THE USE OF MINERAL OIL AS A TRAPPING AGENT FOR VOLATILES PRODUCED BY *Ceratocystis moniliformis* (Hedg.)

by

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(ABSTRACT)

Ceratocystis moniliformis grows well in a semi-synthetic liquid medium containing 30:1 glucose:yeast extract, vitamins, and minerals. At 25°C and 100 rpm on a rotary shaker, the culture grows and produces many pleasing and potentially useful fruity aroma-bearing compounds. A sensory evaluation panel described the aromas "banana," "citrus," "fruity," "peach," and "floral" in samples of this culture. However, yields of the compounds that cause these aroma perceptions are low, as their production is limited due to toxicity, or perhaps a feedback inhibition mechanism.

Seventeen volatile aroma compounds were tentatively identified by Gas Chromatography - Mass Spectrometry (GC-

MS), and of these, the production of isoamyl acetate, β -myrcene, acetophenone, 1-octanol, and geraniol was studied using GC analysis. In an attempt to increase the production of these compounds relative to the production level of aroma-bearing compounds obtained by traditional incubation methods, mineral oil was added to the growth medium as a thin (2 mm) layer. Difficulties in analyzing oil-free cultures made it impossible to directly compare yields in oil-free versus oil-containing cultures. However, the mineral oil acted as a trapping agent, and production of aroma compounds continued in the presence of mineral oil for up to 8 days, yielding from 246 to 2071 ug/100 ml culture of the aroma compounds.

The above-mentioned compounds are similar to natural and synthetic essential oil components that are currently used as food flavorants. Thus, aroma-bearing compounds that are produced by Ceratocystis moniliformis may prove to be valuable alternative sources for food flavoring.

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DEDICATION

This thesis is lovingly dedicated to the memory of

Florence A. McDonnell

Educator

Scholar

Friend

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I. INTRODUCTION

Many species of microorganisms are known to produce pleasing, desirable aroma compounds, both in nature and in culture. Microbially-produced aroma compounds are responsible for the characteristic flavors of foods such as cheese, beer, and oriental soy products. These naturally occurring flavorants have been enjoyed by man for centuries. In the past twenty-five years, there has been an increasing interest in the microbial production of economically important flavor compounds through laboratory scale fermentations.

Much of this work has centered around the use of filamentous fungi, in particular, species of the genus Ceratocystis. Researchers have found that the species C. moniliformis produces the valuable "character impact" monoterpenes linalool, geraniol, nerol, and citronellol when grown in a synthetic medium containing 3% potato-dextrose broth. Other aroma components are alcohols, esters, and lactones. Additional studies have shown that by altering the carbon and/or nitrogen source, the aroma of the culture broth changes as well. Trained sensory evaluation panelists have described the aromas "banana" and "peach" from cultures of C. moniliformis grown with

glucose as the carbon source, while cultures grown with glycerol as the carbon source were described having a "citrus" or "floral" aroma.

It is believed that these aroma compounds can be extracted from the culture medium, and concentrated to give essential oils. These essential oils are remarkably similar to those currently used in food products as flavoring ingredients. At present, these aroma compounds are extracted from plant materials, or produced synthetically. However, some feel that there is a need for an alternative "natural" source of these compounds. This is because plant materials are not always available for agronomic or political reasons. In addition, since the FDA does not readily approve synthetic ingredients for use in food products, aroma compounds of fungal origin may prove to be an alternative "natural" source of flavor compounds for the five billion dollar per year commercial flavor and fragrance market.

There have been problems, however, with the culture conditions under which these aroma compounds are produced. Studies have shown that these aroma compounds, as metabolic endproducts, halt their own production by feedback inhibition or other toxic effects. Because of this, total aroma compound yield is limited to only 50

mg/l under normal incubation conditions. Other studies have shown that if a trapping agent such as Amberlite XAD 2 is used, the endproducts are removed from the culture during incubation. This allows for the continued maintenance of the culture, and enables total aroma compound yields to increase to up to 2.0 g/l.

C. moniliformis grows well and produces aroma compounds when the surface of the culture medium is covered with a layer of mineral oil. The oil acts as a trapping agent by removing the toxic endproducts of secondary metabolism from the aqueous phase of the medium. The following goals were established for this research:

1) To identify flavor compounds of interest to the food industry that are produced by C. moniliformis; confirm earlier studies regarding growth conditions.

2) See if and under what conditions aroma compounds are produced in the presence of oil.

3) To study the potential of using mineral oil as a trapping agent during the production of volatile aroma-bearing compounds.

4) Estimate yields of these aroma compounds to be expected by recovery of the oil fraction.

II. REVIEW OF THE LITERATURE

A. MICROBIAL PRODUCTION OF AROMA COMPOUNDS

1. Flavor

Flavor is a complex sensation. Texture, color, aroma and taste all contribute to our perception of flavor (Flath et al., 1981). However, the most important component of flavor is aroma (Heath and Reineccius, 1986). The perception of aromas in foods is complicated, since aromas detected by the human nose are actually composed of many major and minor volatile components which react synergistically to produce a total effect (Sharpell, 1985). Furthermore, these individual components vary greatly in their odor threshold (Maga, 1976), the amount of the individual compound present in the aroma complex, and the chemical nature of each compound. Flavor-active metabolites produced by microorganisms belong to many classes of chemical compounds, and include esters, ketones, aldehydes, acids, lactones, pyrazines and alcohols (Latrasse et al., 1985). When these compounds accumulate in a food, they influence its final flavor (Gatfield, 1986).

2. History of Microbially Produced Food Flavors

Since Neolithic times, man has enjoyed foods flavored by the action of microorganisms. Some examples are cultured milk products such as yogurt and cheese (Rutloff, 1982), beverages such as wine and beer (Phaff, 1981), breads (Scharpf et al., 1986), and oriental soy products such as tempeh and soy sauce (Gatfield, 1988). These various foods were produced and consumed for hundreds of years before microorganisms were known to exist. Today, the microbial role in food production is fairly well understood, and microbially flavored foods and drinks comprise a large sector of the food industry (Gatfield, 1986).

It has been known for over sixty years that specific aroma chemicals are produced by microorganisms (Sharpell, 1985). In 1923, Omelianski described microorganisms that produce aromas ranging from "fruity" to "glue-like" (Omelianski, 1923). Microbiologists have used the aroma characteristics of microorganisms as taxonomic classification markers (Badcock, 1939; Hutchinson, 1971). As mentioned above, many of the microbially produced aromas are synthesized during classical fermentation processes, and it is now known that microorganisms are also capable of producing "character impact" compounds.

3. Character Impact Compounds

Character impact compounds are those compounds which are generally present in a food or food flavorant in a small amount, yet have a significant impact on the overall flavor perception (Chang, 1989). Some character impact compounds are present in an aroma complex in parts per trillion, but most are found in the parts per million to parts per billion range (Lindsay, 1985). Examples include nootkatone in grapefruit, 1-octene-3-ol in mushrooms, and methyl N-methyl-anthranilate in the concord grape (Chang, 1989). Character impact compounds belong to all of the chemical classes mentioned previously, and can occur naturally in a food. These flavors may also be generated from precursors during thermal processing, enzymatic reactions, or through microbial fermentation by bacteria, yeasts, and other fungi. Duplicates of naturally occurring flavor compounds can be synthesized from petroleum products, or they can be derived from other naturally occurring substances.

4. Bacterial Production of Aroma Compounds

While it would be impractical to discuss all of the bacterial species that produce desirable aroma compounds

in foods, it is worthwhile to mention some of the more important ones. Species of the genera Lactobacillus, Streptococcus, Pediococcus, and Leuconostoc contribute to the production of volatiles such as diacetyl, lactic acid, acetoin and acetaldehyde in dairy products and in bread (Scharpf et al., 1986 ; Berger et al., 1988). Leuconostoc oenos is the organism involved in malolactic fermentation in wine (Lafon-Lafourcade, 1986). Pseudomonas fragi is capable of producing a strawberry aroma in culture (Breed et al., 1957). Pseudomonas perolens produces pyrazines, which are important components of "roasted" and "toasted" aromas (Latrasse et al., 1985).

5. Fungal Production of Aroma Compounds

i. Deuteromycetes (Fungi Imperfecti)

There are about 15,000 species in the subdivision Deuteromycotina, including yeasts and molds. Yeasts are best known for their role in the production of bread, wine and other fermented beverages. In addition to causing the fermentation process, aroma components are also produced by species of Saccharomyces during fermentation. These compounds include lower alcohols in bread, higher alcohols and lactones in beer (Scharpf et

al., 1986), and monoterpenes and esters in wines (Rapp and Mandery, 1986).

Other aroma components are generated by yeasts outside of food fermentations. As early as 1963, Okui and coworkers reported that Candida guilliermondi was able to oxidize ricinoleic acid, the major fatty acid found in castor oil, yielding 4-hydroxy decanoic acid-γ-lactone (Okui et al., 1963). In a similar finding, it was shown that Yarrowia lipolytica is also capable of transforming castor oil to a precursor of γ-decalactone, which is lactonized in situ by lowering the pH of the culture medium (Farabood et al., 1985; Tyrrell, 1990). γ--decalactone is a major character impact compound found in peaches (Heath, 1981). Rather than synthesizing or degrading aroma compounds, some yeasts are able to transform them. For example, Candida reukaufii can transform citronellal to citronellol, an important monoterpene (Sharpell, 1985).

Another imperfect fungus that produces a desirable aroma is Trichoderma viride, a common soil fungus. This species produces a characteristic coconut-like aroma in nature, and when grown in liquid potato-dextrose medium (Collins and Halim, 1972b). Analysis by gas chromatography (GC), infrared spectroscopy (IR), mass

spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), and elemental analysis showed that the major character impact compound responsible for this aroma was 6-pentyl- α-pyrone.

Molds produce a variety of economically important secondary metabolites, such as citric acid, which is produced by Aspergillus niger, and Penicillium chrysogenum, which produces medicinal penicillins (Phaff, 1981). However, some molds also produce aroma compounds of interest to flavorists. For example, the mold Penicillium decumbens has a distinctive odor resembling perfume, when grown in a stationary liquid culture. Its major essential oil components are thujopsene, nerolidol, and 1-octen-3-ol (Collins, 1976).

ii. Ascomycetes

Members of the subdivision Ascomycotina produce sexual spores within a structure called the ascus. Many ascomycetes are able to synthesize aroma compounds, including species of the genera Ceratocystis and Penicillium (Collins, 1979). In 1939, Badcock published a list of the odor properties of certain fungal cultures. From this list, and from further work conducted at a number of laboratories, it is evident that at least fifty

desirable flavor compounds have been identified from at least one hundred species of fungi, including the filamentous fungi.

Some of the compounds of economic and sensory importance that are produced by filamentous fungi are citronellol (Sharpell, 1985), 6-pentyl- α -pyrone, geraniol, methyl salicyclate (Maga, 1976), γ -decalactone (Sarris and Latrasse, 1985), and 2-heptanone (Dwivedi and Kinsella, 1974). Many important character impact compounds are monoterpenes, which are produced by several species of the filamentous genus Ceratocystis. Currently, there are no commercially available foods that derive their characteristic flavor from the addition of microbially produced terpenes. However, many feel that there is potential for the commercial production of terpenes via fermentation (Heath and Renecccius, 1986).

iii. Basidiomycetes (Mushrooms)

Basidiomycetes are fungi that produce basidiospores, and whose mycelia form clamp connections during elongation (Miller, 1987). The largest number of odor-producing fungi is found in the Basidiomycetes (Collins, 1976), and some of these odors are desirable aromas that

are associated with food. For example, Myocoacia uda produces an almond-like odor when grown in several different liquid media (Sastry et al., 1980). Earlier studies by Halim and Collins showed that the almond odor was largely due to the production of para-tolualdehyde and para-methylacetophenone (Halim and Collins, 1975). Halim and Collins also examined volatile production by Trametes odorata, and found that its major odorous constituent, methyl para-methoxyphenylacetate, produced an anise-like odor (Halim and Collins, 1971).

The basidiomycete Polyporus durus has been shown to produce up to 7.4 mg/l of γ-octalactone, which has an intensive fruity aroma resembling coconut (Drawert et al., 1983). Several species of Phellinus produce desirable aromas whose primary compounds include linalool, methyl benzoate, and methyl salicyclate (Collins and Halim, 1972a). Despite these findings, however, Basidiomycetes are generally difficult to grow in synthetic media. Fruiting bodies are not always formed, and the period of growth required for aroma production is approximately six weeks (Collins, 1979). Because of these considerations, in vitro aroma production by basidiomycetes for industrial purposes is generally impractical.

B. SECONDARY METABOLISM IN FUNGI

1. Description of Secondary Metabolism

The biosynthesis of volatiles by fungi has been examined in detail by many authors (Collins, 1979; Collins and Halim, 1970, 1977; Hutchinson, 1971;). It is well established that fungal volatiles such as monoterpenes, alcohols and esters are all products of secondary metabolism. Although some of the pathways of secondary metabolism in fungi have been elucidated (Lanza and Palmer, 1977), their exact physiological roles and functions have not (Martin and Demain, 1975).

Simply put, secondary metabolism is anabolic and dispensable, and it is generally restricted to plants and microorganisms. By contrast, primary metabolism is essential for cellular growth, and includes all of the catabolic and anabolic processes that keep an organism alive (Bennett, 1983).

At least one thousand secondary metabolites are known to be produced by fungi, yet these compounds are synthesized from only a few key precursors, in a limited number of biochemical pathways (Bu'Lock, 1965). For example, polyketides are derived from acetate during fatty acid metabolism (Sharpell, 1985), and terpenes are derived from isopentyl pyrophosphate via the mevalonic

acid pathway (Lanza and Palmer, 1977). In microorganisms, secondary metabolism normally occurs when active growth has ceased, either permanently or temporarily. In addition, anatomic differentiation, especially sporulation, can occur simultaneously after growth has ceased (Bennett, 1983).

2. Purpose of Secondary Metabolism

The question that is still debated is, "Why are secondary metabolites produced?" There have been a number of possible explanations as to why they are produced by fungi:

- A number of fungi produce volatile compounds which stimulate spore germination (Collins, 1979).
- Certain fungi produce volatiles that act as insect attractants. These insects can in turn help to disseminate spores.
- Volatile compounds may be produced as a result of a carbohydrate "overflow mechanism." This mechanism would provide an orderly way in which extraneous intermediates may be disposed of. Otherwise, accumulation of these compounds may be toxic to the organism (Collins, 1979).
- The operation of secondary metabolism keeps

cellular functions in working order when growth is not possible (Bu'Lock, 1965).

Whatever the reasons for secondary metabolism, many believe that it is often triggered during periods of stress. This situation can be advantageous, since one can alter the nutrient availability, especially in vitro, in order to select for the formation of particular secondary metabolites (Lanza et al., 1976). Primary and secondary metabolism are processes that "compete" for key intermediates, and cannot be regarded as mutually exclusive activities; these two forms of metabolism are also known to alternate over time (Martin and Demain, 1975).

C. TRAPPING AGENTS

1. Inhibitory Effect of Volatile Oils

It is well known that volatile oils produced by fungi through secondary metabolism can inhibit growth of the fungus, or have a fungicidal effect if the concentration of the metabolites becomes too high (Berger et al., 1988). Earlier studies have shown that essential oil components interfere with the biochemical processes within the cells, especially within the phospholipid bilayer of cellular membranes. In addition, terpenoid

compounds are known to inhibit electron transport, phosphorylation, and other enzyme-dependent steps. However, the mode of action by essential oil components is largely unknown (Knobloch et al., 1986). Kurita and coworkers reported on the antifungal activity of forty different essential oil components (Kurita et al., 1981). In this study, the duration of growth inhibition of seven fungi by various concentrations of these forty compounds, including citronellol and geraniol, was examined and found to be significant. Knobloch and coworkers found that, in general, the antiseptic activities of terpenoids was dependent upon the compounds' solubilities in water (Knobloch et al., 1988).

