To the Graduate Council:

I am submitting herewith a dissertation written by Coesha Ancoinette Fairley entitled “Application of Solid Phase Micro-extraction With Gas Chromatography-Mass Spectrometry for the Determination of Geosmin and 2-Methylisoborneol in Processed Navy Beans (Phaseolus vulgaris). I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

David A. Golden

Major Professor

We have read this dissertation and recommend its acceptance:

P. Michael Davidson

Phil Perkins

Bonnie H. Ownley

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures on file with official student records)
APPLICATION OF SOLID PHASE MICRO-EXTRACTION WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY FOR THE DETERMINATION OF GEOSMIN AND 2-METHYLISOBORNEOL IN PROCESSED NAVY BEANS (PHASEOLUS VULGARIS)

A Dissertation
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Coesha Ancoineet Fairley
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Dedication

I would like to dedicate this dissertation to my parents, Pauline and Artis Fairley, my twin sister Rokesha, and to my brothers, Karlos and Solomon. You have shown support through your prayers, words of encouragement, and love throughout this endeavor. I love you.
Acknowledgments

I can certainly count this experience as one of those that has allowed me to mature both mentally and professionally. But I could not have attained this level of achievement without the assistance of a few great people. To my advisor, Dr. David A. Golden, I really can't express the appreciation that I have for the experience of working with you. I thank you for your guidance and support throughout the terms of Ph.D. studies at the University of Tennessee. To my committee members, Dr. Phil Perkins, Dr. Bonnie Ownley, and Dr. P. Michael Davidson, I express my gratitude to each of you for words of advice and expertise given on this project.

To the bean industry, I liked to thank you for allowing me to work for you on a project such as this. I appreciate the opportunity of assisting in the efforts of resolving the issue of musty product. I would also like to thank Dr. Paul Angelino and Sharon S. Melton for teaching me how to operate the gas chromatography-mass spectrometer. Thank you so much for the knowledge.

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Abstract

This investigation was conducted to address the issue of musty bean products. The main objectives of this study were to: (1) identify the source of the musty, off-flavor in processed navy beans, (2) determine and identify the associated off-flavor compounds (geosmin and 2-methylisoborneol (2-MIB)), and (3) isolate the microorganism(s) responsible for production of the compounds.

To confirm the presence of geosmin and 2-MIB in processed baked beans, an automated gas chromatographic method employing solid phase micro-extraction (SPME-GC-MS) was used. Artificially musty baked beans, known musty beans, and raw ingredients (bacon, navy beans, and water) were analyzed. Results of this investigation revealed that navy beans were the source of the musty compounds. 2-MIB and geosmin were detected in artificially musty baked beans, and 2-MIB also was detected in control beans (non-musty). Neither geosmin nor 2-MIB was detected in smoked bacon or water. Results of the analysis identified geosmin and 2-MIB as the musty-odor compounds associated with processed navy beans, with moldy navy beans being the source.

Aspergillus flavus, Penicillium commune, and Penicillium expansum were isolated from moldy navy beans. An additional experiment was performed to determine the production of geosmin and/or 2-MIB by the isolated molds. Non-moldy navy beans were inoculated with spore suspensions from A. flavus, P. commune, or P. expansum, incubated for 10 days at 25°C, and samples were analyzed for mold counts, geosmin, and 2-MIB.
Geosmin was detected in navy beans spiked with *A. flavus, P. commune, and P. expansum*. *A. flavus* produced geosmin after three days of incubation, with concentrations increasing during incubation, corresponding with increasing mold counts. Maximum concentrations of geosmin produced by *A. flavus, P. commune, and P. expansum* were 216, 144, and 19.5 ng/kg, respectively. 2-MIB was not produced by any of the molds, indicating that its presence in beans is due to another source (e.g., other, unidentified microorganisms).
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Introduction

The issue of musty off-flavors and/or odors in the bean industry has been ongoing, specifically for baked bean products. While the definite cause of the problem has not been confirmed, navy beans have been implicated as the source of musty odors and flavors in the finished product. Products exhibiting detectable levels of off-odors and off-flavors are not widely accepted. As a consequence, companies may endure economic loss and a decline in consumer confidence.

The main objective of this literature review is to provide an introduction to musty odors and flavors, specifically geosmin (trans-1, 10-dimethyl-trans-9-decalol) and 2-methylisoborneol (2-MIB), and the effect these compounds have on food quality. Microbial producers of geosmin and 2-MIB are discussed as well to provide an understanding of why and how these compounds are synthesized. A discussion comparing sensory and instrumental methods used for the detection of geosmin and 2-MIB in food and water samples concludes the review.
PART ONE: REVIEW OF LITERATURE
I. Musty Off-Flavors in Foods

Two bi-cyclic terpenoids, geosmin and 2-MIB (figure 1), are commonly associated with the presence of “musty,” “moldy,” and “earthy” flavors and odors in municipal water (Wnorowski, 1992; Howgate, 2004; Whelton and Dietrich, 2004; Zaitlin and Watson, 2006). These compounds create a major concern for the water treatment industry because they are tertiary alcohols whose structure makes them resistant to oxidation, a process normally used for water purification (Wnorowski, 1992). Other foods that have been affected by these compounds include fish (Persson, 1980; Grimm et al., 2004; Zierland et al., 2004; Robin et al., 2006), fruit (Zierland et al., 2004; La Guerc he et al., 2005; Boutou and Chatonnet, 2007), and vegetables (Whitlfield, 1998; Lu et al., 2003).

