THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF FOOD SCIENCE

DETECTION OF MOLD GROWTH ON *THEOBROMA CACAO* UTILIZING GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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ABSTRACT

The ability to detect the presence of mold growth on *Theobroma cacao*, or cocoa beans, using gas chromatography/mass spectrometry (GC/MS) was evaluated in this study. Two volatile compounds, 1-octen-3-ol and p-cresol, are associated with molds that characteristically grow on cocoa beans. 1-octen-3-ol is generally produced as a volatile organic compound (VOC) by molds, while p-cresol can be utilized by molds that thrive on cocoa, such as *Aspergillus fumigatus*, as a carbon and energy source. Chromatograms for 1-octen-3-ol and p-cresol were collected using gas chromatography and mass spectrometry. Additionally, chromatograms from samples of both fresh commercial cocoa beans with no visible mold and pre-moistened commercial cocoa beans with distinct mold growth were analyzed using GC/MS to determine if mold growth on *Theobroma cacao* can be detected by the presence of 1-octen-3-ol and p-cresol. The results indicate that it is possible to detect characteristic VOCs on *Theobroma cacao* by identification using MassHunter Software, and that differences in concentration of these volatiles and subsequently the amount of mold growth can be detected by GC/MS analysis.

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Chapter 1

Introduction

1.1 Problem Statement

The cultivation of *Theobroma cacao*, commonly known as cocoa beans, is one of the largest food product industries worldwide, with a 4.95 million metric ton trading volume in 2011. The major cocoa producing countries are Cote d'Ivoire, Ghana, Nigeria, Cameroon, Indonesia, Malaysia, Papua New Guinea, Brazil, Ecuador, and Colombia[1]. These countries depend on the revenue provided through the cocoa trade to support their citizens. Cocoa serves as a source of income for 40-50 million people, and small cocoa farms provide more than 90% of the cocoa grown across the globe. In the United States alone, cocoa bean imports totaled $1,228,060,000 in 2009, and some of the largest confectionary companies worldwide include Mars Inc (USA), Nestle (Switzerland), Meiji Holdings Co Ltd (Japan) and Arcor (Argentina)[1]. However, the cocoa industry is reliant on healthy crops in order to make a profit, and these efforts can be spoiled by the introduction of mold through poor growing conditions and storage practices that can destroy tons of cocoa beans yearly. If this mold could be detected early in its progression, it may be possible to prevent the beans from entering the production system.

1.2 Hypothesis

It is expected that gas chromatography and mass spectrometry can be used to detect the presence of mold growth on cocoa beans by analyzing the chromatograms of samples of both fresh and moldy cocoa beans to standards of 1-octen-3-ol and p-cresol.

1.3 Objectives

- To detect the presence of mold on infected cocoa beans utilizing gas chromatography and mass spectrometry.
- Analysis of both fresh and moldy cocoa beans as a comparison to the chromatograms of two standards, p-cresol and 1-octen-3-ol.

Chapter 2

Review of Literature

2.1 History and Origin of *Theobroma cacao*

The genus *Theobroma* first appeared millions of years ago in South America, and has been divided into twenty-two separate subcategories, where *T. cacao* is the most well known[2]. The Mayans were the first to cultivate cacao as a crop, and it was often used to create a drink for various ceremonies. The first person outside of the Maya and Aztecs to experience chocolate in the form of a liquid was Christopher Columbus, but the individual who brought the recipe for the Aztec *xocoatl,* or chocolate drink, back to the Old World was Hernan Cortes upon returning from an exploration mission to South America[2]. It did not become popular in Spain until the addition of sugar, upon which time its popularity increased and it became more well-known as the chocolate we are familiar with today[2].

2.2 Current Production of Cocoa

The current top producers of cocoa beans around the globe include Cote d'Ivoire, Ghana, and Indonesia, although it is also produced in other countries such as Nigeria, Cameroon, Malaysia, Papua New Guinea, Brazil, Ecuador, and Colombia[1]. *Theobroma cacao* can be grown and thrive between 10° N and 10° S of the equator, where the conditions are ideal for the cultivation of the cacao pods[2]. Cocoa beans are a significant industry globally and can be considered a cash crop for growing countries looking for economic development. The production of chocolate from seed to finished product is a labor-intensive, multi-step process involving many individuals and skilled techniques to obtain a high-quality product. A typical pod contains 20-50 beans, and approximately 400 beans are required to produce one pound of chocolate[1]. This equates to approximately 8 to 20 pods required for one pound of chocolate.