It is known that the choice of carbon and nitrogen sources, pH value, length of incubation, and gas exchange can profoundly affect the production of essential oil components in fungi (Schindler and Schmid, 1982). It has also been shown by several authors that if the toxic metabolic endproducts can be removed from the culture, it is possible to increase their production through continuous action of the healthy cells.

2. Methods of Volatile Trapping

There have been reports of several methods of

volatile trapping and/or continuous bioproduction of volatile compounds. Dale and coworkers suggested the continuous stripping of volatiles from the output gas stream of a fermentor (Berger et al., 1988). Cohen and coworkers designed a bubble column reactor for the study of continuous production of terpenic alcohols by Ceratocystis moniliformis. However, they found that this system did not produce greater quantities of terpenic compounds than in a traditional batch culture system. This was attributed to the formation of culture pellets instead of the typical filamentous state (Cohen et al., 1987).

In studies conducted by Schindler and coworkers, it was determined that the respiration of glucose by C. variospora was inhibited by levels of 0.1% geraniol in the culture. This obstacle was bypassed by the addition of the lipophilic adsorber Amberlite XAD to the shake culture. Amberlite welded in perlon bags was added to the cultures in the late growth phase. In this study, the production of geraniol, for example, was increased from 35.5 mg/l to 220.3 mg/l in the presence of 18 g/l of Amberlite (Schindler and Bruns, 1980). In another study, the terpene production behavior of C. variospora was examined in a 20 l fermentor. It was found that by

circulating the fermentor broth through an external vessel containing Amberlite XAD2, the total yield of the metabolites linalool, citronellol, nerol, and geraniol was increased to nearly 1.9 g/l. This figure compares with yields generated through the extraction of traditional plant materials (Schindler, 1982).

D. ECONOMIC IMPORTANCE OF MICROBIALLY PRODUCED FLAVOR COMPOUNDS

1. Commercial Value

In 1987, the value of worldwide commercial flavor and fragrance sales was estimated to be five billion dollars (Berger et al., 1988). Flavor, or aroma compounds, represent 10-15% by weight of the world use of food additives, which amounts to approximately one quarter of the value of the total food additives market (Armstrong and Yamazaki, 1986). According to Schindler, the worldwide demand for flavors is continuously increasing (Schindler, 1982). In addition, many consumers show a preference for "natural flavors" (Berger and Drawert, 1987), since they tend to link natural flavors with purity, safety, and wholesomeness (Chang, 1989).

2. Production Methods

Most of today's flavor compounds are produced by chemical means. These compounds are considered to be "synthetic" or "artificial." While these compounds are less expensive to produce, the obvious drawbacks are legislative concerns and reduced consumer acceptance (Sharpell, 1985; Armstrong, 1986). Traditionally, flavor compounds, particularly the essential oils, were extracted from plant sources, yet the use of botanical sources has its problems. For example, there may be seasonal variation in availability, quality, and consistency of the plant source (Schindler and Schmid, 1982). Furthermore, the supply of these botanicals has dwindled due to social, economic, and political factors (Scharpf et al., 1986).

It stands to reason, therefore, that aroma compounds of microbial origin may offer an alternative method for the production of natural flavors. Logically, the industrial success of these products depends upon high yield of these products, upon the manufacture of unique products, or upon the production of compounds that are no longer available from traditional sources (Sharpell, 1985).

According to Bedoukian, the flavor market is

expected to increase at an annual rate of about 30% (Armstrong and Yamazaki, 1986). With this increase, it is probable that microbially and biotechnologically produced aroma compounds can capture a portion of the market.

E. LEGAL ASPECTS OF FLAVOR COMPOUNDS DERIVED FROM MICROBIAL SOURCES

1. Definition of "Natural Flavor"

The term "natural" has been a topic of hot debate in recent years. In the United States, flavors which are generated during heating, enzymatic reactions, fermentation, or processing, or which occur naturally in a food are considered to be "natural" (Chang, 1989). Legally, the United States defines the term "natural flavor" as:

> the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis which contains the flavor constituents derived from a spice, fruit or fruit juice, vegetable, vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy product, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional (Code of Federal Regulations, 21 CFR 101.22.a.3.).

This definition would therefore encompass products

modified by or produced by microbial cells or their enzymatic components.

2. GRAS and Labeling

Many of the food flavor compounds produced by traditional means have received GRAS (Generally Recognized As Safe) status, and some feel that those compounds produced biotechnologically by GRAS microorganisms should receive this status as well (Armstrong and Yamazaki, 1986). Where labeling is concerned, added flavors may be declared as "spice," "natural flavor," or "artificial flavor," according to Part 101.22 of Title 21 of the Code of Federal Regulations, depending upon the situation. Flavor ingredients may be declared individually, or be cited as approved for food use by the FDA. According to 21 CFR 101.22 (i) (1) (iii), an ingredient may also be listed as a characteristic natural flavor "with other natural flavors" (Chang, 1989).

Although many microorganisms which are known to produce desirable and valuable aroma compounds are not recognized as GRAS, it would seem logical that these compounds would nonetheless be legally qualified as "natural flavors," due to the definitions given above.

F. GAS CHROMATOGRAPHY IN THE FLAVOR INDUSTRY

1. General Considerations

Because of its high degree of sensitivity, excellent accuracy, and considerable speed of analysis, the gas chromatograph is the instrument of choice in the food industry, where volatile compounds are detected, separated, and identified daily. Despite the fact that the gas chromatograph can detect volatiles in the parts per billion level (Vercellotti et al., 1988), odor perception by the human nose is the ultimate judge of aroma. It has been postulated that the nose has a theoretical odor detection limit of about 10^{-19} moles (Heath and Reineccius, 1986). Furthermore, Stuiver calculated that as few as eight molecules of an extremely potent odorant can trigger one olfactory neuron, and that as few as forty molecules can provide an identifiable sensation (Teranishi et al., 1981).

Food flavors are typically quite complex. For example, more than 700 volatile components have been identified in cooked meat (Reineccius, 1984). Although human perception of flavor is comprised of a well integrated combination of color, texture, aroma and taste, it is the volatile aroma component that plays the greatest role in defining a particular flavor character

(Flath, 1981b). Flavor compounds constitute a very small percentage of the composition of any given food, and are unevenly distributed throughout the food matrix (Heath and Reineccius, 1986). Thus, the flavor chemist is faced with the challenging task of separating and identifying aroma compounds that behave differently according to such parameters as the type of food in which they are present, and the physical state of the food itself.

2. GC Analysis of Microbial Flavors

Gas chromatography is used to analyze the volatile components of many foods that are flavored through the action of microorganisms. For example, Rapp and Mandery analyzed the changes in wine aroma during the yeast fermentation (Rapp and Mandery, 1986), and Dwivedi and Kinsella characterized the methyl ketone profile of blue-type cheese flavored by a submerged fermentation of P. roqueforti (Dwivedi and Kinsella, 1974). It is clear that one standard method of separation and identification cannot be used to analyze the aroma fraction of all foods (Jennings and Rapp, 1983).

3. Sample Preparation

Perhaps the most challenging step in volatile

analysis is sample preparation. Typically, the flavor isolate is obtained through one of the following methods: direct headspace sampling, distillation, solvent extraction, and direct sampling.

Briefly, direct headspace sampling involves sampling the gaseous headspace above a food that is in a closed container. Sprecher and Strackenbrock used this approach in 1963 to analyze the volatile production of C. coerulescens (Ko, 1971). The headspace method allows the volatile flavor to be analyzed in the same context as it is perceived by the human senses. However, flavor compounds are present in the headspace in low quantities at room temperature, so a concentration step is usually necessary (Koller, 1987). In addition, errors often occur in the estimation of the total volatile content in the sample, especially in dense, complex samples (Gregoire, 1984). Nonetheless, this method of sampling is a rapid method, and is useful in quality control.

Distillation is a common method of flavor isolation. One of the most efficient distillation methods for food flavor isolation is the Nickerson-Likens method. However, Ko found that this method was not suitable for the isolation of microbially produced volatile constituents (Ko, 1971). Another drawback of

distillation processes is the use of high heat, which may cause thermal degradation of the volatile compounds, as well as thermally induced chemical reactions.

Extraction techniques have been widely used in flavor isolation. Organic solvents such as pentane are normally used. One drawback with the use of organic solvents is the existence of residual solvent in the final isolate (Takeoka et al., 1985). Recently, the use of supercritical CO_2 as an extraction fluid has become widespread (Moyler, 1988). The advantages to supercritical CO_2 extraction include high selectivity, use at ambient temperatures, and the absence of residual solvent in the product (Krukonis, 1985; Jennings, 1979).

Direct chromatographic sampling of oils has been reported by Dupuy (Dupuy et al., 1973; Dupuy et al., 1976; Dupuy et al., 1985). Basically, the volatile-containing oil sample is placed directly onto a GC inlet liner packed with glass wool. This method eliminates lengthy sample preparation, and flavor profile sensory scores have been shown to correlate with results obtained by this GC method (Dupuy et al., 1976).

A fairly recent method of sample preparation is the "stripping" of volatiles onto an adsorbent trap at mild temperatures, under vacuum This method yields a

moisture-free, solvent-free sample, that has undergone little, if any, thermal degradation (Legendre et al., 1978; Vercellotti et al., 1988). Furthermore, volatiles in the parts-per-billion level may be detected.

4. Recent Developments in Columns

One of the most revolutionary developments in gas chromatography in recent years has been the invention of fused silica capillary columns. Fused silica capillary columns are extremely inert, and they usually produce 3000-5000 theoretical plates/meter (Reineccius, 1984). In addition to the development of fused silica capillary columns, bonded fused silica columns have been used extensively (Perry, 1981). These columns contain internally cross-linked phases which are present as thin films. One such example is the HP ultra performance capillary column, which is coated with a 0.52 μm film of crosslinked 5% phenyl- methylsilicone. This type of column was used by Dupuy and coworkers in their study of volatiles in vegetable oil (Dupuy et al., 1985).

5. Thermal Desorption

Gas chromatographic analysis involving the thermal

desorption of volatiles, which have been contained in a variety of trapping devices, has been reported. This involves the use of an External Closed Inlet Device (ECID), which introduces the sample onto the top of the cold column by the flow of the carrier gas (Legendre et al., 1978). Volatiles from large food samples may be analyzed in this way, after "stripping," as described above.

Many of the methods mentioned above have or can be applied to the analysis of volatiles produced by microorganisms.

G. GC-MS IN THE FLAVOR INDUSTRY

1. General Considerations

Separation and component identification are two critical steps in any complete flavor research program. Gas chromatography is considered to be the most effective technique currently available for the separation of volatile mixtures. Mass spectrometry is an extremely powerful tool for the elucidation of volatile structures, so it is logical that by coupling a gas chromatograph with a mass spectrometer, a superb analytical tool is the result (Jennings and Shibamoto, 1980).

The obvious function of the gas chromatograph in the

GC-MS system is to separate the sample volatiles, while the purpose of the mass spectrometer is to identify the compounds. In this process, the sample is ionized, and the ions are accelerated into the analyzer by a magnetic field (Perry, 1981). This produces a mass separation of the ions, thus providing a typical fragmentation pattern, which may be used as a "fingerprint' to compare with reference files of known mass spectra (Flath, 1981a). This last step is normally done by a computer database search. A compound's spectrum is not necessarily unique, however, and further analysis, such as NMR, may be needed in order to unequivocally confirm an unknown compound's identity. Nonetheless, MS is widely considered to be a reliable identification tool.

An integral part of the GC-MS system is the interface between the two components. The interface device must transfer as much sample as possible from the GC to the MS, without the introduction of too much carrier gas (Flath, 1981a).

2. Instrumentation

The most common union of the gas chromatograph and the mass spectrometer involves the use of a capillary column GC and a quadropole MS, in which the GC column

exit and the MS inlet are directly coupled. Typically, a short length of small-bore glass-lined metal tubing or platinum tubing is used to direct the column effluent into the MS (Flath, 1981a).

In a quadrupole mass analyzer, four parallel bars are arranged in such a way that a space of roughly eight cm exists in the center of the bundle. Direct and alternating currents, typically of 300v and 2,000v, respectively, are simultaneously applied to the bundle of bars. However, while all four bars exist at the same voltage, a bar's sign of direct voltage, as well as its phase of alternating current, is similar only to its diagonally opposite neighbor (Perry, 1981). The amount of voltage applied to the bars defines the resolution of the MS; resolution increases with the ratio of direct voltage to alternating voltage. It is through this electromagnetic field that the sample ions pass. As the ions pass through the field during the mass scan, the resolution is varied in order to separate one mass from the next.

H. THE GENUS Ceratocystis

1. General Description

The genus Ceratocystis, an ascomycetous fungus, was established by Ellis and Halsted in 1890 (Hunt, 1956). However, its taxonomic history has been confounded by the description of various other genera formerly thought to belong to Ceratocystis, including Endoconidiophora (Munch, 1907), and Ophiostoma (Sydow and Sydow, 1919). These genera are now considered to be synonymous with Ceratocystis. Excellent taxonomic monographs of the genus are given by Hunt (1956) and Upadhyay (1981).

Species of the genus Ceratocystis possess several common, distinguishing features when viewed microscopically. The following morphological features are the determining factors for their taxonomic grouping. The perithecia, within which eight hyaline ascospores are contained in each evanescent ascus, are found singly or in clumps. The flask-shaped or globose bases of the perithecia are brown to black, with elongated necks. The asci normally disintegrate to form oily, gelatinous masses upon maturity. At this stage, hydrostatic pressure forces the smooth ascospores from the tips of the perithecia along with the oily ascus remnants (Hunt, 1956).

The genus has been divided by Hunt into two parts based upon the types of conidia present, either endoconidia or exoconidia. These conidia are produced during the imperfect stage of the life cycle (Hunt, 1956). All species of Ceratocystis may be grown on a variety of synthetic media, preferably within the temperature range of 22-25°C (Collins and Morgan, 1962, Collins and Halim, 1970). The young mycelial mat is normally white, changing to dark brown and black with age. Cultures are easily maintained on potato-dextrose agar slants at 20°C (Collins and Kalnins, 1965). Many, but not all of the species, particularly those in the endoconidial group, produce "sweet," "fruity" or "banana-like" odors in pure culture (Hunt, 1956) and on their natural substrates (Davidson, 1935).

2. Economic Roles

As a genus, Ceratocystis contains species with a variety of economic roles, some of which are pathogenic. A majority of the species are found on or in wood. C. fagacearum is the causal organism associated with oak wilt (Fowler, 1953), while C. ulmi (Ophiostoma ulmi) is the causative fungus of Dutch elm disease (Gibbs, 1981). The abovementioned species have had an extremely

detrimental effect upon standing oak and elm trees in the United States. Some species of Ceratocystis, such as C. ips, are the major cause of blue stain on logs and lumber (Boyce, 1951). The dissemination Ceratocystis is primarily associated with insects such as the ambrosia beetle or the bark beetle (Hunt, 1956). C. fimbriata causes black rot of the sweet potato (Halsted , 1890). Other species, such as C. moniliformis, are non-pathogenic, and seem to have no established economic role.

