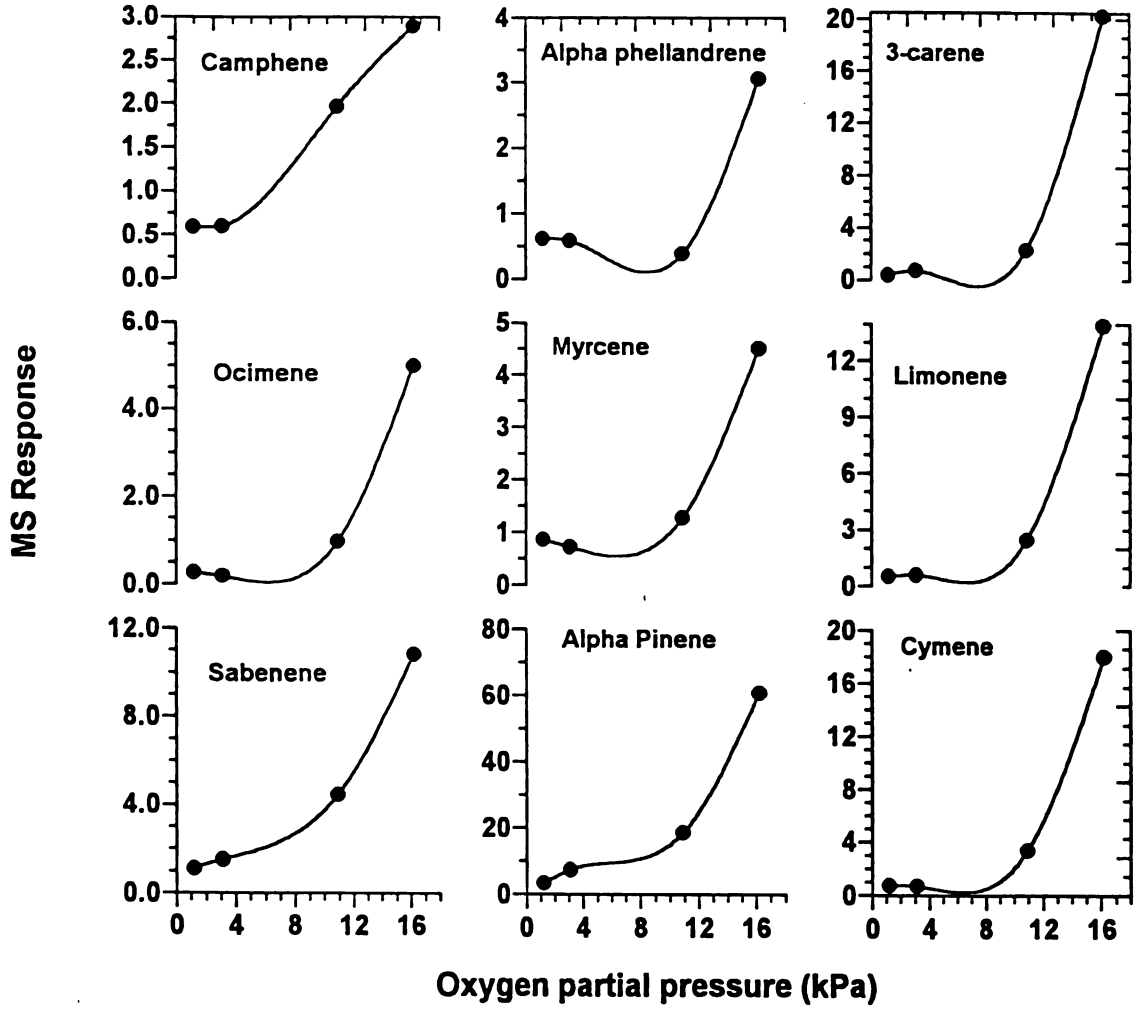

Figure 3. Selected volatile compounds produced by MA-stored carrot as affected by different levels of in-package O_2 partial pressures.