![Figure 1.1. Molecular structure of geosmin and 2-MIB.](image-url)

Figure 1.1. Molecular structure of geosmin and 2-MIB.
A. Geosmin

Geosmin is a clear, neutral oil with a molecular weight of 182.31 g/mol and chemical formula of \( C_{12}H_{22}O \). The aqueous odor threshold concentration (minimum concentration required for an individual to detect an odorant) for geosmin is 6 to 10 ppb. (Persson, 1980; Krasner et al., 1983; Rashash et al., 1997). Geosmin was originally isolated from actinomycetes by Gerber and Lechevalier (1965) and later in cyanobacteria and algae as the main component imparting an earthy odor to soil and water. Geosmin is the major odor substance produced by actinomycetes, but it has also been reported to be produced by several species of \textit{Aspergillus} and \textit{Penicillium} (Zaitlin and Watson, 2006).

B. 2-Methylisoborneol

2-MIB is a white crystalline solid with a molecular weight of 168.28 g/mol and chemical formula \( C_{11}H_{20}O \). The aqueous odor threshold concentration for 2-MIB is 2 to 20 ppb (Krasner et al., 1983; Rashash et al., 1997). 2-MIB, like geosmin, has been identified as a metabolite of actinomycetes. Gerber (1969) was the first to isolate the compound from actinomycete cultures. Several species of cyanobacteria and fungi are also known to produce 2-MIB (Watson et al., 2000; Westerhoff et al., 2005).

II. Microbial Producers of Geosmin and 2-Methylisoborneol

Odors and off-flavors can be microbial metabolites produced as a result of food degradation or spoilage. Growth of off-flavor producing microorganisms is affected by the environment and chemical composition of the foods they contaminate. Factors
affecting growth of microorganisms in foods include water activity ($a_w$), pH, atmospheric conditions, and temperature under which food is stored (Whitfield, 1998). Geosmin and 2-MIB are produced by certain actinomycetes, cyanobacteria, and molds (Dionigi et al., 1999).

A. Bacteria, Cyanobacteria, and Fungi

1. Bacteria

Actinomycetes, particularly the genus *Streptomyces*, are known to produce geosmin and 2-MIB (Wnorowski, 1992; Schöller et al., 2002; Zaitlin and Watson, 2006). They are identified by their branched, thread-like structure and can survive under aerobic or anaerobic conditions. Abundant in mud of shallow lakes and streams and ever-present in soil, these bacteria play a major role in the decay of organic matter. High nutrient levels, presence of plant debris, structure of reservoir banks, allowing for sedimentation and accumulation, and aerobic conditions in sediments are relevant to the production of earthy taints along with nitrogen by actinomycetes in water (Wnorowski, 1992).

Geosmin was originally isolated from actinomycetes by Gerber and Lechevalier (1965). Volatile metabolites produced by actinomycetes contribute to the earthy odors of soil and certain vegetables, such as beets. Actinomycetes are also believed to be responsible for production of geosmin in dry white navy beans. Buttery et al. (1976) reported that actinomycetes, either on the beans themselves or in the water supply used while growing them, may be a source of geosmin. At least ten species of actinomycetes are known to produce geosmin and seven species produce 2-MIB; the genera
Micromonospora, Nocardia, and especially Streptomyces are known to produce a variety of volatile metabolites.

2. Cyanobacteria

Cyanobacteria are the most common cause of taste and odor problems in surface waters (Watson et al., 2000; Westerhoff et al., 2005). Cyanobacteria, also called blue-green algae, include some genera (Anabaena, Oscillatoria, Symploca, Lyngbia, and Microcytis) that reportedly produced musty, earthy odors. Cyanobacteria are widely distributed in fresh water and growth is encouraged by high concentrations of dissolved organic matter. Izaguirre and Taylor (2004) described 41 different species or morphologically distinct forms of cyanobacteria that have been shown to produce geosmin or 2-MIB.

3. Fungi (Mold)

2-MIB has been reported to be synthesized by some penicillia, including Penicillium commune, Penicillium roqueforti, and Penicillium crutosum (Jeleń and Wasowicz, 1998). 2-MIB from Penicillium caseicolum was related to musty-earthy tones in Brie and Camembert cheeses (Karahadian et al., 1985).

Molds can produce volatile compounds as they colonize nutrient rich substrates such as grains in storage (Magan and Evans, 2000). In a study performed by Jeleń and associates (2003), geosmin and 2-MIB were identified as odor causing compounds in stored wheat grain. The compound was detected using sensory and instrumental methods.
Penicillium aethiopium and Penicillium vulpinum were linked to the production of geosmin. Aspergillus niger and Penicillium aurantioserum were identified as producers of 2-MIB (Jelen et al., 2003).