Given that in 2009 the top importer of cocoa beans was the Netherlands at a value of $2.75 B, and the second top importer of that year was the United States with an import value of whole beans at $1.23 B, the cocoa industry is a massive business that depends on the health of the beans produced[1].

2.3 Varieties of Cocoa Beans

There are three major varieties of cocoa beans, each with their unique advantages and uses within the industry. The three varieties are Criollo, Forastero, and Trinitario. Criollo cocoa was the dominant variety until the middle of the 18th century, but is no longer as popularly used. Trinitario is a hybrid of Forastero and Criollo. While Criollo and Trinitario beans are prized as flavor-beans, or those with fine flavor, bulk cocoa beans are often of the Forastero variety, and are the most commonly used variety for mass chocolate production. This has become the case due to Forastero's hardy nature, meaning it is likely to survive the growing season, and has a strong flavor. The most popular Forastero variety of cocoa beans is Amelonado, which is widely grown in West Africa and Brazil. This variety commonly produces a smooth yellow pod containing 30 or more purple beans. Criollo, which has a mild chocolate flavor, is most commonly grown in Indonesia, Central and South America. This variety is not as hardy as Forastero beans, and produces a softer, red pod with 20-30 white, ivory, or pale purple beans. Trinitario, a blend of both Criollo and Forastero varieties, is mostly cultivated in the Caribbean, Cameroon, and Papua New Guinea. These plants are variable in color and contain approximately 30 beans that also vary in color.

2.4 Growing Conditions for Cocoa Beans

There are a variety of conditions that must be met for cocoa to grow successfully. Cocoa thrives in environments with relatively high temperatures, with a minimum average between 18-21° C and a maximum average of 30-32° C[2]. The yield of cocoa trees varies from year to year, and can be highly

affected by the rainfall of that growing season. An annual rainfall of between 1,500 mm and 2,000 mm is preferred for cocoa growth. Since a hot and humid atmosphere is beneficial for cocoa growth, a relative humidity of as high as 100% during the day and 70-80% at night is tolerated[2].

2.5 Cocoa Fermentation and Drying

After the pods are allowed to grow and develop to maturity, harvesting occurs. This is a labor-intensive process and is done by hand, where workers use knives to cut the pods from the trees. The freed pods are then split open, and the beans and white pulp, or mucilage, are removed[3]. The curing process for cocoa involves two stages: fermentation and drying. Two common fermentation methods are the box method and the heap method. The heap method is most often used in West Africa, where the wet beans are piled onto banana or plantain leaves and covered by more leaves, where they will remain for 5-6 days, allowing fermentation to occur[3]. The goal of fermentation is to allow the pulp and astringency of the beans to dissipate as the sugar in the pulp is converted to alcohol. The box method of fermentation is most often used in the West Indies, Latin America, and Malaysia. Wooden boxes with drainage holes that allow for the passage of air and removal of liquids are filled with the wet cocoa beans. Fermentation using this method requires 6-8 days[3]. After fermentation is complete, regardless of method, drying occurs, traditionally through spreading the beans out in a thin layer in the sun, although alternative mechanical methods can be used. From here, beans are packaged and shipped to production facilities that will use them for processing into chocolate or other chocolate products[3].

2.6 Importance of Fermentation and Drying

If poor fermentation and drying occur, cocoa bean quality can suffer due to mold growth. While the flavor of the cocoa or chocolate produced from the beans depends on successful fermentation, the success of the drying process that succeeds fermentation is critical; if slowed down or executed incorrectly, mold will develop on the beans and result in extremely unpleasant flavors. The ideal moisture

content for cocoa beans should be no more than 6% in equilibrium with relative humidity of 65% at 30° C, but it is more practical to obtain the trade standard of 7-8% moisture[4]. Mold in cocoa beans results in an increase in free fatty acids, and reduces the chance that a processor will accept the product[4]. Failing to accept the product results in a loss for the cocoa suppliers, and can cause serious economic loss. Some of the most common molds to grow on *Theobroma cacao* include *Aspergillus niger, Aspergillus flavus, Botryodiplodia theobromae, Fusarium* spp*., Mucor* spp*., Neurospora* spp*., Penicillium* spp*., and Phytophthora palmivora*[5].