I. Ceratocystis moniliformis

1. General Description

C. moniliformis (Hedg.) was first described from hardwood lumber in Texas and Arkansas (Hedgcock, 1906). Ceratostomella moniliformis, Ophiostoma moniliforme, Endoconidiophora bunae, Endoconidiophora moniliformis, and Ceratocystis wilsoni are all synonyms for Ceratocystis moniliformis (Hunt, 1956). In his 1935 study, Davidson isolated C. moniliformis from hardwood lumber in a variety of Southern locations. Although he noticed that C. moniliformis produced a dark brown stain in fresh sapwood, this stain lightened as the hardwood lumber dried. Thus, C. moniliformis was not considered

to be a major contributor to hardwood stain, even though it is often found growing in association with other species which do cause dark stains in hardwood lumber. C. moniliformis was not found to cause any staining on pine lumber (Davidson, 1935). Later, Gordon (1950) identified C. moniliformis as the causative agent in the blue staining of recently cut stumps of short-leaf pine (Pinus echinata) at Duke University.

Morphologically, C. moniliformis is similar to C. fimbriata, C. variospora (Hunt, 1956), and C. virescens (Davidson, 1935). Some consider C. variospora to be synonymous with C. fimbriata (Webster and Butler, 1967), while Hunt determined that C. coerulescens is synonymous with C. virescens. C. virescens is the type species of the genus. The only major morphological differences between C. moniliformis and these other species are its pear-shaped base ornamented with spines (Hunt, 1956), and the production of hat-shaped spores (Gordon, 1950).

2. Aroma Production

Despite confusion in the nomenclature, all of the above mentioned species produce a characteristic banana oil and fruit-like odor when grown in pure culture and on their natural substrates (Collins and Morgan, 1962;

Collins and Halim, 1970; Collins and Halim, 1977). All species of Ceratocystis may be grown on a variety of artificial media (Hunt, 1956). Furthermore, many species of Ceratocystis produce yeast-like cells when grown in submerged culture, thus making them ideal candidates for large-scale fermentations (Collins, 1979).

In their work with C. moniliformis, Lanza and coworkers (1976) used a basal medium described by Wilson and Lilly (1958) containing urea, micronutrients and vitamins, and any of a variety of carbon sources such as galactose, fructose, corn starch, and glycerol. C. moniliformis has also been shown to grow well on natural media such as malt agar, nutrient agar, and potato-dextrose agar (Gordon, 1950).

Gordon (1950) used a chemical method to isolate and identify ethyl acetate in cultures of C. moniliformis grown in a basal medium containing various carbon sources. Using potato-dextrose broth as the growth medium, Collins and Morgan (1962) identified several volatile components produced by C. moniliformis using gas chromatography. These compounds included ethyl acetate, ethanol, and isoamyl acetate. All of these compounds are constituents of banana oil. Ko (1971) used combined GC-MS to identify the volatile components in the headspace

above cultures of C. moniliformis, and found the same compounds as did Collins and Morgan. Using combined GC-MS, Lanza, Ko and Palmer (1976) analyzed both the headspace and the Freon-11 extract of the culture broth of C. moniliformis, and identified the monoterpenes geranial and citronellol, as well as gamma and delta-decalactones in both the headspace and the extract. These monoterpenes are known to be character impact compounds in citrus fruits (Heath, 1981), and the decalactones are responsible for the characteristic peach aroma (Sharpell, 1985).

Lanza and Palmer (1977) expanded this study, and using combined GC-MS, identified the monoterpenes nerol, neral, linalool, geraniol, and alpha-terpineol in the culture broth of C. moniliformis. These compounds, as secondary metabolites, reached their peak of production only after the nitrogen source was significantly depleted (Lanza and Palmer, 1977). This is a typical pattern in the biosynthesis of secondary metabolites by fungi (Schindler and Schmid, 1982). Furthermore, using radiolabelled precursors, Lanza and Palmer determined that the monoterpenes are biosynthesized via the mevalonic acid pathway. This is the same pathway that is utilized by higher plants in the production of terpenes.

It seems likely that cultures of C. moniliformis can be manipulated to produce desirable fruity-type volatiles that would have uses in food product flavoring.

III. MATERIALS AND METHODS

A. CULTURE MEDIA

1. Glucose Yeast Extract Agar (GYEA)

Five grams of anhydrous dextrose (reagent grade) and 1 g yeast extract (Difco, Detroit, Michigan), and 15 g agar (Difco) were added to 1000 ml distilled water. The medium was autoclaved at 15 psi, 121°C for 15 minutes. Plates were poured, cooled, and subsequently stored at 4°C.

2. Potato Dextrose Streptomycin Agar (PDSA)

This agar was prepared by Jean Ratliff, Plant Pathology, Physiology, and Weed Science, Virginia Tech. It was standard Difco potato dextrose agar with 100 mg streptomycin sulfate added after autoclaving at 15 psi, 121°C for 15 minutes. Plates were stored at 4°C.

3. Basal Medium With Yeast Extract

This medium is based on that of Wilson and Lilly (1958), but yeast extract (Difco) was used as the nitrogen source. A stock solution of the minerals (reagent grade) was prepared separately, and added to the

broth prior to autoclaving. No vitamin sources were added to the broth, since the vitamins biotin, thiamine, and inositol are present in the yeast extract. The carbon source was either glycerol (gYEB) or glucose (GYEB), and the pH of the medium was 5.5. The medium was sterilized by autoclaving at 15 psi, 121°C, for 20 minutes. Composition of this medium is shown in Table 1a.

4. Basal Medium With Urea

This basal medium is based on that of Wilson and Lilly (1958), but urea was used as a nitrogen source. The same stock solution of minerals described above was used. Stock solutions of the vitamins thiamine, biotin, and inositol, and the nitrogen source urea were prepared and sterilized separately by filtration through 0.2 µm millipore filter units (Whatman). The vitamins were needed for the culture medium, since C. moniliformis is deficient for these nutrients (Gordon, 1950). The sterile urea, thiamine, inositol and biotin were aseptically added to the culture broth after the carbon and mineral sources were sterilized by autoclaving at 15 psi, 121°C, for 20 minutes. The pH of the medium was 5.5. The carbon source was either glycerol (gUB) or

glucose (GUB). Composition of this medium is listed in Table 1b.

5. Oil Sources

The heavy mineral oil used in this study was added to the basal media, where indicated, prior to sterilization. The oil (Squibb Inc., Princeton, NJ) was obtained from a local convenience store. Tween 80 was used as a surfactant, where indicated.

Castor oil (Swan Brand) was also added to the basal media, where indicated, prior to sterilization. Tween 80 (Fischer) was used as surfactant, where indicated. The oil was obtained from a local convenience store.

B. CULTURAL TECHNIQUES

1. Maintenance of Stock Culture

A lyophilized culture of Ceratocystis moniliformis (ATCC 12861) was rehydrated and plated onto potato-dextrose-streptomycin agar, incubated at 25°C until a full mycelial mat was formed, and subsequently stored at 4°C until needed. These plates are henceforth referred to as the sources of pure culture.

Table 1a. Composition of basal medium with yeast extract. [a]

Component	per 100 ml
dextrose, or equiv.	3.00 g
yeast extract	0.10 g
KH_2PO_4	0.10 g[b]
$MgSO_4*7H_2O$	0.05 g[b]
$FeSO_4*7H_2O$	0.20 mg[b]
$MnCl_4*4H_2O$	0.10 mg[b]
$CaCl_2$, anhydrous	10.00 mg[b]
$ZnSO_4*7H_2O$	0.20 mg[b]

[a] based on Wilson and Lilly, 1958.
[b] prepared as a mineral stock solution.

Table 1b. Composition of basal medium with urea. [a]

Component	per 100 ml
dextrose, or equiv.	3.00 g
urea	0.10 g[c]
KH_2PO_4	0.10 g[b]
$MgSO_4*7H_2O$	0.05 g[b]
$FeSO_4*7H_2O$	0.20 mg[b]
$MnCl_4*4H_2O$	0.10 mg[b]
$CaCl_2$, anhydrous	10.00 mg[b]
$ZnSO_4*7H_2O$	0.20 mg[b]
thiamine	10.00 µg[c]
biotin	0.50 µg[c]
inositol	0.50 mg[c]

[a] based on Wilson and Lilly, 1958.
[b] prepared as a mineral stock solution.
[c] filter sterilized, aseptically added to broth after heat sterilization.

2. Preparation of Inoculum

In preparing the inoculum, 3 sterile loopfuls of the pure culture were mixed with 2 ml sterile distilled water, and spread on a 5:1 glucose:yeast extract agar plate. The plate was incubated at 25°C for 4 days. Number 6 cork borer "plugs" from the agar culture served as the inoculum for liquid cultures.

All liquid cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of culture broth. When an oil source was added to the culture medium, 10 ml (approximately 9 g) of the oil were always used. The flasks were covered with 50 ml inverted beakers; this allowed for ample gas exchange (Stipes, 1989). Cultures were grown at 25°C, either standing, or shaking at 100 rpm on a rotary shaker.

C. ASSAY METHODS

1. Cell Mass Determination

Cells of the culture were recovered from the growth medium, dried and weighed as follows:

Cells were harvested at 1, 2, 3, 4, 5, , 7, 8, and 10 day intervals. The harvest was performed by filtration of the culture broth through a 47 mm x 3.0 µm glass fiber filter disc (Gelman, Ann Arbor, Michigan).

The filter disc was held by a Millipore (Bedford, Massachusetts) filter funnel, and the cell-free culture broth was collected under vacuum in a 500 ml sidearm flask. Gelman filters had been previously dried at 110°C overnight, held in a desiccator, and weighed just prior to use. Cells were collected on the filter disc, washed three times with 100 ml distilled deionized water, and dried at 110°C until a constant weight was obtained.

2. Determination of Nitrogen Depletion

A Micro-Kjeldahl method was used to determine nitrogen depletion by C. moniliformis during the incubation period. The method used was a modification of the Kjeldahl-Wilforth-Gunning method (Pomeranz and Meloan, 1978), using sodium sulfate and copper sulfate as the catalysts in a 16:1 ratio. Samples of the culture broth (GYEB) were taken at 1, 2, 3, 4, 5, 6, 7, 8, and 10 days after inoculation. The GYEB culture broth was filtered as described previously, and divided into 20 ml aliquots.

For the analysis, 10 ml of each sample was dispensed into a separate 100 ml Kjeldahl flask. To each of the control and sample flasks, the following reagents were added:

0.16 g sodium sulfate, reagent grade

0.01 g copper sulfate, reagent grade

5.0 ml concentrated sulfuric acid, reagent grade

Five drops of Antifoam Agent B containing 10% silicone (Sigma) was added to each sample flask. The samples and controls were digested for approximately 2 hours on a benchtop Micro Kjeldahl digestion unit (Labconco Co.), until digestion was complete as indicated by a persistent green color. The flasks were cooled, and 30.0 ml distilled water was added. The flasks were cooled again, and 10.0 ml 50% w/w sodium hydroxide, reagent grade (Fisher) was added to each flask. The flasks were distilled using a standard Micro Kjeldahl distillation apparatus, and the samples were titrated using a syringe microburette (Micro-metrics Co., Cleveland, OH).

3. Determination of Glucose Depletion

A Sigma Diagnostics quantitative enzymatic assay using a glucose oxidase/peroxidase mix was used to determine glucose depletion of the culture medium by C. moniliformis during incubation. This assay is basically a micro Glucostat assay, largely based upon the method of Raabo and Terkildsen (1960). The reagents are available

as a whole blood, serum or plasma glucose determination kit manufactured by Sigma, St. Louis, MO (Anon., 1988). A similar method was used by Ko in his study of glucose depletion by C. moniliformis (Ko, 1971).

Cell-free samples of the GYEB culture broths were taken at 1, 2, 3, 4, 5, , 7, 8 and 10 days post-inoculation. These samples were diluted 500 fold for the assay. A 20-fold dilution of the original 1 mg/ml glucose standard was also used. The enzyme-chromogen mix was prepared, and all subsequent procedures were followed as per the instructions provided with the kit. A Bausch and Lomb Spectronic 20 was used for the colorimetric analysis. Absorbance readings were taken at 450 nm.

4. Sensory Evaluation Procedure

Sensory evaluation was performed using 12 panelists from the department of Food Science and Technology, Virginia Tech. For this study, sensory evaluation was performed purely to generate descriptive data of the aromas, rather than to determine the relative intensities of aromas of the samples.

Cultures which had been incubated in the glucose yeast extract broth for 5 and 8 days, respectively, were evaluated for aroma by each panelist. Samples were

prepared and presented as follows:

The contents of each culture flask were divided in half, yielding 50 ml samples. These samples were presented to the panelists in wine glasses at room temperature (22 - 25°C). Each glass was capped with a 100 mm watch glass to prevent the escape of volatiles.

The wine glasses were placed on the counter in the Test Kitchen, and the panelists were given sensory evaluation question sheets with the sample numbers arranged in a random order. Each panelist was told to sniff the samples in the order in which they appeared on their evaluation sheets. Panelists were instructed to handle only the stem of the glass, so as not to change the temperature of the samples. They were also instructed to remove the watch glass only long enough to thoroughly sniff each sample at a distance of approximately 2 -3 inches above the sample.

5. Sensory Evaluation Questions

Panelists were instructed to answer the following questions for each sample during each sensory evaluation session:

1. What does this sample smell like to you? Please choose any and all descriptors that apply from this list:

rancid	tropical	fatty	citrus
floral	musty	peach	beefy
banana	apricot	soapy	fruity

2. Which descriptor represents the most dominant note?
3. Do you detect any aromas not listed? Y/N
4. If so, how would you describe the aroma(s)?

Panelists were advised that the descriptor "tropical" referred to a combined tropical floral, coconut and cocoa butter aroma similar to many suntan oil aromas. The term "soapy" was meant to be defined as the aroma of unperfumed soap. The panelists were presented with eight samples on day one, and eight samples on day two of the test period. The samples were as follows:

- GYEB, no inoculum (control), day one
- gYEB, no inoculum (control), day two
- castor oil (control), day one
- mineral oil (control), day two
- GYEB, 5 and 8 day incubations, day one

- GYEB + mineral oil, 5 and 8 day incubations, day one
- GYEB + castor oil, 5 and 8 day incubations, day one
- gYEB, 5 and 8 day incubations, day two
- gYEB + mineral oil, 5 and 8 day incubations, day two
- gYEB + castor oil, 5 and 8 day incubations, day two

Panelist were told only that the samples were from a fermentation, and they were not told the differences in treatments, or that there were two control samples that were not fermented. As for the oil samples, panelists were not told that the samples were oils. No attempts were made to mask color differences of the samples, if color differences did exist. Panelists were asked to describe the samples purely in terms of aroma.

D. PREPARATION OF STANDARDS

1. Internal Standard

An internal standard for GC analysis was prepared for use in the oil and aqueous portions of the culture medium. Limonene was chosen as the internal standard,

since no limonene was produced by the cultures, and it would not mask any significant peaks in the gas chromatograms. The pure limonene was obtained from Analytical Standard Kit #85C, Essential Oil Fingerprint Kit (Polyscience Corp.).

In preliminary work with the 2, 4, 6, and 8 day samples, the internal standard was added at a final concentration of 0.1 mg/ml in the aqueous samples, and at 0.1 mg/mg in the oil samples. These levels were found to be too high. Therefore, for subsequent analyses, the internal standard was used as follows:

Two stock solutions of the standard were prepared, one for use in the oil fraction of the samples, and one for use in the aqueous fraction of the culture medium. For the oil fraction, a solution of 8 mg limonene per 1 g autoclaved mineral oil was used. For the aqueous fraction, a solution of 40 µg limonene per ml of methanol (GC grade) was used. These stock solutions were added to the samples so that the final concentration of the limonene in 50 mg samples of the oil fraction was 40 µg/ 50 mg, and in 10 ml aqueous fraction samples, the final concentration was 40 µg/ 10 ml. These concentrations were chosen so as not to overload the GC flame ionization

detector, and so that the standard concentration would be within the range of the predicted concentrations of some of the unknown compounds in the samples.

2. Standard Solutions of Known Aroma-Bearing Compounds

These solutions contained mixtures of components which had been tentatively identified in cultures via GC-MS analysis, and which have been reported to have an aroma impact. Each solution also contained the limonene internal standard.