Common soil fungi from the genera Penicillium and Aspergillus are known to produce geosmin and 2-MIB (Zaitlin and Watson, 2006). Roberts and Matheis (1992) investigated these volatile compounds in stored pome fruit (e.g., apples). Penicillium expansum was identified as being responsible for production of geosmin, the pungent, earthy odor associated with decay of pome fruits.

B. How are these compounds synthesized?

It has been concluded by several investigators that the biosynthesis of geosmin and 2-MIB by different organisms occurs via two common pathways: (1) the melvonic acid (MVA) pathway, and (2) the 1-deoxy-D-xylulose 5-phosphate 2-c-methyl-derythritol 4-phosphate (DOXP/MEP) route (Watson, 2003; Zaitlin and Watson, 2006). Both geosmin and 2-MIB are speculated to be produced by actinomycetes from the MEP pathway. It is unclear why these compounds are produced. However, it has been suggested that geosmin may function as a quorum sensing signal molecule. Also, geosmin and 2-MIB may serve as antagonistic agents against competing microflora (Watson, 2003; Zaitlin and Watson, 2006).
III. Methods for Detecting Geosmin and 2-Methylisoborneol

The techniques used for detection and quantification of flavor and odor causing compounds in food and water include sensory analysis using human subjects and instrumental methods (Zervos and Albert, 1992; Bao et al., 1998).

A. Sensory Analysis

The human senses have been recognized as the most sensitive tools for taste and odor detection and quantification (Wnorowski and others, 1992). Food flavors are composed of a broad array of chemicals which interact with olfactory and lingual receptors to impart a specific organoleptic (sensory) impression (Zervos and Albert, 1992). As a result, humans are able to identify, describe, compare, and rank characteristic features of the taste, odor, texture, and color of foods (Zervous and Albert, 1992). Expert tasters are commonly employed by food processors to detect off-odors and/or off-flavors. According to Gimm et al. (2004), professional flavor checkers are employed for the sensory analyses of earthy, muddy aroma in catfish. Even though the sample loads may exceed 100 samples per day, the checkers are highly sensitive to the flavors, and fish are rejected if there is any indication of geosmin or 2-MIB (Grimm et al., 2004).

Although the human nose can readily detect 2-MIB and geosmin at the sub parts per billion range (<1 μg/kg), humans readily succumb to sensory overload (Grimm et al., 2004). Another drawback of the sensory analysis method is that thresholds (or limits of sensory capacity) may vary from person to person (Persson P.E. 1980; Bett and Johnsen, 1996; Grimm et al., 2004). Adaptation is the loss of sensitivity due to continuous
exposure to chemical stimuli, during which the sensory system continues to function, but some compounds may elicit qualitative changes (Whelton et al., 2004). For example, panelists’ initial perception of trimethylamine is a fishy odor, but after continuous exposure, the smell is described as ammonia-like (Amerine et al., 1965). Adaptation may be overcome rapidly, whereas fatigue requires lengthy recovery time for accurate sample evaluation. Fatigue is caused when the sensory system becomes less responsive to stimuli over repeated evaluations. Another concern is carry-over from one sample to another. Enhancement or suppression, the effect on intensity of descriptors by the presence of another substance, may also cause differences in flavor perception (Bett and Johnsen, 1996).

B. Gas Chromatography / Mass Spectrometry

Gas chromatography/mass spectrometry (GC-MS) methods are routinely used for the qualitative identification of unknown compounds and their accurate quantitative determination (Massucci and Caldwell, 2004). GC-MS has been used for the determination of fatty acids, cholesterols, gases, water, alcohols, simple sugars, pesticides, herbicides, food additives, and flavor aromas, just to name a few (Reineccius, 1994). Because aroma compounds must by nature leave the food matrix and travel through the air to be perceived, they are usually good candidates for gas chromatography analysis (Wampler, 2002). The analysis of food samples is accomplished by an extraction step, followed by concentration, chromatographic separation, and detection (Grimm et al., 2002).
1. What is Gas Chromatography?

As an analytical tool, gas chromatography can be used for the direct separation of and analysis of gaseous samples, liquid solutions, and volatile solids after they have been obtained at the desired volume (Reineccius, 1994). The method involves the analysis of volatile organic compounds, materials that exist in the vapor phase at the typical GC operating temperature between 40 and 300°C (Harmon, 2002). These components distribute themselves between two phases, one stationary and the other mobile. The mobile phase, usually helium, is inert and does not participate in the chemistry of the chromatographic processes, but only serves to carry the sample through the column (Miller, 2005). The stationary phase may be a solid or a liquid supported on a solid (Reineccius, 1994; Grobb and Barry, 2004).