2.7 Further Cocoa Processing

Following fermentation and drying, the beans are shipped to chocolate and cocoa manufacturers across the globe. Once they arrive at the factory, the beans are cleaned to remove any extra material that may be present. The next step in the process is roasting, which brings out the chocolate flavor and color that is familiar to consumers. The temperature, time and degree of moisture involved in the roasting process are dependent on the type of beans used (either Criollo, Forastero, or Trinitario) and the form of chocolate or other product that the cocoa is destined to become. The next stage is winnowing, where the shells are removed from the beans leaving only the cocoa nibs[6].

Often the nibs subsequently undergo alkalization, which assists in the development of flavor and color. The common alkaline agent in this process is potassium carbonate. Milling follows alkalization, where the nibs are ground to create cocoa liquor, which is essentially cocoa solids suspended in cocoa butter. If more than one bean type is used in the product, blending occurs following milling. Cocoa liquor is pressed to extract the cocoa butter, leaving a presscake of remaining solids. Cocoa butter is used in the manufacture of chocolate, while the presscake can be ground to form cocoa powder[6].

Cocoa liquor can be used to make chocolate through the addition of cocoa butter. Other ingredients are commonly added, such as sugar, milk, and emulsifying agents. This mixture undergoes refining, where rollers travel over the product until it forms a smooth paste. The next step, conching, is a

smoothing process that results in the desired flavor and texture. This mixture is tempered to prevent fat bloom and discoloration, then used as an enrobing agent or filled into molds to form the finished chocolate product[6].

2.8 1-octen-3-ol and p-cresol

Two volatile compounds, 1-octen-3-ol and p-cresol, are commonly associated with mold growth. In a study designed to isolate volatile compounds produced by molds of the *Aspergillus, Penicillium,* and *Fungi imperfecti* varieties, 1-octen-3-ol ($C_8H_{16}O$, MW 128.21), which is also called "mushroom alcohol," was found predominant[7]. Below is the chemical structure of this compound.

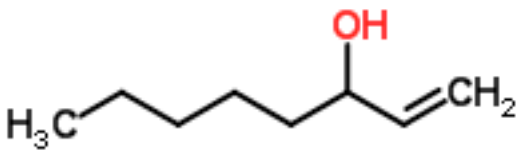

Figure 2-1: 1-octen-3-ol Chemical Structure

1-octen-3-ol is produced naturally by some fungi, plants and molds, It is a metabolite of linoleic acid, and is also found in human breath and sweat[8].

Several yeasts and molds have been discovered to be capable of the utilization of aromatic compounds as growth substrates. One of these molds, *Aspergillus fumigatus*, can use p-cresol[9] (C_7H_8O, MW 108.14)[10] as a substrate, and is commonly found on cocoa beans[5]. Therefore, the presence of p-cresol may be an indication of potential *Aspergillus fumigatus* contamination due to the favorable conditions provided by the presence of p-cresol. Additionally, p-cresol is known to be a common volatile for other

foods commonly affected by mold growth, such as Turkish Motal cheese[11]. Below is the chemical structure of p-cresol.

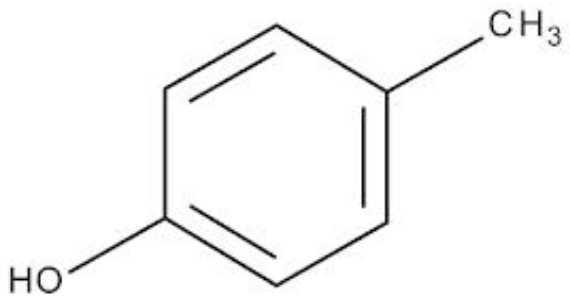

Figure 2-2: P-cresol Chemical Structure

For these reasons, 1-octen-3-ol and p-cresol will be the focus of this study, and their presence may indicate the infection of the cocoa bean samples with mold.