One set of solutions was prepared in autoclaved mineral oil. Aliquots were then added to mineral oil, so that final concentrations subjected to GC analysis were in the range of 20 to 80 µg in the 50 mg samples normally analyzed. Table 2 shows the composition of these mixes.

A second set of solutions containing the selected aroma-bearing compounds was prepared in methanol. When 1 ml of these stock solutions was added to 9 ml of water, the final concentration of each component was in the range of 20 to 80 µg in the 10 ml aqueous samples normally analyzed. Table 3 shows the composition of these mixes.

As described later (Section I), these mixes were

Table 2. Composition of oil standard mixes.[a]

Mix No.	Components [b]	Concentration[c] (µg/50 mg sample)
Mix 1	Geraniol	80
	Isoamyl acetate	40
	Limonene	40
Mix 2	1-Octanol	80
	Citronellol	40
	Limonene	40
Mix 3	Acetophenone	80
	β -Myrcene	40
	Limonene	40
Mix 4	Geraniol	80
	1-Octanol	80
	Acetophenone	80
	β -Myrcene	40
	Isoamyl Acetate	40
	Limonene	40
Mix 5	Geraniol	40
	1-Octanol	40
	Acetophenone	40
	β -Myrcene	20
	Isoamyl Acetate	20
	Limonene	20

[a] Prepared in mineral oil

[b] Sources: geraniol, β -myrcene, isoamyl acetate, 1-octanol from Sigma; citronellol from American Tokyo Kasei; acetophenone from Polyscience, Inc.

[c] Concentration in samples as analyzed by GC

Table 3. Composition of aqueous standard mixes.[a]

Mix No.	Components [b]	Concentration [c] (µg/10 ml sample)
Mix 6	Geraniol	80
	1-Octanol	80
	Acetophenone	80
	β -Myrcene	40
	Isoamyl Acetate	40
	Limonene	40
Mix 7	Geraniol	40
	1-Octanol	40
	Acetophenone	40
	β -Myrcene	20
	Isoamyl Acetate	20
	Limonene	20

[a] Prepared in methanol

[b] See Table 2 for sources

[c] Concentration in samples as analyzed by GC

used to tentatively identify and estimate the concentration of selected components in cultures of C. moniliformis.

E. ISOLATION OF VOLATILE COMPOUNDS FOR GC ANALYSIS

1. Volatiles From The Oil Fraction

The contents of a culture flask containing oil were centrifuged at 10,000 x g at 22°C for 20 minutes to separate the aqueous and oil phases, and to precipitate the cells. The oil layer was removed as completely as possible using a Pasteur pipette. Four and one half grams of this oil was weighed into a glass vial, and 0.5 g of the limonene-in-mineral oil internal standard stock solution was added to the oil. The final concentration of the limonene standard was 40 µg per 50 mg sample. This was mixed thoroughly, and the oil/internal standard mix was frozen until it was analyzed.

2. Volatiles From The Aqueous Fraction

This method is based on that of Vercellotti et al. (1988). The method involves "stripping" the volatile components from the sample under reduced pressure, at temperatures ranging between 50-65°C. Briefly, the volatiles in the aqueous portion of the abovementioned culture sample were collected on a Tenax trap. This trap

consists of a Pyrex glass liner similar to that mentioned above, filled with about 200 mg of 60/80 mesh Tenax-TA (Alltech Associates, Deerfield, IL). The Tenax had previously been conditioned by heating at 340°C for two hours in the presence of a stream of nitrogen flowing at 20 ml/min. The glass liner was plugged at both ends with volatile-free glass wool, leaving the same clearance as mentioned previously.

For the stripping, 10 or 20 ml of this aqueous sample was added to a 50 ml round bottom boiling flask, along with 1 ml of the 40 µg/ml limonene/methanol internal standard. The flask was fitted with a 24/40 standard taper tap- water-cooled condenser. A 24/40 standard taper center-bored teflon plug (custom made by Joe Agnew, Laboratory Support Services, VPI & SU) was fitted into the top of the condenser, and the Tenax trap was fitted snugly into the top of the plug. The Tenax trap was connected to a vacuum. The sample was heated to 55°C at approximately 400 mm Hg, and the volatiles were collected for two hours. The glass liners containing the samples were then plugged at both ends with corks, placed in a screw-cap test tube, and frozen until it was analyzed.

3. Volatiles From the No Oil (Control) Samples

As a control, the culture was grown in the glucose-yeast extract basal medium, without any oil added. Volatile stripping was done in the same manner as the aqueous fraction samples, as described previously. Twenty ml or 10 ml aliquots were used for the final analysis of 5 and 8 day samples.

F. ISOLATION OF VOLATILE COMPOUNDS FOR GC-MS ANALYSIS

The volatiles of the aqueous fractions, as well as the no-oil samples, were collected by the same methods as mentioned previously, with one exception. The pyrex glass liners containing the samples measured 20.2 cm long x 0.6 cm outer diameter x 0.4 cm inner diameter. The liners containing glass wool for oil samples were packed to within 4.0 cm of each end. The liners containing Tenax-TA were packed within 5.0 cm of each end with the Tenax-TA, and 1.0 cm plugs of glass wool were used to keep the Tenax-TA in place. Samples were from 2, 4, and 6 day incubation periods. These samples were also frozen as described above until needed.

G. SEPARATION OF VOLATILE COMPOUNDS BY GC

1. Instrumentation

All gas chromatography work was performed in collaboration with Dr. Genaro Arganosa, VPI & SU, Department of Food Science. A Hewlett Packard 5890 Gas Chromatograph equipped with a Flame Ionization Detector (FID) set at 275°C was used. A Hewlett Packard ultra-performance 2 crosslinked 5% phenyl-methylsilicone column, measuring 50 m long by 0.32 mm inner diameter, with a film thickness of 0.52 μm, was used. The GC was modified by interfacing with an External Closed Inlet Device (ECID, Scientific Instrument Service, River Ridge, LA) (Vercellotti et al., 1988). Flow rates for air and hydrogen were 240 ml/min., and 30 ml/min., respectively. Volatiles were desorbed using helium as the carrier gas, at a flow rate of 1.34 ml/min. The auxiliary make-up gas, also helium, was set at a flow rate of 30 ml/min. The "split" mode was not used.

2. Analytical Procedure For Aqueous Samples

This method was used for the no-oil (control) and aqueous samples, as well as for the aqueous standard mixes. The inlet valve and the rotary valve of the ECID were both preheated to a temperature of 180°C. Prior to

and during desorption, a portion of the column was immersed in an acetone and dry ice bath, and cooled to -78.5°C, in order to temporarily trap the sample "plug." The glass liner containing the sample which had been trapped on the Tenax-TA, was placed in the inlet valve. Desorption was started by switching the valve handle to the "purge" mode. After a two minute period of desorption, the valve handle was returned to the "run" mode. The acetone/dry ice bath was removed, and the oven temperature was brought from 30 to 150°C, at a rate of 2.5°C per minute, and then to 250°C at a rate of 5.0°C per minute. The final hold time was 30 min. This method is based upon that of Vercellotti et al. (1988).

3. Analytical Procedure For Oil Samples

Prior to analysis by GC, the oil samples were thawed for 5-10 minutes at room temperature. Each sample was collected on a separate glass wool trap (Pyrex fiber glass silver 8 um, Corning Glass Works, Corning, NY) as follows: Approximately 50 mg of sample was weighed into a Pyrex (Corning Glass Works, Corning, NY) glass liner (8.3 cm x 0.9 cm OD x 0.7 cm ID) packed with volatile-free glass wool. The glass wool had been treated by heating at 200°C for 24 hours to remove unwanted

volatiles. The glass liner was packed so as to leave a clearance of approximately 0.75 cm at the bottom, and 1.25 cm at the top of the tube. Thermal desorption of all oil samples, including the oil-based standard mixes, was carried out for a period of twenty minutes, with the ECID inlet temperature at 140°C. The rotary valve temperature was 180°C. Volatiles were temporarily trapped in the column, which was cooled to -78.5°C prior to and during the desorption period. The same oven temperature program as described above was used for these oil samples.

H. IDENTIFICATION OF SELECT VOLATILE COMPOUNDS BY GC-MS ANALYSIS

1. Instrumentation

The analyses were performed by Dr. Mark Malcomson, Department of Chemistry, University of Arizona. Because not all samples could be analyzed, nor could all peaks be identified due to time and cost restraints, only several selected samples were subjected to analysis. A Hewlett Packard model 5890 gas chromatograph, interfaced with a Hewlett Packard model 5970 benchtop quadrupole mass selective detector, was used. An RTE-6/VM data system which employed the NBS-Wiley Library of mass spectra was

used. The column was a J&W Scientific (Folsom, CA) DB-1 dimethylsilicone bonded phase fused silica capillary column. It measured 20 m in length, with an internal diameter of 0.18 mm, and a film thickness of 0.25 μm.

The gas chromatograph was modified by the addition of a desorption furnace and a cryogenic trap. The carrier gas was helium with a column head pressure of 12.5 psig, and a flow rate of approximately 0.7 ml/min. The capillary column of the gas chromatograph was directly linked to the mass spectrometer, and the interface was set at 280°C. A split injector was used with a split ratio of 70:1.

2. Analytical Procedure

Samples were thawed at room temperature. The glass liner containing either an oil sample on glass wool, or an aqueous sample trapped on Tenax-TA, was placed in the desorption furnace. With the 6 port valve in the "inject" position, the cryogenic trap was cooled in a Dewar of liquid nitrogen at -78.5°C. The sample liner was purged for 30 seconds to displace air. The 6 port valve was then switched to the "sample" position and the desorption furnace was turned on. The furnace was heated from room temperature to 200°C over a period of 3

minutes. The furnace was held at 200°C for 5 minutes. The 6 port valve was then returned to the "inject" position, and then the trap was quickly moved from the liquid nitrogen to a metal beaker of boiling water. The GC oven temperature program was then started as follows:

The oven was held at an initial temperature of 50°C for 2 minutes, and then raised to 110°C at a rate of 30° C per minute. The rate was then changed to 2°C per minute until a temperature of 140°C was reached, at which time the rate was switched to 10°C per minute until the final temperature of 250°C was reached.

3. Detection and Identification of Compounds

Compounds in both the oil and the aqueous fractions of the samples were detected by total ion current, and the mass spectrum of each compound was generated through mass separation by the quadrupole mass analyzer. These spectra were referenced with the spectra of known compounds contained in the computerized NBS-Wiley library of mass spectra. Identity of the unknown compound was given in terms of the probability of a match with a spectrum or spectra found in the library.

In preliminary studies, it was found that the volatiles present in the oil samples were not fully

desorbed from the Pyrex glass liner sample holders. Based on this finding, data from oil samples generated through GC-MS analysis were not conclusive. However, data from the aqueous samples provided useful information as to the identity of some seventeen volatile aroma compounds produced by C. moniliformis.

I. IDENTIFICATION AND QUANTITATION OF SELECT VOLATILE COMPOUNDS

1. Identification

The GC-MS analysis identified a variety of volatile aroma-bearing compounds in cultures of C. moniliformis. The yields of five of these compounds were analyzed, and in particular, the effect of oil as a trapping agent in respect to compound yields, was studied.

The GC-MS analysis employed a GC column and GC conditions which were different from those used in our laboratory. Therefore, it was necessary to first determine which peak corresponded to each of the selected components, when GC separation was carried out using our system. To accomplish this, the peak retention times relative to the limonene internal standard were determined, for both the aqueous and oil fractions, and also for the no-oil (control) cultures. These relative

retention times were then compared to the relative retention times obtained upon the analysis of the standard mixes listed in Tables 2 and 3. If the relative retention times for a particular peak agreed within ± 1%, then the component responsible for the peak in culture samples was identified as the component responsible for the peak in the standard mix. These must be considered tentative identifications, since the identities were not confirmed by further analysis.

2. Calibration of Gas Chromatograph

The GC system was calibrated by introducing a series of standard mixes prepared either in mineral oil (Table 2) or in methanol (Table 3).

Approximately 50 mg of each oil standard mix was introduced into the GC, as described earlier. The oil standard mixes were used only to calibrate the GC in response to the oil samples.

Our original assumption was that the components of the standard mixes would yield approximately equal peak areas when introducing equal quantities into the GC. However, some components, especially the higher boiling ones, yielded appreciably lower peaks than limonene, when identical quantities were injected. This suggested that

desorption from the oil was incomplete. Nevertheless, it was possible to calculate for each standard mix the response of individual components relative to limonene. These relative responses were reasonably consistent over a number of runs with standard mixes containing the same concentration of limonene, but varying concentrations of standard mix components. Table 4 summarizes the response data for the components of interest. In GC analyses of oil samples from cultures, the samples were "spiked" with a known concentration of limonene prior to GC analysis. The amount of a component X in these samples was then calculated as follows:

$$\text{ug component X} = \frac{(\text{counts for X})\ (\text{ug/count for limonene})}{(\text{response factor for component X})}$$

Attempts to obtain similar response factors or calibration data for aqueous standards met with failure. Samples of the aqueous standard mixes prepared in methanol (Table 3) and subsequently diluted 1:10 with water were carried through the "stripping," Tenax absorption, desorption and GC analysis, as described earlier (Sections E2, G2). In contrast to the oil standards, the response factors calculated from the peak areas relative to limonene were highly variable. In

Table 4. Average responses of compounds in standard mixes [a,b]

Compound	Response [c]	Variation
Isoamyl Acetate	1.00 ± 0.093	9.3%
β -Myrcene	1.20 ± 0.120	10.4%
Acetophenone/ Octanol	0.79 ± 0.046	5.8%
Geraniol	0.23 ± 0.047	20.0%

[a] Prepared in mineral oil
[b] Average for all runs of mixes 1 through 5
[c] Mean response relative to limonene

fact, the peaks expected from the higher boiling compounds (compounds eluting after limonene) often did not appear at all on the chromatograms. These results indicated problems with the "stripping" and/or desorption procedures. Whatever the problems, it was clearly not possible to quantitate flavor component concentrations in aqueous or no-oil (control) samples using the "stripping"/GC procedure.

IV. RESULTS

A. GROWTH CHARACTERISTICS OF C. moniliformis

1. Growth and Morphological Changes

Preliminary studies were conducted in order to compare the basal medium with urea and the basal medium with yeast extract, in relation to the growth and morphology of C. moniliformis. During these studies, it was found that the culture grew faster and more uniformly when the culture flasks were incubated while shaking at 100 rpm on a rotary shaker. The cultures grown in the shaking flasks grew most often as single cells, and frequently as long strands of mycelia measuring up to 14 cm in length, or in small clumps which measured as large as 2 mm in diameter. Single cells appeared to be yeast-like, as described by Hunt (1956). These findings are based on both microscopic and macroscopic observations. Sporulation was apparent after 96 hours of incubation, and the culture darkened to a greenish-black color as the proportion of spores increased.

The cells grown in stationary flasks tended to aggregate in large clumps or "pellets," measuring from 5 to 10 mm in diameter. According to Cohen et al (1987),

the formation of "pellets" in cultures of C.moniliformis is undesirable, because aroma production is decreased when this occurs.

2. Comparison of Culture Media

Early studies were conducted to assess the use of inorganic nitrogen sources in relation to the rate of growth of C. moniliformis. Ammonium nitrate, ammonium sulfate, and potassium nitrate, when used instead of urea in the basal medium shown in Table 1b, were each unable to support growth of the organism.

The ratio of carbon to nitrogen greatly affected the growth of the culture. Preliminary studies using ratios of 2.5:1, 5:1, 10:2, and 30:1 carbon:nitrogen showed that, when the medium supported growth, the ratio of 30:1 produced the most rapid growth. Therefore, all later studies were performed using a 30:1 carbon:nitrogen ratio. Results are summarized in Table 5.