The major components of the GC system are the gas supply and regulators, injection port, oven, column, detector, electronics, and recorder/data handling system (Miller 2005). The type of column, its length and diameter, temperature program for the oven, detector type and carrier gas flow are important parameters that should be kept constant for reproducible results (Harmon, 2002). Carrier gas is important since differences between its thermal properties and the analytes determines response. Hydrogen, helium and nitrogen are carrier gases (Reineccius, 1994; Barry, 2004). Of the three gases, hydrogen is the best choice but is also flammable. As an alternative, helium is commonly chosen.
2. **Mass Spectrometry**

The mass spectrometer (MS) performs three basic functions (Smith, 1994). Operating under a high vacuum, analytes are eluted from the chromatograph and are directed into the spectrophotometer ion source where they are ionized. The molecules are exposed to high energy electrons and broken down into charged molecular fragments. By the quadrupole, the different fragments are separated depending on their mass to charge ratio and detected (Smith, 1994; Miller, 2005). This process allows for the detection and identification of an unknown compound.

Numerous detectors are available for GC analysis, each offering advantages in sensitivity or selectivity. These include flame ionization (FID), thermal conductivity (TCD), electron capture (ECD), flame photometric (FPD), and photoionization (PID) (Colón and Baird, 2004; Miller, 2005). Of these, FID is considered the detector of choice for food analysis. With FID, as compounds elute from the analytical column, they are burned in a hydrogen flame. Overall, it has very good sensitivity, and dependability. It works well when a specific detector is not desired or when sample destruction is acceptable (Miller, 2005).

The GC-MS combination is by far the most popular technique for the identification of volatile compounds in foods and beverages (Maarse, 1991). However, GC analysis cannot work well without sample preparation. The constituent(s) of interest must be isolated from the food matrix in a manner that permits concentrations at detectable limits for the GC. Therefore, the analyst(s) must perform some type of sample preparation consisting of component isolation and concentration prior to the GC analysis.
The isolation procedure may involve headspace analysis, distillation preparative chromatography, simple solvent extraction, or some combination of the basic methods. Solvent extraction is often the preferred method for the recovery of volatiles from foods. However, recovery of the volatiles will depend upon solvent choice and the solubility of the solutes being extracted (Reineccius, 1994).

C. Solid Phase Micro-extraction

Solid phase microextraction (SPME) offers significant advantages as an extraction/concentration technique prior to gas chromatography analysis (Harmon, 2002). The technique is ideally suited for the characterization of unknown mixtures of volatile organic compounds because it is an equilibrium technique that requires no solvents for extraction or pre-concentration steps (Bao et al., 1998; Harmon, 2002).

1. What is SPME?

SPME devices have been introduced commercially for both manual and automatic injections by Supelco. The device is essentially a syringe having a spring-loaded plunger and a barrel, with the plunger held in an extended position during the extraction phase and during the injection/desorption period. The needle functions to puncture the septa sealing both the sample container and the GC injection port. It also protects the fused silica fiber during storage and use (Harmon, 2002). A short, thin, solid rod of fused silica, coated with an absorbent polymer is employed to extract volatile compounds. Commercially, SPME fibers are coated with polymers ranging from the nonpolar
polydimethilsiloxane (PDMS) to the more polar Carbowax (Supelco). For most analyses, especially of volatile aroma compounds, a fiber having a 100 um coating of PDMS is often the preferred choice (Harmon, 2002). Non-polar thick film fibers, such as PDMS, provide good recovery for most aroma compounds (Bao et al., 1998; Jeleń et al., 2003; Lu et al., 2003).

2. Mechanics of SPME

After the sample has been prepared, it is placed into a vial that is sealed with a septum (cap). The SPME needle pierces the septum and the fiber is extended through the needle. During headspace sampling, the fiber is extended into the vapor phase of the sample. Headspace analysis is logical for the study of volatile compounds of foods and beverages (Bao et al., 1999; Harmon, 2002; Jeleń et al., 2003). It reveals the identity and the concentration in the vapor phase of those compounds that are directly responsible for the aroma of the product. Headspace techniques benefit from the addition of salt or adjustment of pH, which enhances the equilibrium of the contained aroma compounds toward the organic phase of the SPME fiber (Bao et al., 1999; Harmon, 2002). Agitation/stirring assists with this as well. The incorporation of an internal standard into the matrix and adherence to specific sampling times result in excellent quantitative correlations (Harmon, 2002; Grob and Barry, 2004).

After a suitable sampling time, the fiber is withdrawn into the needle. The needle is then withdrawn from the septum and is inserted directly into the injection port of a gas chromatograph (Nilsson, 1996; Harmon, 2002). Any manner of injection is suitable for
SPME, but a split ratio of slit/splitless capillary injection port should be set around 10:1. A splitless injection mode transfers more of the absorbed material to the analytical column for those applications that require higher sensitivity (Harmon 2002). In addition, use of an injection port liner with a diameter of 1 mm or less is recommended, since this provides sharper peaks for highly volatile compounds (Harmon, 2002).