2.9 Ochratoxin

Ochratoxin is one of the most widespread and hazardous mycotoxins, which are fungal secondary metabolites, and can pose serious health risks. Ochratoxin A is a naturally occurring contaminant of food products, drinks, and animal feeds, and is known to be nephrotoxic, teratogenic, and immunotoxic, particularly for the renal system[12]. The fungal species that most commonly produce this toxin are *Aspergillus carbonarius* and *Penicillium viridicatum,* particularly in cocoa beans. It has been classified by The International Agency for Research on Carcinogenics as a potential human carcinogenic, and it is absorbed through the intestinal tract after consuming infected products or through aspiration of airborne toxin. Preventing this toxin from forming on products such as cereals, wine, spices, coffee, grape juice,

dairy products, and specifically cocoa can have a great impact on the insurance of the health and safety of consumers[12]. Experiments have shown that this toxin develops on cocoa beans during the drying period following fermentation, and if this process is incorrectly performed, the mycotoxin can form[12]. Additionally, detection of this toxin before it enters the food system would significantly aid in the prevention of human illness due to OTA

2.10 Significance and Extension

The development of a method to accurately detect the presence of mold growth on cocoa beans would be extremely useful in the processing of higher quality products with a superior flavor, as well as the prevention of human illness due to toxin formation. Utilizing gas chromatography and mass spectrometry techniques may be the answer to these problems. If GC/MS analysis can be shown to accurately diagnose the presence of mold on a variety of products, rejecting the product before it enters the processing line or makes its way to consumers becomes much easier. One way that GC/MS technology could become more accessible to cocoa producers and chocolate manufacturers is through the development of a smaller, more transportable mass spectrometer. One company pursuing the manufacture of these machines is 1st Detect Corporation of Webster, Texas. 1st Detect is working on the development of its MMS-1000, the aim of which is for use in industrial applications such as quality control and food safety testing[13]. A smaller mass spectrometer may be able to revolutionize the food safety and quality industry by allowing producers to more easily detect volatiles such as those produced by molds prior to accepting and beginning production with contaminated raw materials.

Chapter 3

Detection of Mold on *Theobroma cacao* Using Gas Chromatography/Mass Spectrometry Analysis

3.1 Materials and Methods

3.1.1 Sample Preparation

The detection of mold on *Theobroma cacao* focused on two compounds, 1-octen-3-ol and p-cresol. Solid phase microextraction, or SPME, was performed by conditioning a 50/30 micrometer layer of divinylbenzel-carboxen-polydimethylsiloxane fiber for 15 minutes at 200°C. One crushed bean from each sample was introduced to the fiber in a 10 mL headspace vial at 75°C in an incubator for 15 minutes by the use of an automated MPS-2 Gerstel unit[14].

The two standards, p-cresol and 1-octen-3-ol, were analyzed using GC/MS to obtain initial retention times and identification of the pure compounds. Following the standards, three cocoa bean samples were analyzed. The "fresh" cocoa beans are a commercial sample grown in a mass production system, while a second sample of beans, denoted as the small batch sample, originated from Peru. A third, moldy sample, was created by soaking the dry commercial cocoa beans overnight for approximately 16 hours, draining the excess water, and holding at 25°C for 14 days. This sample showed a thick coating of multicolor mold after incubation.

3.1.2 Gas Chromatography and Mass Spectrometry

After the incubation period was completed, analysis was carried out using a 7890A Agilent GC with a DB5-MS column, paired with an Agilent 5973-MS. The set parameters included an inlet temperature of 200°C, desorption time of 900s, splitless mode, an oven profile of 40°C to 60°C at a ramp rate of 10°C

per minute, followed by an increase from 60°C to 200°C at a ramp rate of 3°C/min, where it was held at 200°C for 15 minutes. The detector temperature was set at 280°C, and helium was used as the carrier gas. The flow rate was set to 1 mL/min. The compounds in question were identified through scan mode (25-200amu) at 70ev and were confirmed using qualitative MassHunter software, which utilizes the NIST database.

4.2 Results

Shown below are the chromatograms for pure compounds 1-octen-3-ol and p-cresol.