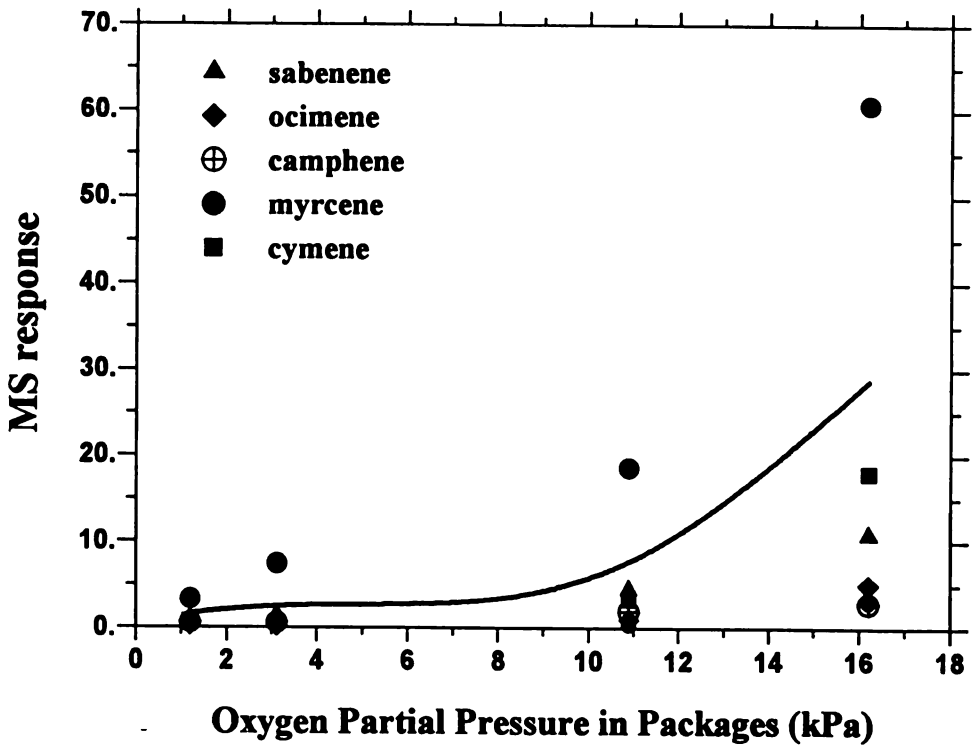

Figure 4. General trend of terpene volatile compounds as affected by different O_2 partial pressure in MA-stored carrot.

SUMMARIES

Senescence-related volatile profiles of strawberry. Our results indicate that, in strawberry, ester volatiles as a whole decrease as fruit senesce and thus a decrease of esters may be used as a general indicator of the physiology of the fruit during storage. Among esters, methyl acetate seems to have unique relationships with physiological processes linked to the senescence of strawberry. The positive correlation of methyl acetate with softening may be related to the release of methanol from the breakdown of the cell wall pectins. Additionally, the acetate moiety of the ester is derived from acetyl CoA, which may result from LOX activity and ß-oxidation processes that are thought to be involved in membrane degradation during senescence. Methyl acetate may be a good candidate as a fingerprint-type volatile to assess senescence in strawberry.

Volatile profiles of broccoli and carrot during storage. Volatiles of carrot sticks decreased diring storage, possibly as a function of tissue aging. Besides the general trend of terpenes over time, ratios among terpene compounds shifted markedly as well, suggesting the ratios may be used as markers of carrot stick age. In broccoli florets, dimethyl disulfide seemed to be related to senescence and may have potential to be used as a senescence index.

Volatile profiles of E. coili and S. typhimurium grown in laboratory media and food products. Both indole and 1-propanol have the potential to be used as fingerprint compounds for *E. coli* in both standard laboratory media and in different produce packages. 1-propanol and indole are volatiles produced by *E. coli* but not by broccoli, carrot or lettuce and this information may be exploited in a more practical way. Indole

was not detected in produce packages, however, due perhaps to the interference of some other carbohydrate sources in produce. Nevertheless, indole may be promising in other types of food such as meet, dairy products and egg. *S. typhymurium*, also produces detectable volatiles which should be explored further. *S. typhymurium* appears to have the enzymatic capacity to breakdown amino acids present in the standard laboratory media, to produce alcohols.

Fingerprint volatiles of microorganisms not only offer a way of identifying microbial development in food but also offer information on the biochemistry and metabolism of the organisms. With the availability of rapid, accurate and more sensitive volatile analysis techniques, it worthwhile to use volatiles produced by microorganisms as a research asset in the development of bacterial detection systems in food packages.

Storage duration, low O_2 and elevated CO_2 alter ester biosynthesis in strawberry. The impact of modified atmospheres on the production of esters by strawberry is a combined inhibitory effect of low O_2 and high CO_2 . The mode of action of O_2 and CO_2, however, is not clear yet. O_2 appears to interfere with the biosynthesis of substrates such as alcohols and acids since the enzyme alcohol acyl transferase has been reported to have little sensitivity to either low O_2 or high CO_2. Nevertheless, the information regarding changes of specific volatiles in packages may be used to assess aroma quality of packaged fruit and for the design of packages to optimize quality at the time of package opening by the consumer.

Aroma production by carrot and broccoli as a function of O_2 partial pressure in modified atmosphere packages. Methanethiol, dimethyl sulfide and dimethyl disulfide were identified in low O_2 headspace of packages (0.8-5 kPa) in a higher concentration

than in headspace with higher levels of O_2. A possible enzymatic pathway for the production of these compounds seems to be activated by low O_2 levels in MAP-packed broccoli that involves the breakdown of L-methionine, S-methylcysteine and S-methylmethionine as suggested by previous workers. Our results indicate that packaging design that would prevent broccoli from producing sulfur volatile compounds should not induce O_2 levels below 5-6 kPa in the packages. In carrot, most of the carrot volatiles detected were negatively impacted by MAP when the in-package O_2 levels were below 3-9 kPa. These results provide evidence that perhaps the formation of terpenes, major component of the carrot volatile profile, is sensitive to O_2/CO_2 levels that limit sensitive carbohydrate metabolism.

General remark. Today, with the availability of rapid, accurate and more sensitive volatile analysis techniques, it worthwhile to use volatiles produced by plants and microorganisms as a research asset. Volatile analysis may be a useful tool to assess some physiological events that take place in plants and microorganisms which today are not fully understood. Furthermore, significant potential exists to exploit the volatiles produced by packaged products and microbial contaminants to assist in developing technologies to non-destructively assess product quality and safety.