Geosmin and 2-MIB can be detected at low concentrations when GC-MS is coupled with solid phase microextraction (SPME). Jeleń et al. (2003) identified geosmin and 2-MIB as odor causing compounds in stored wheat grain. The compounds were analyzed by a GC-MS analysis method employing SPME. Four fibers were tested for their efficiency in isolating the compound: Carboxen/PDMS, carboxen/divinylbenzene/PDMS, divinylbenzene/PDMS, and PDMS. The highest recoveries of geosmin and 2-MIB from water were observed with the PDMS/DVB fibers using an extraction time and temperature of 30 minutes and 50°C. Concentration as low as 1 ng/kg were detected for both geosmin and 2-MIB.

SPME-GC-MS has been used to detect 2-MIB and geosmin in water at a detection limit of 10 ppb (Watson, et al., 2000; Grimm et al., 2004). SPME-GC-MS analysis was compared to sensory detection of off-flavors in catfish by Grimm et al. (2004). A high correlation (R=0.9) existed between the instrumental method and trained sensory panelists. Nilsson et al. (1996) applied head-space SPME-GC-MS for the analysis of volatile metabolites of *Penicillium* species. SPME provided a valid and fast method for determination of fungal metabolites, showing great potential for the analysis of geosmin and 2-MIB.
III. Objectives of the Current Studies

The aim of this study was to identify the source or cause of musty off-flavors in processed navy beans. Most of the research was performed at process facilities where the problem has been known to occur. Original product characterized as musty was analyzed for musty off-flavors and odors using sensory analysis. Product ingredients were collected during normal production hours and analyzed using sensory and instrumental methods for musty off-flavors and off-odors. An instrumental method employing gas chromatography-mass spectrometry with solid phase microextraction (SPME-GC-MS) was used to detect musty compounds geosmin (trans-1, 10-dimethyl-trans-9-decalol) and 2-Methylisoborneol (2-MIB). Microbial producers of these compounds were also investigated.
V. References


PART TWO: IDENTIFICATION OF POTENTIAL SOURCES OF MUSTY OFF-FLAVORS IN PROCESSED NAVY BEANS (*PHASEOLUS VULGARIS* L)
Abstract

An investigative study was conducted at a bean processing plant where issues with “musty” products were known to occur. The primary purpose of this study was to sample ingredients used for the production of baked beans. Collection of ingredients took place during normal hours of production as they were being used. The ingredients were analyzed for musty off-flavors and/ odors. Retained samples were appropriately stored for later analysis.

An additional portion of the trip involved visits to facilities of navy bean suppliers. Processing equipment and storage conditions were considered, but the occurrence of clumps of moldy navy beans was of particular interest. Formation of the clumps was speculated to be due to poor storage conditions under which temperature and moisture were not adequately controlled. Remnants of the navy-bean clumps were collected and later analyzed.

The study concluded with the artificial production of a musty product. Moldy navy beans were combined in a mixture of known levels (0, 1, 2, and 3% musty beans) with non-moldy navy beans and processed under commercial conditions in 794 g aluminum cans in a brine (salt-water) solution. After processing, the product was allowed to stabilize at room temperature (20-25°C) and was analyzed by experienced tasters.

During the investigative study, two incidences of mustiness in finished product occurred. Musty odors or flavors were not detected in the collected ingredients during those times. However, after reviewing process records, the navy bean supplier isolated
moldy navy beans from the bean lot used. As for the musty product created, the evaluators detected differences between the products (0, 1, 2, and 3% musty). This means that the level or intensity of mustiness detected correlated with the percentage of mold-navy beans (0, 1, 2, and 3%). The 2% musty product was described as having a slight musty flavor, while the 3% musty product exhibited a strong odor as well as flavor.

Because of prior speculation and their use in products characterized as musty, three ingredients (smoked bacon, water, and navy beans) were identified as potential causes of the musty problem in finished products.
I. Introduction

The occurrence of musty off-flavors in the bean industry is ongoing. A majority of the documented incidences have been reported to occur in late spring or early summer. To maintain consumer satisfaction, one bean processing company (confidential information) has established a 4-point rating system to rate the level of mustiness (0-3) in musty product based on the severity of mustiness the product was either, released for distribution, donated or destroyed. The rating system is as follows: 0 = no musty flavor or odor; 1 = slight musty flavor; 2 = moderate musty flavor; 3 = strong musty flavor and odor. A recent internal study at this company established that all products exhibiting off-flavors are to be rejected.

The objective of this study was to identify the cause of musty off-flavors in processed navy beans. An investigation of the problem took place at process facility “B” from June – August, 2006 (a 10-week period). Previous issues of “musty product” were identified during the late spring and early summer. Identifying the source of off-flavors will help to reduce the production and distribution of musty product.

The History of Navy Beans

*Phaseolus vulgaris* (or common beans) were originally domesticated in Central and South America over 7000 years ago (Jenner, 1989; Mathews, 1989; Graham and others, 1997). They are referred to as “common beans” because they are speculated to have been derived from a common bean ancestor that originated in Peru. From there, seeds were collected by early explorers of the New World and were being grown in
Europe by 1542. They have since spread to every corner of the world, and have become an important part of human nutrition (Maiti et al., 2007).