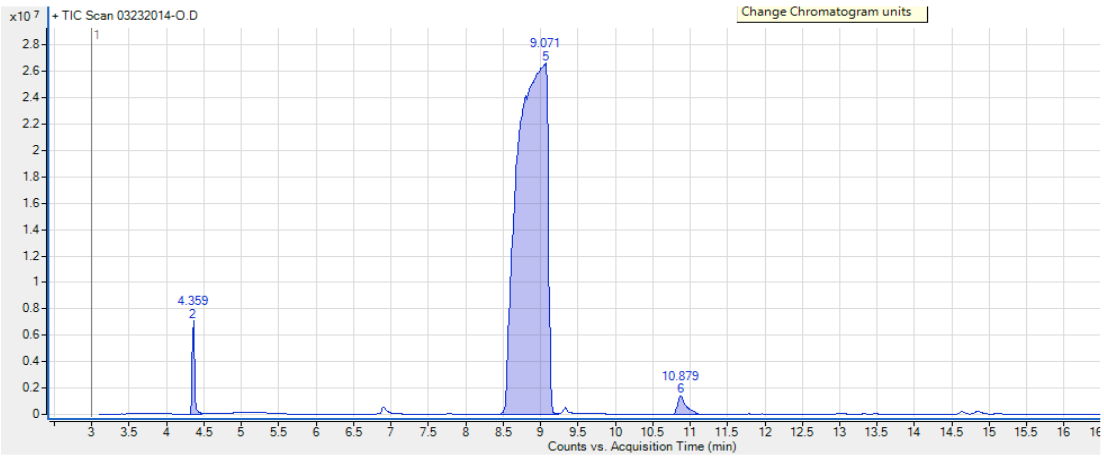

Figure 3-1: Chromatogram of 1-octen-3-ol obtained through GC/MS analysis

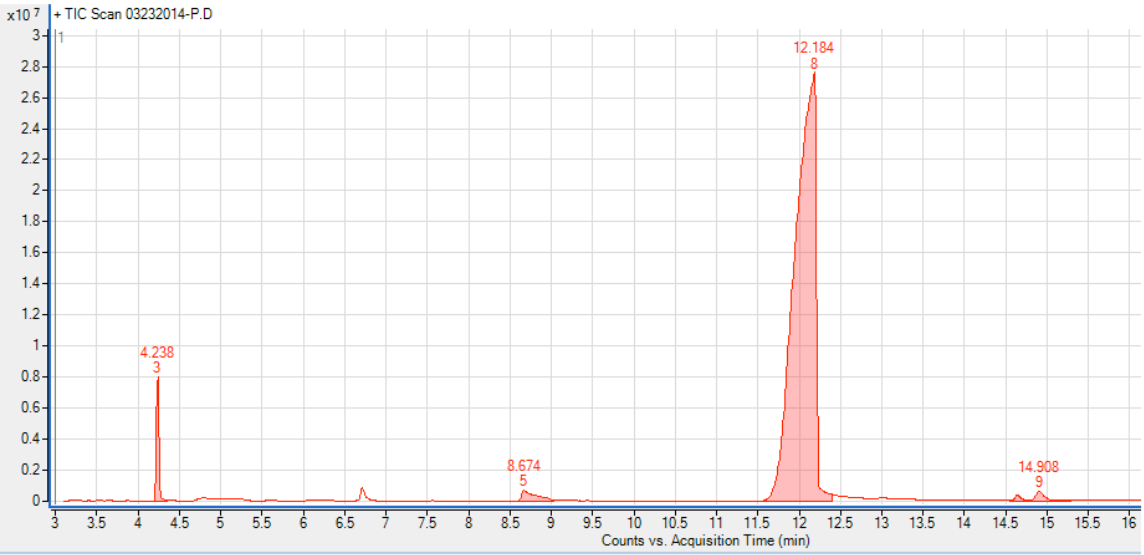

Figure 3-2: Chromatogram of p-cresol obtained through GC/MS analysis

From these graphs, characteristic peaks can be identified for each compound. 1-octen-3-ol's characteristic peak occurs at an approximate retention time of 9.071, while the characteristic peak for p-cresol occurs at an approximate retention time of 12.184. However, these peaks are not a definitive indication of the presence of these compounds in the cocoa samples. Instead, MassHunter Software was used to identify the compounds based on mass and chemical structure.