Incubation temperatures of 20°C, 25°C, 28°C, and 30°C were investigated. Cultures grew too slowly at 20°C. At the other three temperatures, growth was faster, yet there was not an appreciable difference in growth rate and morphological change between the three temperatures. Therefore, as a convenience, 25°C was

Table 5. Effect of carbon:nitrogen ratios on growth of C. moniliformis, 8 day incubation, 25 C.

Ratio	Growth [a]
2.5 : 1	(-)
5 : 1	+
10 : 2	+
30 : 1	++++

[a] (-) = no growth; + = poor growth; ++ = fair growth; +++ = good growth; ++++ = excellent growth. Based upon visual observations

chosen for all subsequent incubations.

The basal media with urea and the basal media with yeast extract were examined in relation to both growth and aroma production of C. moniliformis. In concurrence with previously published results (Lanza et al., 1976), the composition of the culture medium affected both the growth and aroma production of the culture.

The glycerol-urea (gUB) broth did not support any growth of the culture, and the glucose-urea broth (GUB) allowed for poor growth and only slight aroma production. In contrast, the glycerol-yeast extract broth (gYEB) supported fair growth with faint aroma production, and the glucose-yeast extract broth (GYEB) supported excellent growth and abundant aroma production. Based upon these results, subsequent work was performed using yeast extract as the nitrogen source in all incubations. Results are summarized in Table 6.

3. Cell Mass

As shown in Figure 1, the phase of rapid exponential growth in GYEB lasted from 12 to 60 hours post-inoculation. After this time, the increase in cell mass slowed before leveling out to approximately 130 mg/100 ml at 160 hours post-inoculation.

Table 6. Effect of carbon and nitrogen sources on growth and aroma production by C. moniliformis, 8 day incubation, 25 C.

Medium	Growth[a]	Aroma[b]
Glycerol/Urea	(-)	0
Glycerol/Yeast Extract	++	2
Glucose/Urea	+	1
Glucose/Yeast Extract	++++	4

[a] (-) = no growth; + = poor growth ++ = fair growth; +++ = good growth; ++++ = excellent growth
Based upon visual observations

[b] 0 = no aroma; 1 = slight aroma; 2 = moderate aroma; 3 = good aroma; 4 = abundant aroma
Based upon preliminary sensory evaluation of aroma

4. Nitrogen Utilization

The amount of nitrogen present in the filtered culture broth (GYEB) declined sharply between 0 to 60 hours post-inoculation to 22 mg/100 ml. After 60 hours, the amount of nitrogen measured by the modified Kjeldahl method fluctuated between approximately 20 mg/100 ml and 45 mg/100 ml. One explanation for this fluctuation may be that, during filtration of the culture medium, cell fragments passed through the millipore filter, and their nitrogenous constituents were measured by the modified Kjeldahl procedure. Nonetheless, it is evident that the nitrogen depletion is directly related to the duration of the exponential growth phase of the culture, and that the nitrogen is most likely the limiting factor in growth, under these conditions.

Based on the glycine standard which was included with each experimental run, there was a nitrogen recovery rate of 94 to 96 %. A standard deviation of not more than 2.66 % existed between replicates. Results are shown in Figure 1.

5. Glucose Utilization

The amount of glucose present in the filtered culture broth (GYEB) declined moderately between 0 and 60

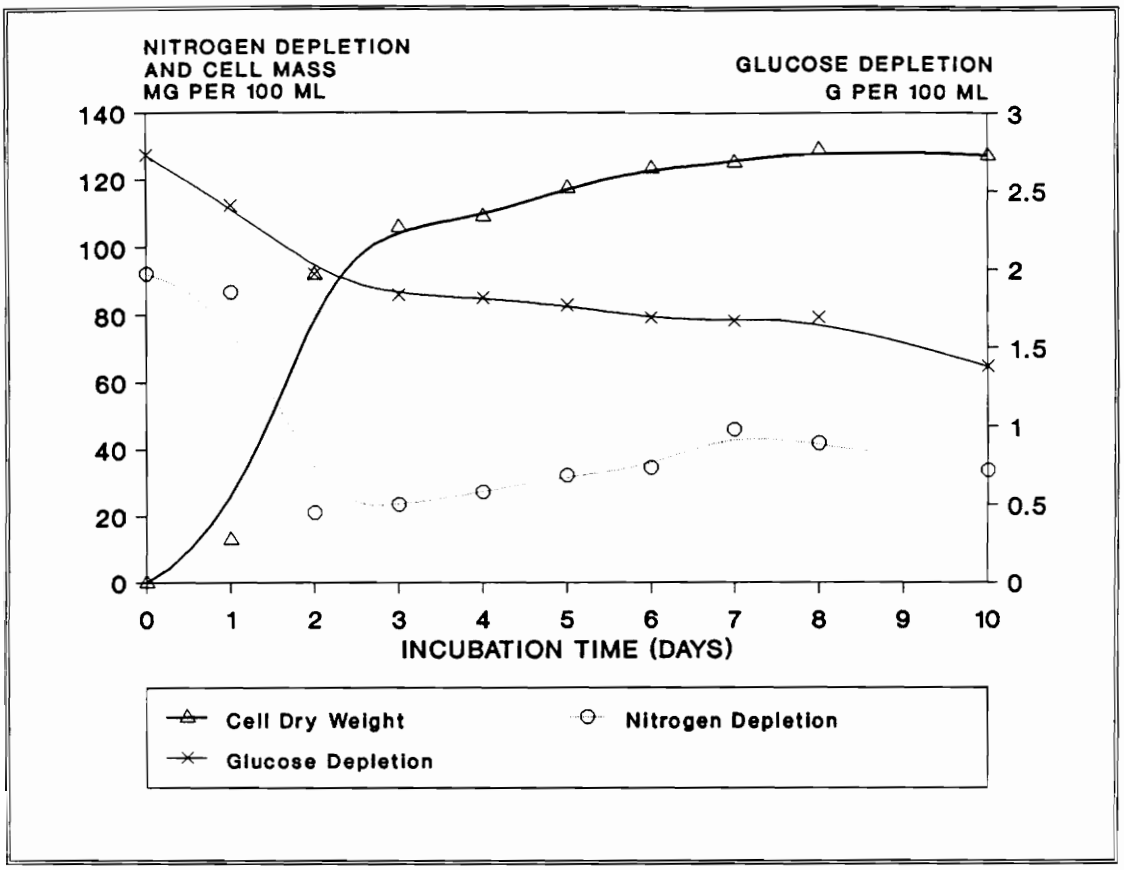

Figure 1. Comparison of growth and nutrient depletion by Ceratocystis moniliformis, basal medium with yeast extract, 25°C, 100 rpm. The data shown are composite data taken from a series of cultures grown over a two month period.

hours post-inoculation, from 3.0 g/100 ml to a level of 1.9 g/100 ml. After 60 hours, the decline in glucose was slight, reaching a level of 1.4 g/100 ml after 240 hours (10 days) of incubation. This level is roughly one half of the starting level of 3.0 g/100 ml, and it is clear that glucose was not a limiting factor under these conditions.

A standard deviation of not more than 0.51 % existed between replicate samples. Results are shown in Figure 1.

6. Effect of Oil Sources on Growth and Aroma Production

Castor oil and mineral oil were each added separately to the gYEB and the GYEB. With both oils, it was found that the culture was able to grow well when a thin layer of oil, measuring approximately 2 mm thick, covered the surface of the culture medium. During the course of incubation, when the flasks were placed on the rotary shaker at 100 rpm, the oil layer was disturbed enough to allow for gas exchange to occur.

Preliminary studies with the two oils showed that the culture also grew well when the surfactant Tween 80 was added to the culture medium. When this was done, the

oil existed as an emulsion in the aqueous phase, as opposed to a thin layer on top of the aqueous phase. While the cultures containing the surfactant did produce pleasing aromas, the use of the surfactant was abandoned because it was difficult to impossible to break the emulsion for further analysis of the two phases.

There were no visible changes in morphological characteristics of the cells with the addition of oil, yet sporulation was visible as much as 24 hours earlier than in the cultures grown without the oil.

Based upon preliminary sensory evaluation of aroma, it was evident that the addition of either oil to the gYEB and the GYEB affected the aroma of the culture medium. Results are summarized in Table 7.

B. SENSORY EVALUATION

1. Panelist Responses

i. Question 1 (descriptor choices)

Descriptor choices by all panelists were very similar within each sample, for all samples. For example, for the GYEB + mineral oil samples, 11 out of 12 panelists chose the descriptors "floral" and "banana," and 6 of the panelists chose the descriptors "citrus,"

Table 7. Effect of oil sources on growth and aroma production by C.moniliformis, 8 day incubation, 25 C.

Medium	Growth[a]	Aroma[b]
gYEB[c] + Castor Oil	+++	2
gYEB[c] + Mineral Oil	++++	3
GYEB[d] + Castor Oil	++++	1
GYEB[d] + Mineral Oil	++++	4

[a] (-) = no growth; + = poor growth;
++ = fair growth; +++ = good growth;
++++ = excellent growth.
Based upon visual observations

[b] 0 = no aroma; 1 = slight aroma;
2 = moderate aroma; 3 = good aroma;
4 = abundant aroma
Based upon preliminary sensory evaluation

[c] gYEB = glycerol yeast extract broth

[d] GYEB = glucose yeast extract broth

"peach," and "fruity." Only one panelist chose the negative descriptors "rancid" and "musty."

Responses given for the 8 day samples were essentially identical to those given for the 5 day samples. Therefore, in summarizing the results, it was not necessary to distinguish between the two days. These results are summarized in Table 8.

ii. Question 2 (dominant aroma)

Answers to this question are summarized in Table 8. The dominant descriptor indicated for each sample is the descriptor chosen most often by panelists.

iii. Questions 3 & 4 (additional aromas)

Most of the panelists answered "no" to this question. However, some responded "yes" to the control culture medium sample, and described the aroma as "smells like agar," or "smells like an autoclave."

C. IDENTIFICATION OF VOLATILE COMPOUNDS BY GC-MS ANALYSIS

1. Sample Analysis

Not all samples that were submitted for GC-MS analysis were actually analyzed by GC-MS, due to time and

Table 8. Sensory evaluation questions 1 and 2, panelist responses for all media, describing aromas of the culture medium.[ab]

Medium	Description of Aroma
GYEB	banana[c], citrus
gYEB	slight citrus[c], fruit
GUB	faint fruit
gUB	no aroma
GYEB + mineral oil	banana[c], citrus, fruity peach, floral
gYEB + mineral oil	citrus[c], rose
GYEB + castor oil	faint fruit[c], musty
gYEB + castor oil	musty[c], rancid, citrus

[a] Summary of responses for 5 day and 8 day samples

[b] GYEB = glucose-yeast extract broth;
gYEB = glycerol-yeast extract broth;
GUB = glucose-urea broth;
gUB = glycerol-urea broth

[c] Indicates dominant note chosen most often by panelists for each sample

cost considerations. Therefore, the following samples were actually analyzed:

Two Day No-Oil Fraction (Control)
Mineral Oil (Control)
Four Day Oil Fraction
Six Day Oil Fraction

These samples were chosen based on preliminary GC analysis, in which sample chromatograms were checked for large and unique peaks that were not present in the mineral oil alone.

Based upon sensory evaluation, and upon the previous findings of Collins and Halim (1970), Lanza et al. (1976), Lanza and Palmer (1977), there was reason to believe that various monoterpenes, alcohols and esters were present in the samples.

Because the column used in the GC-MS analyses performed by Dr. Mark Malcomson of the University of Arizona (Tucson, AZ) was not the same as that used in the GC analyses performed in our labs at VPI & SU, no direct comparisons could be made between the GC and the GC-MS analyses. Therefore, the GC-MS data were used to identify compounds of potential interest which were present in the samples. Standard samples of the components identified by GC-MS were then separated via GC

in our laboratory. Comparison of the retention times of these standards with the retention times of peaks obtained on analysis of Ceratocystis cultures permitted tentative identification of components in the cultures.

2. Compound Identities

i. Two Day No-Oil (control) Sample

Based upon the 17 aroma compound peaks detected by GC-MS analysis, and upon their respective spectra, it was evident that many desirable aroma compounds were present in this sample. Compounds ranged in structure from simple alcohols to monoterpenes, and in functionality from alcohols to aldehydes and ketones. These compounds are listed in Table 9, and of these 17 compounds, 5 were chosen for more detailed study. Based upon these data, compounds found in this sample were chosen as the basis against which other subsequent sample chromatograms were compared. Two representative mass spectra from this sample, along with reference spectra against which sample spectra were compared, are shown in Figures 2 and 3.

ii. Four and Six Day Oil Fractions

Based upon sensory evaluation, and upon data obtained through GC analysis, it was evident that

Table 9. Potential aroma compounds identified by GC-MS analysis [a]

Compound	Probability of Positive Identification (%)
Isoamyl alcohol	84%
Isoamyl acetate[b]	83%
β -Myrcene[b]	93%
Phenylacetaldehyde	90%
Acetophenone[b]	71%
1-Octanol[b]	93%
Linalool	89%
Citronellol	80%
Geraniol[b]	83%
Geranial	81%
1,2,3,4 Tetrahydronapthalene	86%
p-Mentha-trans-2,8-diene-1-ol	35%
Trans-pinocarveol	42%
Limonene glycol	25%
α -Myrcene	27%
2-α Pinene	15%
Cis-Carveol	20%

[a] Chromatogram peaks from two day no-oil (control) sample
[b] Compounds selected for more detailed study

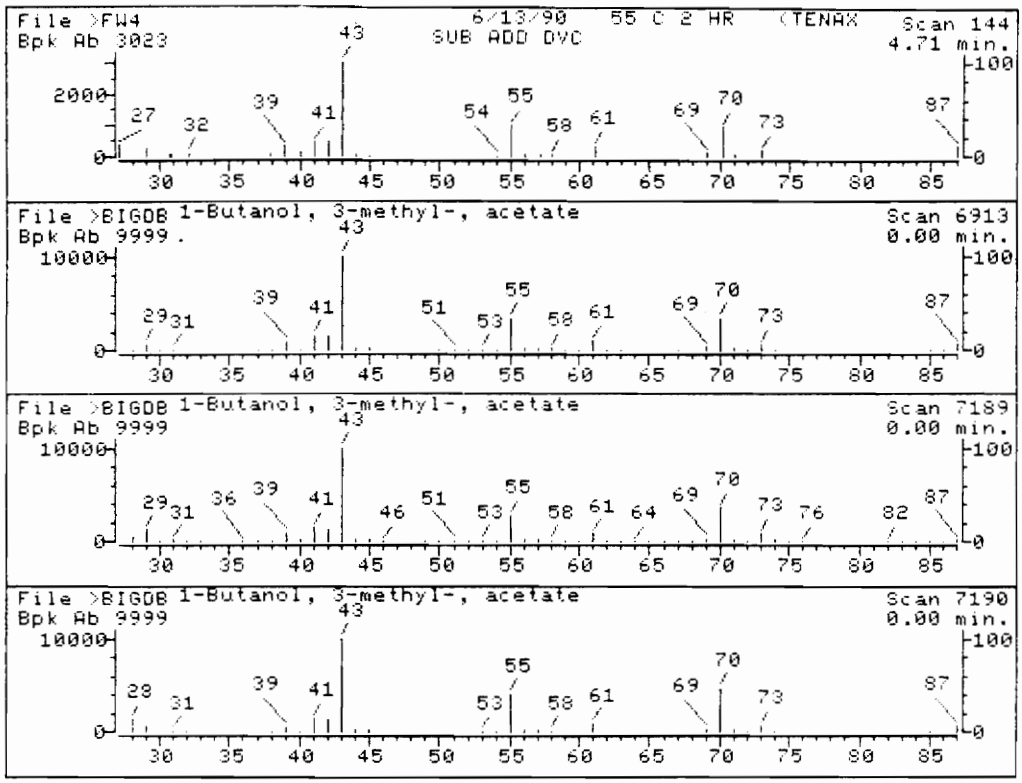

Figure 2. Mass spectrum of isoamyl acetate from two day no-oil sample, and reference spectra of isoamyl acetate. Top scan shows the spectrum of a sample compound detected at 4.71 minutes, which was identified as isoamyl acetate(1-butanol 3-methyl-acetate)