The largest commercial producers of dry beans are Brazil, India, China, Burma, New Mexico, and the United States (USDA, 2005). In the U.S., North Dakota, led in the production of edible beans followed by Michigan, Nebraska, Minnesota, Idaho, California, and Idaho between 2002 and 2004. The leading varieties of edibles produced in the U.S. are pinto, navy, black, and great northern beans (USDA, 2005). Other varieties of edible beans include red kidney, pink cranberry, small red, and small white beans (Hartwig, 1989).

**Cultivation and Agricultural Practices**

In the Mid-West, navy beans are grown in the fertile Red River Valley. However, during their growth cycle, beans are exposed to a large array of constraints that can affect crop yield; these include heat and drought stress and pests (Graham and Ranalli, 1997; Maiti, 1997). Water management is a critical aspect of bean production. Well drained soils with good water holding ability are needed for a successful crop.

Under normal conditions, navy bean crops are not planted one after the other. Seed-borne diseases are the major yield-reducing factors in beans. Charcoal rot and bacterial blight are just a few examples of seed-borne diseases (Mathews, 1989). It is recommended that a three year crop rotation system is employed. Navy beans may follow or succeed many crops except for other edible beans, sugar beets, sunflowers, snap beans,
mustard and canola. The practice of intercropping may can contribute to weed control and also reduce the spread of disease (Hartwig, 1989).

In late August or early September, navy beans are usually harvested. Harvesting machines such as combines are used. After harvesting, beans are allowed to dry under the sun for two to three days. Sun drying is the most common method of drying beans, after which, beans are transferred to storage. A moisture range of 18-22% is desired to protect quality. Before storage, beans are separated from foreign material (e.g., leaves, stones, and dirt). Equipment used includes the following: scalpers are used for the removal of leaves, bean hulls, etc.; density gravity machines separate larger beans from the smaller beans along with dirt balls; blowers remove excess dirt and other contaminants. After cleaning, beans are sorted for imperfections (i.e., blemished or dark colored beans). An electron-eye sorter is used for this process; however, all processing plants are not equipped with this machine. The process of cleaning is performed again before final distribution.

From six months to a year, beans may be stored in elevator bins or silos made of cement, steel, or wood. During the warmer months of bean storage, temperature inside the storage bins rises and falls with the heat of day and cooling of night. As a result, an increase in moisture occurs and the formation of a ‘crust-like’ substance on the top of bean piles is observed. To control the problem of humidity, cooling fans are installed to encourage an even flow of air in/out of storage areas. In addition, silos are checked for bean-crust formation. If present, the crust is removed from the pile of beans.
II. Materials and Methods

A. Ingredient Sampling

The investigation of musty products took place at a bean processing/canning facility in the Midwestern United States. Samples of ingredients (Table 2.1) were collected daily during the first and second shifts of navy bean production. The ingredients were obtained from their respective containers as they were being used in the production areas. Sealable jars or plastic bags were used for the sample collection. Immediately after collection, samples were labeled and stored either at ambient temperature (20-25°C) or frozen, as appropriate for each ingredient. Ingredient identity, lot numbers, and date of sampling were also documented. Organoleptic evaluation (e.g., smell, taste, and visual inspection) of samples was conducted by a trained analyst. Ingredients were retained in storage for one week and destroyed thereafter if there were no incidences of mustiness. Defects, if discovered, were reported to the respective area managers and Quality Assurance. A total of 960 ingredient samples were evaluated over a 10-week period.

Table 2.1. Ingredients evaluated for musty characteristics.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Ingredient</th>
<th>Ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacon</td>
<td>Calcium chloride</td>
<td>Caramel color</td>
</tr>
<tr>
<td>Honey</td>
<td>Maple syrup</td>
<td>Molasses</td>
</tr>
<tr>
<td>Mustard</td>
<td>Navy beans</td>
<td>Onion</td>
</tr>
<tr>
<td>Paprika</td>
<td>Salt</td>
<td>Seasoning</td>
</tr>
<tr>
<td>Starch</td>
<td>Sugar</td>
<td>Tomato paste</td>
</tr>
<tr>
<td>Vinegar</td>
<td>Water</td>
<td></td>
</tr>
</tbody>
</table>
1. Collection of liquid samples (e.g., water, sauce)

Liquid samples were collected and stored at ambient temperatures or refrigerated in sealable, plastic jars. All samples except for water were collected from containers provided by the respective ingredient suppliers. Process water was collected at plant facility B.

2. Collection of non-liquid samples (e.g., bacon and beans)

Conditions of product and package containers were noted and documented. Bacon samples were not examined by tasting, but were examined by smell and appearance for evidence of oxidation and spoilage. Oxidation was indicated by discoloration or graying of meat. Spoilage was indicated by gas formation (bubbles) along with sour odor. Navy beans were collected from the process lines after blanching. It was assumed that the application of heat would aid in the detection of musty off-flavors and odors if present.

B. Tour of Bean Processing Facilities

Bean processing facilities in North Dakota were visited. Cultivation practices and conditions were discussed while walking through fields of black dirt and green vegetation.