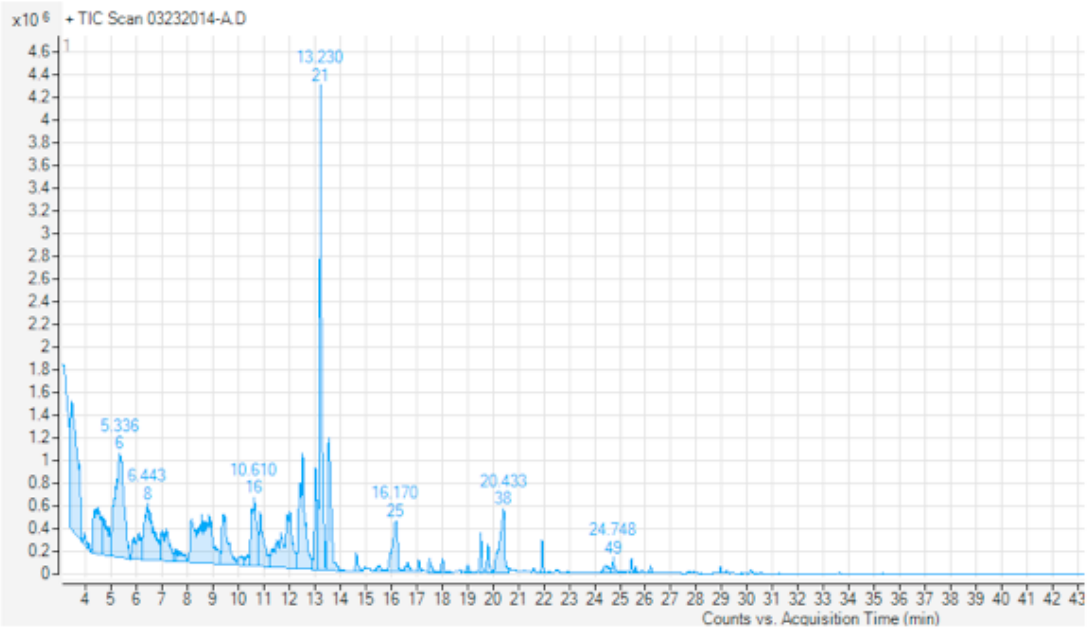

Figure 3-3: Commercial Sample Chromatogram

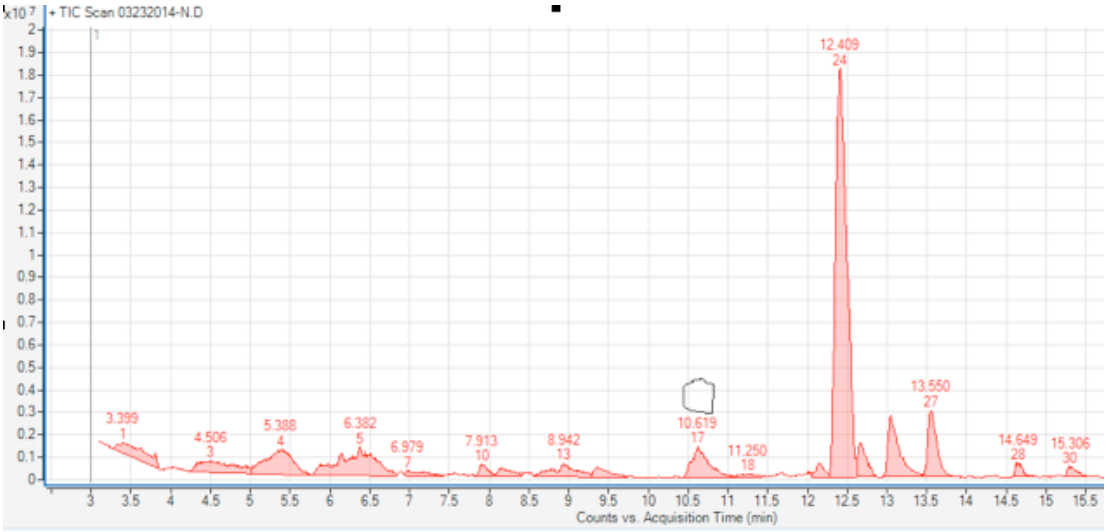

Figure 3-4: Small Batch Sample Chromatogram

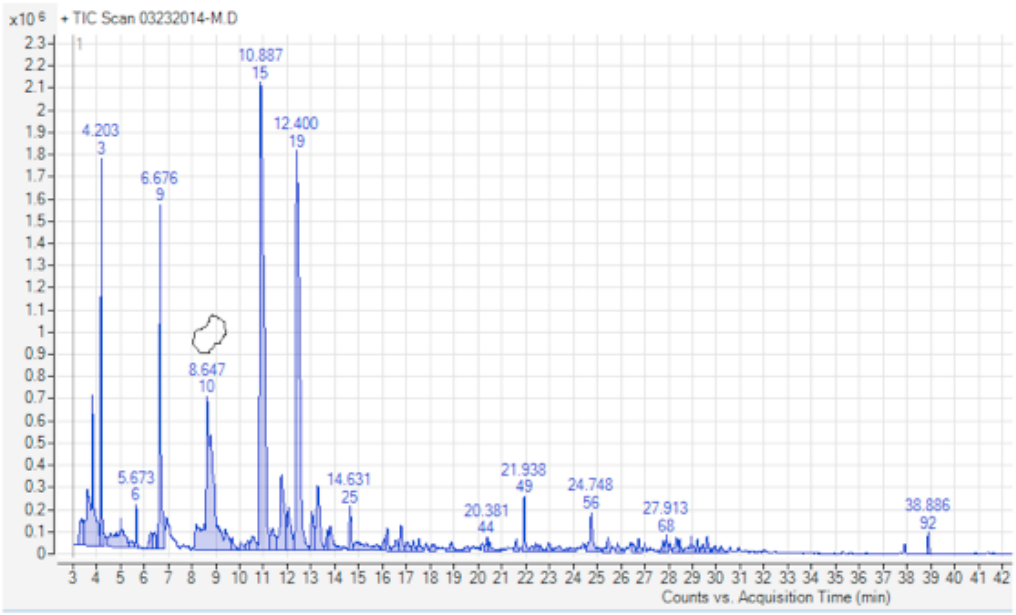

Figure 3-5: Moldy Commercial Sample Chromatogram

Figure 3-3 represents the chromatogram collected from GC/MS analysis of the fresh commercial sample of cocoa beans. While many compounds are present, MassHunter software identified the compound at a retention time of 6.114 min as 1-octen-3-ol, with an approximate total ion count of 0.3 x 10^6. P-cresol was not detected in this sample.

Figure 3-4 shows the chromatogram collected for the small batch sample of cocoa beans after GC/MS analysis. MassHunter positively identified the compound at the retention time of 10.878 with a total ion count of approximately 0.15 x 10^7 as 1-octen-3-ol. Again, p-cresol was not present.

Figure 3-5 shows the chromatogram collected during analysis from the moldy commercial cocoa bean sample. MassHunter identified the compound at a retention time of 8.647 as 1-octen-3-ol, with a total ion count of 0.7 x 10^6. This value is much higher than the ion counts of either the fresh commercial sample or the small batch sample, which may be due to the strong presence of mold on this sample.

Chapter 4

Conclusions and Future Research

4.1 Summary and Conclusions

From these results, it can be determined that while both the fresh commercial sample and the small batch sample show the presence of 1-octen-3-ol in varying concentrations prior to moistening, it is possible to detect this compound in the moldy sample at a higher concentration than the beans that showed no physical mold growth. P-cresol was not detected on any of the samples. These results may be an indication of the presence of mold spores on both the fresh samples and the sample that was exposed to moisture and favorable conditions for mold growth. Because the ion count for 1-octen-3-ol of the moldy sample was much higher than those of the fresh beans, it is likely the spores existed on the fresh beans, but did not thrive and produce the high concentration of VOCs until favorable growth conditions were introduced.