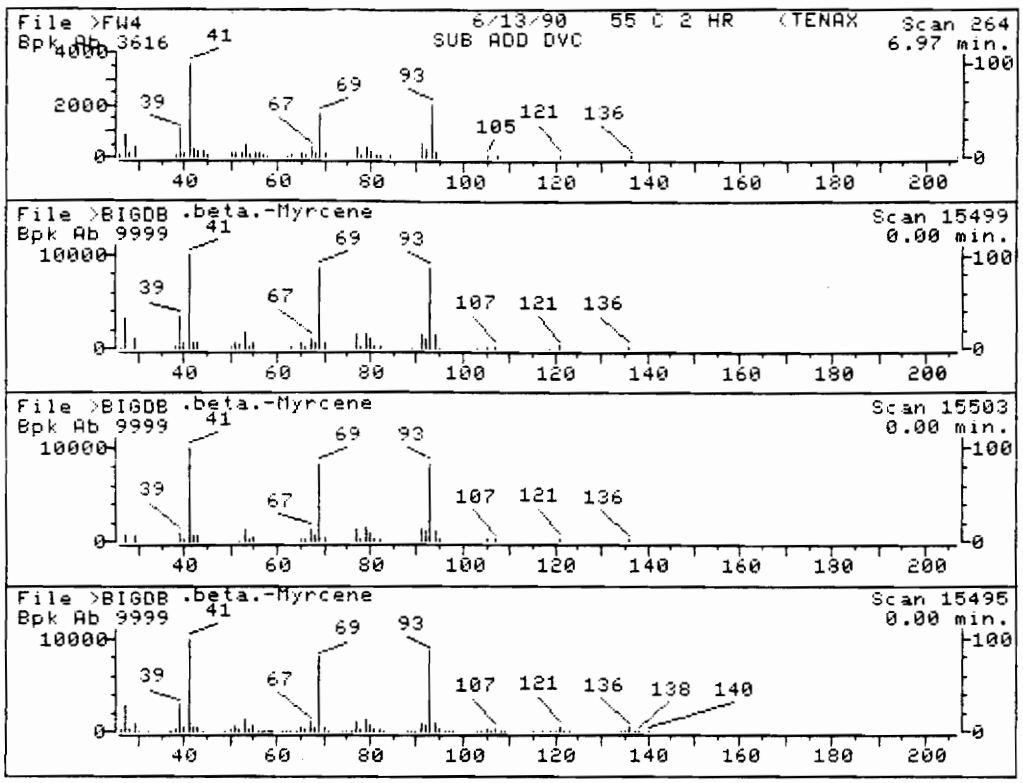

Figure 3. Mass spectrum of β-myrcene from two day no-oil sample, and reference spectra of β-myrcene. Top scan shows the spectrum of a sample compound detected at 6.97 minutes, which was identified as β-myrcene.

compounds having a pleasant, fruity aroma were present in these samples, yet none were detected by GC-MS analysis. Because only hydrocarbon compounds were detected, as evident from the GC-MS chromatograms and spectra of these samples, the volatiles detected by preliminary sensory analysis and by GC analysis were apparently not fully desorbed from the glass wool during the thermal desorption stage of the GC-MS analysis. Thus, GC-MS data from these samples are inconclusive.

iii. Mineral Oil Control

This sample was analyzed in order to make sure that none of the compounds of interest that were found in the samples were also present in the mineral oil. Based upon the spectra of compounds found in the mineral oil sample, none of the compounds listed in Table 9 were found.

D. GC ANALYSIS OF VOLATILE COMPOUNDS

1. Five and Eight Day Sample Analyses

Of the 17 volatile aroma compounds identified by GC-MS analysis of the Two Day No-Oil (control) sample, it was decided that the compounds isoamyl acetate, β-myrcene, 1-octanol, acetophenone, and geraniol were of greatest interest in this study. These compounds

represent four classes of flavor compounds, with different functionalities and aromas. Therefore, the presence or absence of these compounds was assessed in the oil and aqueous fractions of both the 5 and 8 day samples, as well as in their respective no-oil (control) counterparts. This was accomplished by comparing the relative retention times of the compounds in the samples to those of the compounds present in the standard mixes. The average relative retention times of the compounds in the standard mixes 1 through 8 are given in Table 10. Based upon these data, compounds present in the 5 and 8 day oil fractions were tentatively identified.

As mentioned earlier in the Materials and Methods section, compounds in the aqueous standard mixes 6 and 7 that were expected to appear after limonene were not detected at all. This could be due to incomplete trapping, or incomplete desorption from the Tenax trap. Because of this, it was not possible to calculate the yields of these compounds in the aqueous and no-oil (control) samples.

Because not all compounds were detected in the aqueous standard mixes, the assumption was made that peaks appearing after the limonene peak in the 5 and 8 day aqueous fractions and no-oil (control samples) could

Table 10. Average retention times of compounds in standard mixes. [a]

Compound	Ret. Time (min)	Std.Dev.
Isoamyl acetate	0.680	6.7×10^{-3}
β -Myrcene	0.913	2.7×10^{-3}
Acetophenone/ 1-Octanol[b]	1.092	1.2×10^{-2}
Geraniol	1.405	3.7×10^{-3}

[a] Relative to limonene
[b] Composite peak

be identified by comparing their relative retention times with those of the oil based standard mixes.

Five day and eight day oil and aqueous fractions, as well as 5 and 8 day no-oil (control) samples, were analyzed for the presence or absence of isoamyl acetate, β -myrcene, 1-octanol, acetophenone, and geraniol. In analyzing the chromatograms, besides comparing relative retention times of sample peaks with those of the standard mixes, overall peak patterns were analyzed as well. It was found that acetophenone and 1-octanol always appeared as a double peak, and were not able to be further resolved. Therefore, these compounds were considered to be a doublet. Isoamyl acetate always gave a characteristic double peak, perhaps due to the presence of an unidentified yet closely related compound. Results of the comparisons of relative retention times are shown in Table 11. Figures 4 - 9 show the chromatograms for these samples.

E. QUANTITATION OF VOLATILE COMPOUNDS

1. Volatile Compound Yields

Calculations were performed in order to determine the yield of the above mentioned compounds in the 5 and 8 day samples. In the absence of usable data on the GC

Table 11. Compounds found in 5 and 8 day samples, their relative retention times, and variance from the average retention times of compounds in standard mixes. [a]

Sample	Compound	Rel. Ret. Time (min.) Avg.	Var. From Std. (%)
5 Day Control	Isoamyl acetate	0.672/ 0.677	-1.500
	β -Myrcene	0.922	+1.010
	Geraniol	1.410	+0.075
5 Day Oil	Isoamyl acetate	0.661/ 0.667	-3.110
	Geraniol	1.382	-1.700
5 Day Aq.	Isoamyl acetate	0.673/ 0.678	-1.500
8 Day No-Oil	Isoamyl acetate	0.671/ 0.675	-1.750
	Geraniol	1.400	+0.390
8 Day Oil	Isoamyl acetate	0.673/ 0.678	-1.740
	β -Myrcene	0.919	+0.550
	Acetophenone/ 1-Octanol	1.039	-4.900
	Geraniol	1.406	+0.075
8 Day Aq.	Isoamyl acetate	0.676/ 0.682	-0.890
	Geraniol	1.403	-0.140

[a] Retention times relative to limonene

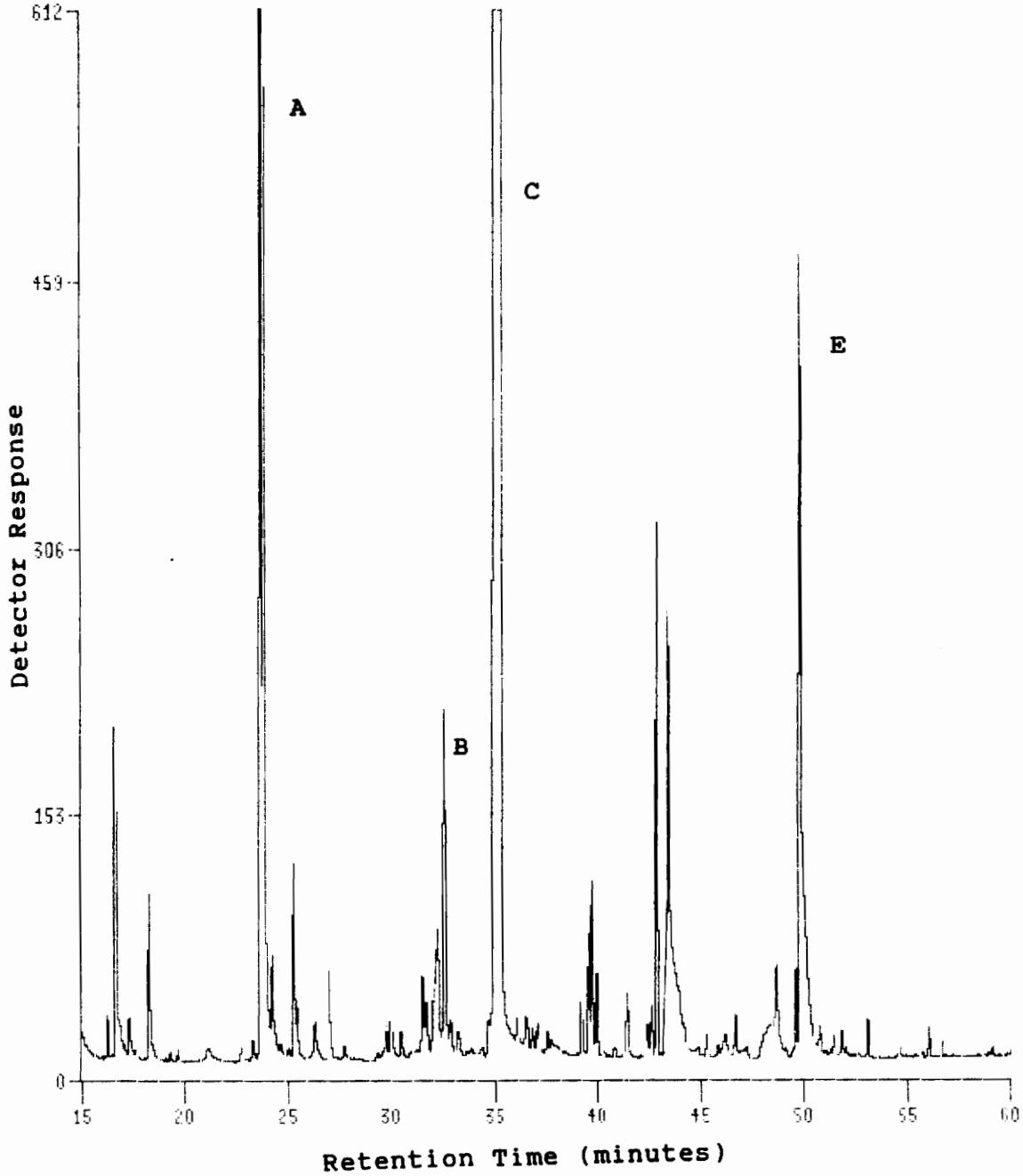

Figure 4. Chromatogram of five day no-oil sample. A= isoamyl acetate, B= β -myrcene, C= limonene internal standard, E= geraniol.

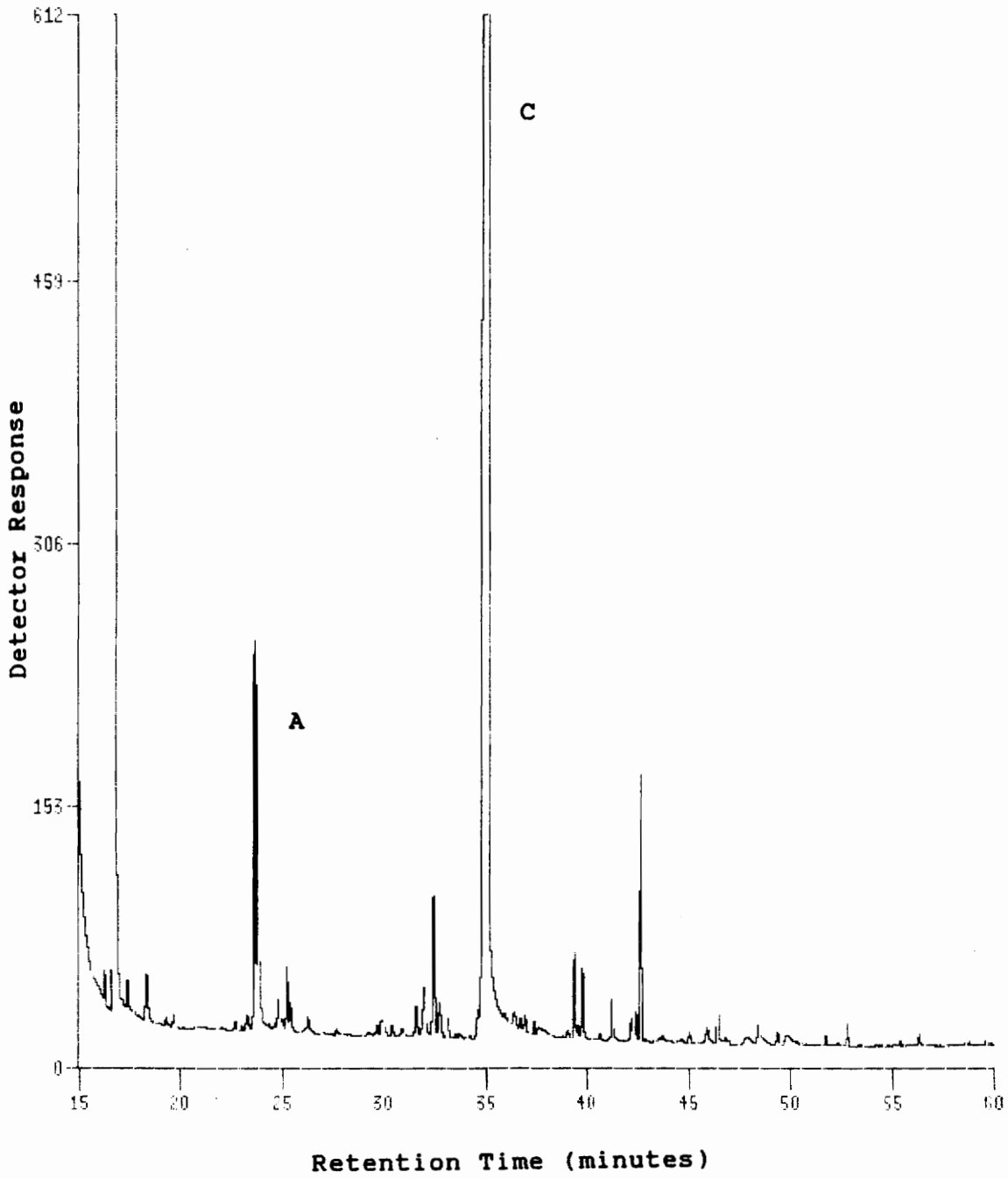

Figure 5. Chromatogram of five day aqueous fraction. A= isoamyl acetate, C= limonene internal standard.