C. Production of Musty Navy Bean Product

Known levels of musty navy bean product were produced to determine the presence of musty off-flavors and/or odors. Various levels of moldy navy beans (0, 1, 2
and 3%) were combined with non-moldy navy beans to make musty product (Table 2.2). Moldy, musty beans were pre-weighed and separated in netted bags. The beans were soaked in water at 49°C for 2.5 hr.

After soaking, 794-g aluminum cans were filled to the appropriate weight with beans and brine (salt + water). No additional ingredients were added. The product was processed for 30 min at 125°C in an Allpax Hydro Mode retort. Cans were allowed to cool to room temperature (20-25°C) before being examined.

Expert tasters (8) were involved in the analysis of the finished product. Before tasting, the net containing the moldy navy beans was removed from the product. A 4-point rating system (musty levels 0, 1, 2, and 3) was established for musty product characterization.

<table>
<thead>
<tr>
<th>Musty product concentration (%)</th>
<th>Musty beans added (g)</th>
<th>Navy beans added (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>none</td>
<td>127.6</td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
<td>126.3</td>
</tr>
<tr>
<td>2</td>
<td>2.6</td>
<td>124.7</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>123.7</td>
</tr>
</tbody>
</table>

### III. Results

#### A. Ingredient Sampling

During the 10-week period of investigation, two incidences of musty odor/flavor issues were observed in finished product. Musty odors and/or flavors were not detected
in the ingredients collected during those times. However, after reviewing process
records, the navy bean supplier collected clumps of moldy navy beans from the lot of
beans that was used for production.

B. Production of Musty Product

The occurrence of moldy clumps of navy beans (Figure 2.1) during critical
months was highlighted during the bean supplier tour. The clumps were speculated as
potential causes of mustiness, and were attributed to poor storage conditions. Samples of
the clumps were collected for further analysis at a later date.

![Figure 2.1. Photograph of moldy clump of navy beans.](image)

C. Production of Musty Product

Musty product was produced to determine if the moldy navy beans were
responsible for musty flavors in processed beans. Evaluators ranked the finished product
by intensity of the musty flavor and/or odor detected (Table 2.3).
Table 2.3. Evaluation of musty product made with brine.

<table>
<thead>
<tr>
<th>Product</th>
<th>Musty flavor/odor description</th>
<th>Musty classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% musty</td>
<td>none</td>
<td>level 0</td>
</tr>
<tr>
<td>1% musty</td>
<td>slight</td>
<td>level 0 or 1</td>
</tr>
<tr>
<td>2% musty</td>
<td>moderate</td>
<td>level 1 or 2</td>
</tr>
<tr>
<td>3% musty</td>
<td>extreme</td>
<td>level 3</td>
</tr>
</tbody>
</table>

A musty odor was detected in all products except the control (0% musty). Can-to-can variation (i.e., two levels of mustiness were detected) was observed in 1% and 2% musty product. 1% musty product was classified as level 0 or 1 (slight musty). 2% product was classified as slight (level) or moderate (level 2) musty product. The 3% musty product exhibited strong musty odor and flavor.

IV. Discussion and Conclusions

In previous incidences in which musty products occurred, retention samples of ingredients were not available for testing. Only cans of processed navy beans were available, thereby precluding the possibility of determining which ingredient was the source of the musty problem. Therefore, a procedure for ingredient sampling was created in hopes of eliminating suspected ingredients.

A majority of the ingredients used for processing are received in bulk, which means that there use spans over a period of weeks or even months, depending on production needs. But, because of their consistent use in products involved in previous
cases of mustiness, smoked bacon, process water, and navy beans were suspected as potential sources of mustiness.

From the results of the artificial musty product evaluations, it was apparent that moldy navy beans were the source of musty off-flavors and odors in finished product. Use of 3% moldy navy beans in processing yielded the strongest off-flavors and/or odors. It takes only 1 or 2 moldy navy beans (equivalent to a slightly musty product; 1%) to produce a musty off-flavored product, thereby rendering it unacceptable to the consumer.

Further analysis of ingredients and processed beans using instrumental analysis is necessary to confirm the true cause of the musty product issue and to identify the associated odor and/or flavor compounds.
V. References


PART THREE: APPLICATION OF HEADSPACE SOLID PHASE MICROEXTRACTION FOR THE DETERMINATION OF GEOSMIN AND 2-METHYLIsoBORNEOL IN PROCESSED NAVY BEANS
Abstract

A gas chromatographic method employing solid phase microextraction (SMPE-GC-MS) was used for the determination of geosmin and 2-methylisoborneol (2-MIB) in processed navy beans and raw ingredients (bacon, navy beans, mold-navy beans, and water). The SPME method used employed a PDMS/DVB fiber for the extraction/adsorption of desired compounds. After extraction, the fiber was desorbed to the GC system.