4.2 Future Research

Further research would be invaluable not only to qualitatively detect mold VOCs via gas chromatography and mass spectrometry analysis, but to additionally be able to quantitatively detect these volatile compounds as a direct correlation to the concentration of mold growth on cocoa bean samples. Other compounds of interest besides p-cresol and 1-octen-3-ol may be more directly relatable to mold growth on *Theobroma cacao*. *Aspergillus niger*, a common mold found on cocoa beans, produces VOCs such as 3-octanone and 3-octanol in addition to 1-octen-3-ol[1], and these compounds might prove to be more advantageous to the quantification of mold growth on cocoa before it is visibly detected.

Additionally, a more customized SPME procedure specifically designed for cocoa could yield more accurate and precise results. The SPME procedure used in this research was originally designed for the analysis of truffle aroma, which shares some similarities to the compounds of interest in this study. Specifically targeting the cocoa VOCs of interest in the SPME procedure would aid in the collection of accurate and precise chromatograms for this research.

As discussed in Section 2.10, further applications for this technique could be expanded to include the growing interest in miniaturized mass spectrometers as a tool for food safety and quality assurance. A method for small mass spectrometers for the detection of volatile compounds in food products that indicate spoilage or human health hazards, such as Ochratoxin in cocoa, would be extremely useful to the food industry. Replicating these results using a smaller mass spectrometer could open up the possibility for this equipment to become mainstream in food safety and quality assurance departments of various organizations and food manufacturers.

Chapter 5

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