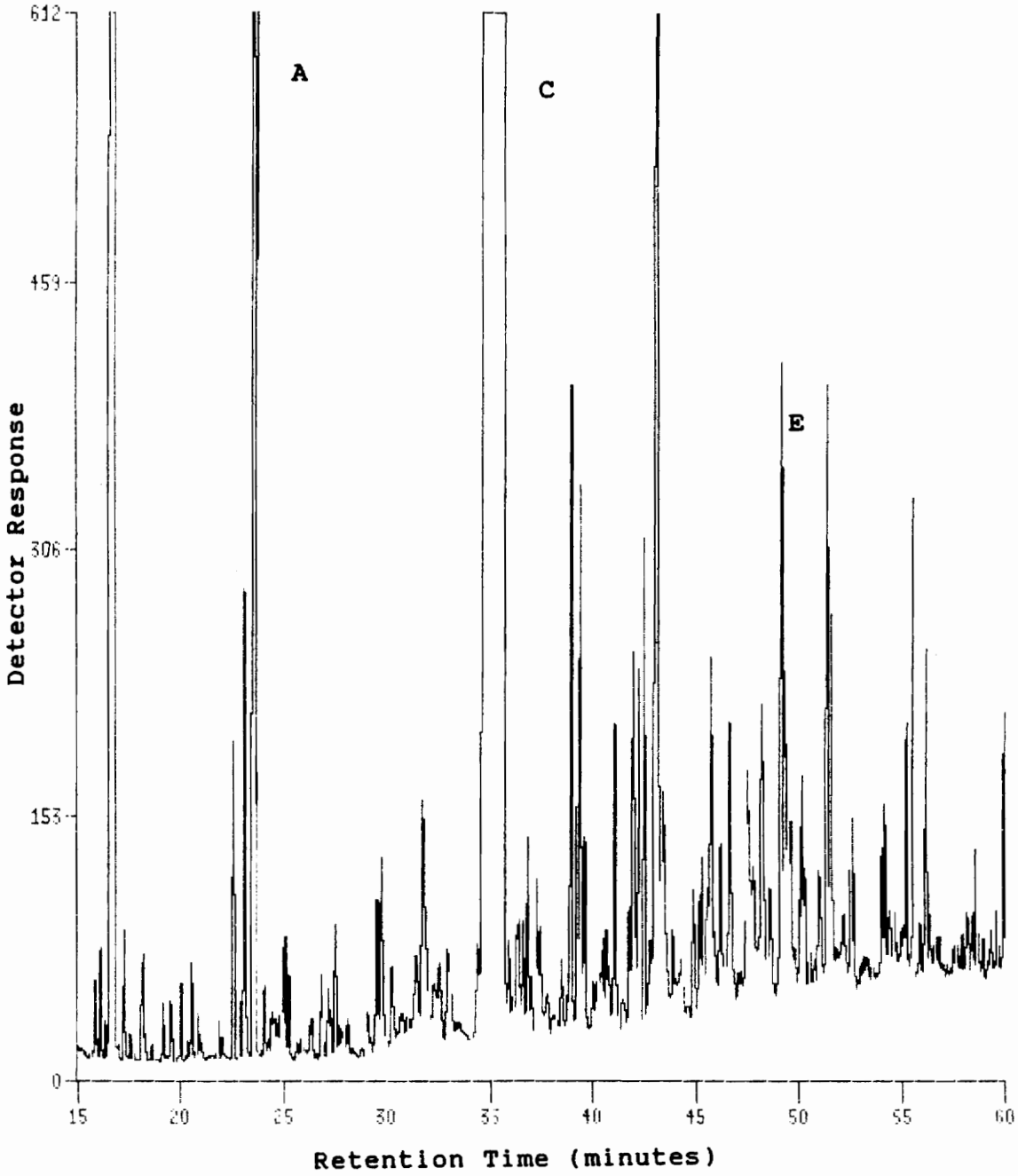

Figure 6. Chromatogram of five day oil fraction. A= isoamyl acetate, C= limonene internal standard, E= geraniol.

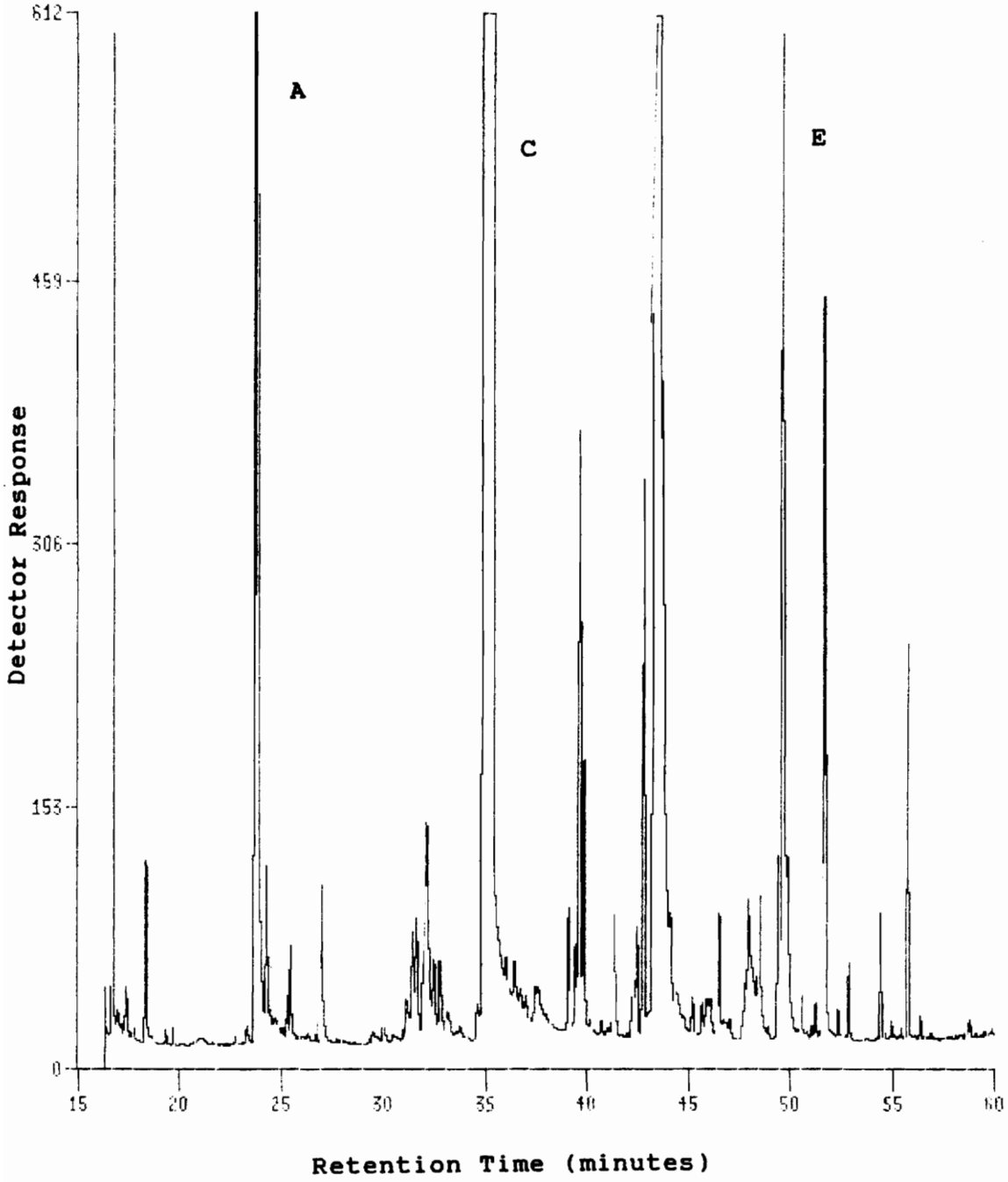

Figure 7. Chromatogram of eight day no-oil sample. A= isoamyl acetate, C= limonene internal standard, E= geraniol.

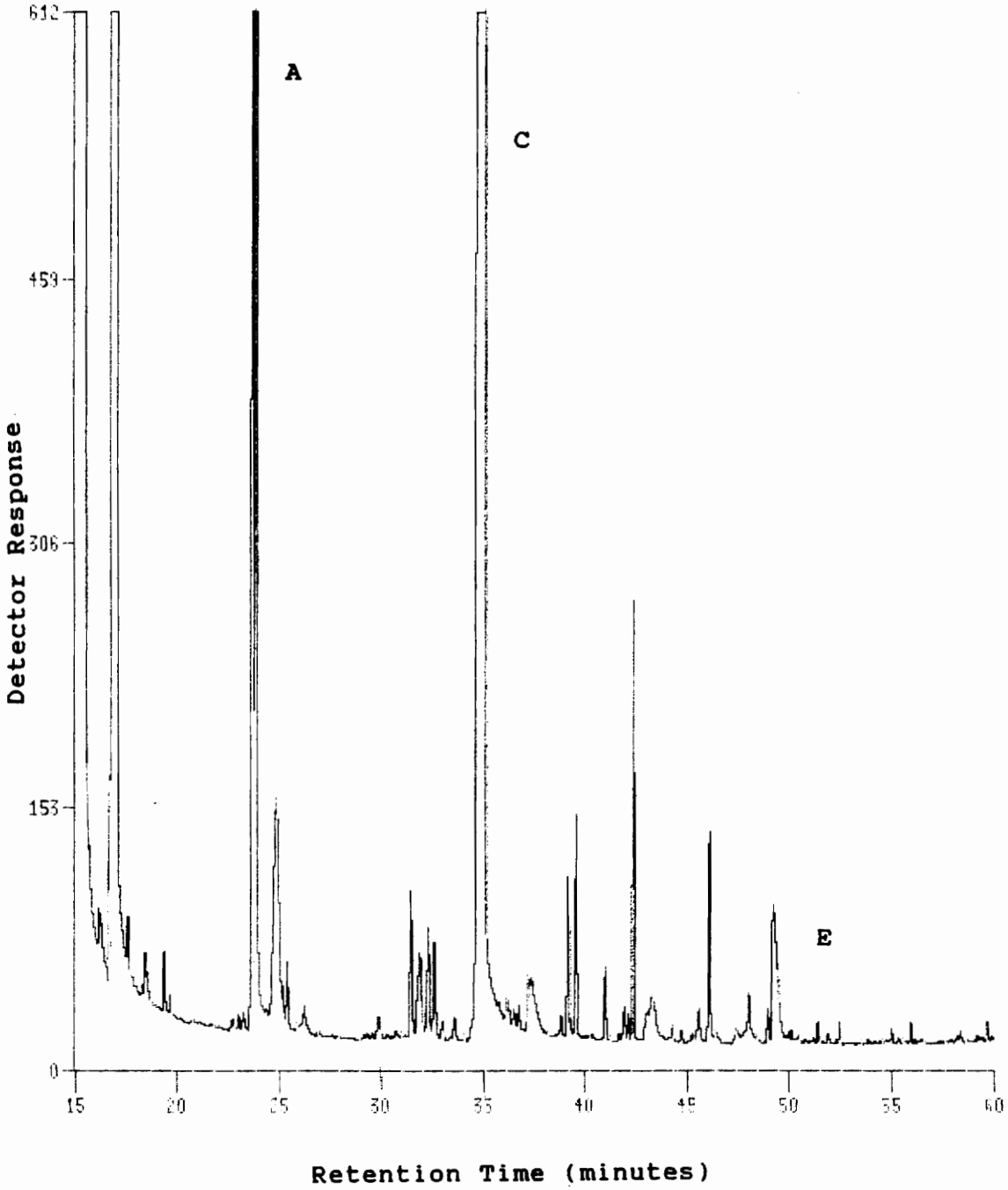

Figure 8. Chromatogram of eight day aqueous fraction. A= isoamyl acetate, C= limonene internal standard, E= geraniol.

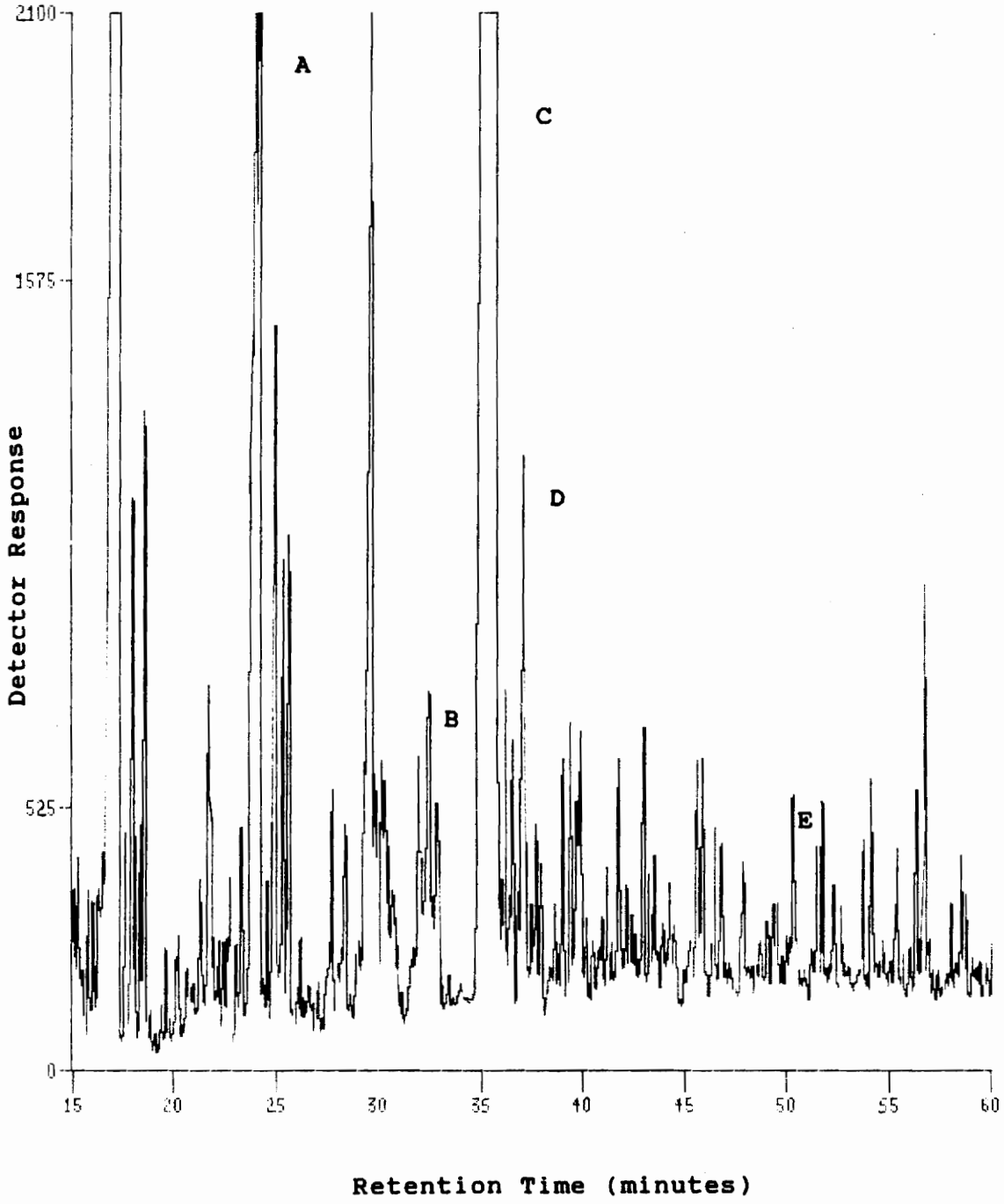

Figure 9. Chromatogram of eight day oil fraction. A= isoamyl acetate, B= β-myrcene, C= limonene internal standard, D= acetophenone/ 1-octanol, E= geraniol.

response factors for the volatiles in the aqueous media, we were only able to calculate the yields in the oil phase. It seems likely that the compounds of interest, being relatively non-polar, would tend to migrate into the oil phase as they were produced. Therefore, as a first approximation, we are assuming that a measurement of the concentrations accumulated in the oil phase provides a reasonable estimate of the total quantity produced. In any case, we would expect that the aroma compounds would ultimately be recovered from the oil fraction, and thus the concentration in that fraction is of the most interest.

Once the amount of each compound present in each oil sample was calculated, results were adjusted in order to account for the entire sample size of the starting sample. Then, these results were further adjusted to account for the average recovery rates of each compound as determined by the standard oil mixes. These adjustments were made to show how much of each compound would be present in the sample if the compound were fully recovered by the experimental methods. Table 12 shows the yields of the compounds detected and determined in the oil fractions of the 5 and 8 day samples.

Table 12. Aroma compound yields in five and eight day cultures [a]

Sample	Compound	Total Yield [b] (ug)	Sample Yield (ug) [c]
5 Day Oil Fraction	Isoamyl acetate	2.0	350.0
	Geraniol	0.3	235.0
8 Day Oil Fraction	Isoamyl acetate	11.6	2071.0
	β -Myrcene	1.6	246.0
	Acetophenone/ 1-Octanol	9.0	670.0
	Geraniol	1.1	2.0

[a] Incubated in GYEB with mineral oil
[b] per 50 mg oil, adjusted for compound recovery rate (see Table 11)
[c] Yield for total starting volume of 10 ml of oil

2. Comparison of Sample Compound Yields With Yields Reported in Previous Studies

Earlier reports of volatile production by filamentous fungi have stated that bioproduction of these compounds has been low, despite the fact that the cultures had intense aromas. Many studies of aroma production failed to quantitate volatile production at all. Nonetheless, some studies have shown that it is possible to increase volatile production in filamentous fungi by optimizing fermentation conditions through the use of bioreactors or trapping agents.

Lanza and Palmer (1977) reported a yield of 1900 µg/100 ml glucose-urea broth for geraniol, after three and one half days of incubation. Cohen et al. (1987) reported a yield of 10.6 mg/l for geraniol, after two days of incubation in a glucose-urea-yeast extract broth. Using a bioreactor for continuous production of aroma compounds, Cohen et al. were able to generate yields of 1.6 mg/l/hr for geraniol in a glucose-urea broth. They did not report the duration of this level of production, but stated that this level of production was not likely to be maintained in this reactor working in steady state.

There seem to be no reports in the literature of yields for any of the other compounds investigated in

this study. A comparison of geraniol yields from this study, and from the work of Lanza and Palmer (1977) and Cohen et al. (1987), is shown in Table 13.

As mentioned previously, we were unable to get useable recovery-response data from the aqueous standards, and thus were unable to calculate compound yields in the aqueous and no-oil (control) samples. We were able to calculate yields from the oil fraction of the samples, yet unable to directly compare these yields with those of the no-oil (control) and the aqueous samples. There is no question, however, that based upon qualitative GC data, and upon sensory data, that aroma compounds were produced in the no-oil (control) system.

Table 13. Comparison of geraniol yields in 5 and 8 day samples with yields reported in previous studies.

Study	Yield (mg/l)
Lanza and Palmer, 1977 [a]	19.0
Cohen et al., 1987 [b]	11.0
Cohen et al., 1987 [c]	1.6
Present, 5 Day Oil [d]	2.3
Present, 8 Day Oil [d]	8.8

[a] 30:1 glucose-urea broth, 25 C, 3 1/2 days, batch culture

[b] 30:3:3 glucose-urea-yeast extract broth, 3 days, batch culture. Geraniol represents approximately 82% of "total volatiles" shown in this report.

[c] 30:3 glucose-urea broth, continuous bioreactor, per hour

[d] 30:1 glucose-yeast extract broth with mineral oil, 25 C, batch culture

V. DISCUSSION

A. GENERAL GROWTH CHARACTERISTICS

It is evident that the filamentous fungus C. moniliformis grows well and produces desirable aroma compounds when grown in a 30:1 glucose:yeast extract culture broth at 25°C, shaking at 100 rpm on a rotary shaker. Furthermore, when 10 ml (9 g) mineral oil is added to the culture broth, the culture continues to grow and produce pleasant and potentially useful aromas.

Under the growth conditions described above, the stationary phase of growth was reached when the nitrogen was fairly well depleted, probably indicating that nitrogen was the limiting growth factor. It was clear that glucose was not a limiting factor.

The addition of mineral oil to the culture medium did not adversely affect growth of the culture. It is possible that some factors, such as gas exchange, were affected by the addition of the oil phase, but further investigation of this aspect is outside the realm of this study.

B. SENSORY EVALUATION

For the purposes of this study, a descriptive sensory evaluation study was chosen. Although panelist responses were limited to a list of specific descriptors for questions 1 and 2, they were given a chance to add their own descriptors in question 3. However, most of the panelists answered "no" to this question, and those who answered "yes" did so only for the control culture medium. This suggests that the list of descriptors available to the panelists was reasonably complete.

It is known that some of the compounds identified in this study are character impact compounds in fruits and flowers, as well as in their respective essential oils. In this study, we were mainly interested in whether or not the panelists could associate the overall aromas of the culture samples with known, familiar descriptive terms. This was well and consistently demonstrated.

C. GC-MS ANALYSIS

It is unfortunate that the data generated by GC-MS analysis could not be directly correlated with the GC data generated in our labs. This was because the same columns and GC programs were not used. Nonetheless, it

was possible to use these data to tentatively identify compounds which were present in the samples. GC-MS, and the capability to scan a very complete library of known compound spectra, provide a powerful analytical tool. Further work in the area of thermal desorption of samples using the external inlet device with this particular GC-MS configuration is needed.

D. GC ANALYSIS

1. Sample Preparation and Instrumentation

The sample preparation methods were straightforward, and were not overly time consuming. Other sample preparation methods that have been used in the past for similar samples, such as distillation and solvent extraction, can be damaging to the sample. Many volatile compounds are degraded by high heat and the action of solvents. The Freon-11 extraction technique used by Lanza and Palmer (1977) was mild, but there are environmental problems associated with disposal of Freon-11. Furthermore, since the boiling point of Freon-11 is so low, special cold room conditions are needed in order to work effectively. The sample preparation methods described in this study are non-destructive, environmentally safe, and non-labor intensive.

In general, it seemed that the GC analysis of the oil-based standards was more reliable than that of the aqueous standards. With the aqueous standards, compounds that were known to be have been added to the mixes were not detected in the GC analysis. This could be due to a number of reasons. First, because the compounds were initially prepared in methanol before being diluted with water, it is possible that the compounds were quickly volatilized and lost into the atmosphere as the samples were transferred to the boiling flasks for stripping. It is also possible that, in the aqueous standard mixes, all of the standard compounds were not stripped from the methanol/water solution onto the Tenax. It is further possible that the Tenax trapping system was incomplete, or may not have been sufficient enough to hold all of the volatiles that were stripped from the samples. It has been suggested that the Tenax may have become overloaded, and that later boiling compounds may not have been able to be adsorbed onto the Tenax.

For the oil fraction of the 5 and 8 day samples, it was clearly evident that many compounds were separated and detected.

In general, the HP 5980 gas chromatograph used in sample analysis performed well. The use of the external

closed inlet device (ECID) provided an excellent means of introducing the sample to the column. Furthermore, the use of an ECID eliminates variation in individual injection methods that can sometimes lead to variations in data due to human error.

2. Peak Identification

The use of relative retention times for peak identification was a reliable method, despite the fact that direct comparison of GC and GC-MS results was not possible. Each reference compound analyzed in oil, whether it was analyzed alone, or as a component of a standard mix, was consistently separated and detected. The use of limonene as an internal standard turned out to be a fortuitous choice, since it behaved consistently well, and did not mask any other peaks of interest in this study. In most cases, relative retention times of sample peaks varied very little from those of the standards.

3. Compounds Identified in Samples

Although there were many compounds present in the samples, this study focused on only five of them. These five compounds, isoamyl acetate, β-myrcene, 1-octanol,

acetophenone, and geraniol, are all used in food flavoring applications. The production of β-myrcene, acetophenone, and 1-octanol by C. moniliformis under these conditions is a novel discovery. Typical sources and applications, as well as regulatory statuses of all five compounds, are discussed below.

a. Isoamyl acetate: This compound is commonly called banana oil, and less often pear oil. In its pure form, it exists as a colorless liquid with a boiling point of 142°C, and a density of 0.876 (Stecher et al., 1968). Isoamyl acetate has been reported to be found in the volatile portion of the banana, cocoa bean, and pear. Lanza, Ko, and Palmer reported its production by cultures of C. moniliformis in 1976. For commercial uses, it is typically prepared by esterification of isoamyl alcohol with acetic acid (Furia and Bellanca, 1975). Although cut and pureed banana is used in a variety of foods, the pulp itself does not provide adequate flavor in prepared foods, and thus concentrated banana oil is commonly used when a more than mild banana flavor is desired (Heath, 1981). Isoamyl acetate is used to flavor non-alcoholic beverages at a level of 28 ppm, gelatins and puddings at 100 ppm, and candies at 190 ppm (Furia and Bellanca,

1975.) When prepared as described above, isoamyl acetate is considered to be a synthetic flavor, and its use is controlled by FDA 121.1164 of the Federal Food, Drug and Cosmetic Act.

b. β -Myrcene: This compound is found in bay leaves, verbena, orange, hemlock, and hops. It exists as an oily liquid with a boiling point of 93°C and a density of 0.794. β -myrcene is normally synthesized by pyrolysis of α-pinene or from linalool (Stecher et al., 1968). Applications include use in ice creams at 6.4 ppm, candies at 0.5 ppm, and in baked goods at 4.9 ppm (Furia and Bellanca, 1975). β-myrcene is covered by FDA 121.1164.

c. Acetophenone: Acetophenone (phenyl methyl ketone) is found naturally in the oil of labdanum, or is synthesized from benzene and acetylchloride in the presence of aluminum chloride (Furia and Bellanca, 1975). This compound is a colorless liquid at room temperature, has a density of 1.025, and boils at 202°C. Its aroma is that of orange blossom, yet it has a bitter taste. Uses include levels of 2.8 ppm in ice cream flavoring, 7.0 ppm in candies, and in perfumes. Acetophenone is governed by FDA 121.1164.

d. 1-Octanol: 1-octanol (caprylic alcohol) has a fresh, rose-orange odor, and a sweet, herbaceous taste (Furia and Bellanca, 1875). It has been reported in the oils of geranium, mint, and lavender. It is synthesized by distillation of sodium ricinoleate with an excess of sodium hydroxide, and if this synthetic form is used, it is governed by FDA 121.1164. The compound in its pure form exists as a colorless, oily liquid, with a density of 0.817 and a boiling point of 180°C. Applications include use in ice cream at 0.6 ppm, and in certain baked goods at 4.0 ppm. 1-octanol is also used in the manufacture of perfumes and esters.

e. Geraniol: Geraniol, an olefinic monoterpene alcohol, is an important compound. Its presence has been reported in over 160 essential oils, including oils of lemongrass, nutmeg, sassafras, rose, and orange. It has a strong rose-like odor. Geraniol can be prepared from the distillation of the abovementioned essential oils, or it can be prepared synthetically from myrcene. The boiling point of geraniol is 229°C, and the density of the pure, colorless liquid is 0.889 (Furia and Bellanca, 1975). Most importantly, this compound is GRAS, and is typically used in beverages at 2.1 ppm, candy at 10 ppm,

and in perfumes.

E. SAMPLE COMPOUND YIELDS

1. Comparisons Between Study Samples

Because the GC detector responses of the compounds were not the same (see Table 4), the compound yield calculations for each sample had to be adjusted in order to estimate yields as if responses had been equal to that of limonene. However, in reviewing the adjusted compound yields, some trends were evident.

The amount of isoamyl acetate in the 8 day oil fraction was nearly six times more than the amount in the 5 day oil fraction. This suggests that the presence of the mineral oil allowed for increased continuous production of isoamyl acetate due to a trapping effect. The mineral oil may also have provided protection from volatilization of the isoamyl acetate into the atmosphere.

The level of geraniol it increased nearly four-fold from the 5 and 8 day oil fractions. This data would also indicate that the mineral oil acts as a trapping agent for the geraniol.

The lesser constituents β-myrcene, 1-octanol, and acetophenone appeared to be produced later in the

fermentation. There was a trace of the β-myrcene in the 5 day no-oil sample, and there was nearly ten-fold more in the 8 day oil fraction. Acetophenone and 1-octanol were not detected until 8 days, and even then, they were only detected in the oil fraction. Because of this, it is hard to draw conclusions regarding their changes in production. However, based upon their structures, one would expect them to be "pulled" to the oil fraction almost immediately upon their synthesis, due to their hydrophobic nature.

2. Comparisons With Previous Studies

It is not possible to compare the yields of each compound investigated in this study with previous studies, since many studies only report yields of "total volatiles," without breaking the final figure into individual components. It is evident that geraniol yields, as shown in Table 13, were less in this study than in the previous studies of Lanza and Palmer (1977) and Cohen et al (1987).

It is unclear as to whether the compounds were detected differently by the FID, and thus apparently recovered at different rates, due to the varying percentages of carbon in each compound. While isoamyl

alcohol contains 64.6 % carbon, β -myrcene contains 88.2 % carbon. Yet it is clear that both of these compounds had high recovery rates, while geraniol, which contains 77.9 % carbon, had an extremely low recovery rate. It is possible, nonetheless, that a "carbon factor" needs to be taken into account when analyzing GC data.

Overall, it is apparent that the growth conditions examined in this study did not allow for greater production of the compounds of interest than did the conditions tested in previous studies.

VI. CONCLUSION

A. SUMMARY

Ceratocystis moniliformis was able to grow well at 25°C, shaking at 100 rpm, in a 30:1 glucose-yeast extract culture medium containing a thin layer of mineral oil on the surface. After the nitrogen source was 80% depleted, the culture reached a stationary phase.

After 5 days of incubation, sensory panelists were able to distinguish the aromas "banana," "citrus," "fruity," "peach," and "floral."

Through the use of GC and GC-MS analysis, this culture was shown to produce a variety of desirable aroma compounds, many of which are key components of the aromas described by the sensory panelists. Further study of the compounds isoamyl acetate, β -myrcene, acetophenone, 1-octanol, and geraniol showed that differing amounts of these compounds were produced by the culture, depending upon the length of incubation and the presence or absence of mineral oil in the culture broth.

C. moniliformis produced the compounds in question even when mineral oil was an ingredient in the culture medium. It seems that the mineral oil acts as a trapping

agent by attracting these fairly lipophilic compounds, and removing them from the aqueous portion of the medium. This action removes compounds that could otherwise be toxic to the organism, and thus allows for their greater production.

It is also possible that the presence of the mineral oil altered the rate of volatility of the aroma compounds; perhaps in the samples that had no mineral oil, the volatile compounds were able to escape into the atmosphere more readily, and thus, their concentrations in the culture broth could be lower.

Although it was apparent that the presence of mineral oil in the culture broth may allow for the increased accumulation or production of several volatile aroma compounds, the production of geraniol in particular was not above and beyond production levels reported in other studies in which oil was not used.

If the compounds investigated in this study could be successfully extracted from the culture broth, and subsequently purified and concentrated, they would make good, natural flavoring ingredients for food products.

B. Recommendations For Future Work

1. Further elucidation of the pathways by which

these compounds are made could help reveal keys to increasing their production.

2. More work is needed in order to refine the sample preparation and GC techniques used in this study, as they relate to the analysis of fungal volatiles.

3. Further quantitation of other compounds that were detected in these samples is needed.

4. Development and use of a continuous bioreactor, which incorporates the use of mineral or other oil as a trapping agent, may increase the production of volatiles by C. moniliformis.

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