Geosmin was detected in musty level 1 and level 3 baked beans at concentrations of 10.2 and 35.2 ng/kg, respectively. 2-MIB was detected only in musty level 3 baked beans at 29.1 ng/kg. Neither compound was detected in musty level 0 (control) product. Geosmin was detected at concentrations of 4.4 ng/kg in 3% musty product with a mean relative recovery of 73.2, while 2-MIB was detected in all of products including the control.

As for the ingredients, 2-MIB was quantified at a mean concentration of 28.4 ng/kg in navy beans and 106 ng/kg in moldy navy beans with a mean relative recovery of 9% and 25% respectively. Geosmin, however, was detected in moldy navy beans at a mean concentration of 249ng/kg with a relative recovery of about 60%. Neither geosmin nor 2-MIB was detected in smoked bacon or water.

Results of this study indicate that geosmin and 2-MIB are compounds responsible for musty off-flavors and odors in processed beans with the source being moldy navy beans.
I. Introduction

Moldy navy beans have been identified as a potential cause of musty, off-flavors and/or odors in processed navy beans, but the associated musty compounds have not yet been identified. Geosmin and 2-methylisoborneol (2-MIB) are associated with earthy, musty odor and/or flavor problems in food and water supplies.

Gas chromatography- mass spectrometry (GC-MS) is the traditional method of choice for analyzing aromas in foods (Nelsen, 1994; Miller, 2005). However, geosmin and 2-MIB are a challenge to analyze because of their low odor thresholds (0.01ug/kg and 0.015 μg/kg, respectively). Methods used for analysis of geosmin in foods have required additional instrumentation and sample preparation steps before analysis by gas chromatography (Watson, 2000; Lu et al., 2003). For example, Tyler et al. (1978) developed a liquid-liquid extraction method for red beets using Freon 113, followed by purification using a florisil column and gas chromatography analysis. Using these methods, samples were sometimes destroyed or degraded by the solvents used, which ultimately resulted in the loss of volatiles.

Solid phase microextraction (SPME ) offers significant advantages as an extraction/concentration technique prior to gas chromatography analysis (Harmon, 2002). It is a solvent-free, inexpensive, rapid, and versatile method for the extraction of organic compounds; a short, thin, solid rod of fused silica, coated with an absorbent polymer is used to extract/adsorb compounds (Harmon, 2002).

Low concentrations of geosmin and 2-MIB can be detected when GC-MS is coupled with SPME. Jeleń et al. (2003) used the SPME technique prior to gas
chromatography analysis for the detection of geosmin and 2-MIB in wheat grain. Concentrations as low as 1 ng/kg were detected when a carboxen/PDMS/DVB fiber was used.

For this study, a gas chromatography method employing SPME was developed to determine the presence of geosmin and 2-MIB in processed baked beans.

II. Materials and Methods

A. Chemicals, Fibers, and Samples

Geosmin (minimum 98% purity) and 2-methylisoborneol were obtained from Sigma. (-)-Menthone (90% purity) was purchased from Aldrich. Methanol was purchased from Fisher Scientific. Sodium chloride reagent (crystals; 99+% purity) was obtained from ACROS. Polydimethylsiloxane-divinylbenzene (PDMS/DVB) fibers were purchased from Supelco.

Cans of processed beans were obtained from a bean processing plant in the Midwestern U.S. (plant B). This included product characterized as musty (baked beans level 0, 1, and 3). Artificially musty product (non-musty beans containing 0, 1 and 3% musty beans) were also analyzed. As for ingredients, five-pound containers of navy beans (non-moldy and moldy) were obtained from another bean processor (plant A). Process water and smoked bacon were obtained from plant B.
B. Solid Phase Micro-extraction

1. Sample preparation

Procedure A: Processed Beans

Bean samples were blended with water to form a slurry. Two grams of the bean slurry were combined into a 10 ml headspace vial with 1.5 grams of NaCl, 2 ml of water, and 20ul of (-)-menthone (72.8 ng/kg). Duplicate samples (n=3) were prepared in the same manner but also were spiked with external standards, geosmin and 2-MIB (40 μl, respectively, each), to yield concentrations of approximately 235ng/kg per sample. Menthone was added as an internal standard to allow for calculation of percent recovery of geosmin and 2-MIB. Percent recovery was calculated as:

\[
\text{Percent recovery} = \frac{\text{Conc. detected in spiked sample} - \text{conc. detected in non-spiked sample}}{72.8} \times 100\%
\]

Procedure B: Raw Ingredients (bacon, beans, and water)

One gram of chopped bacon was combined into a 10 ml headspace vial with 1.5 g of NaCl, 3 ml of water, and 20 μl of (-)-menthone. Four ml of processed water was combined into a 10 ml headspace vial with 1.5 grams of NaCl, and 20 μl of (-)-menthone. Raw beans (control navy and moldy navy beans) were prepared in the same fashion. Whole beans were finely ground with a Black & Decker® Smart Grinder. One gram of beans was combined into a 10 ml headspace vial with 1.5 grams of NaCl, 3 ml of water, and 20ul of (-)-menthone.
2. Headspace SPME

The SPME extraction process was accomplished using an automatic sampler system (Combi-PAL) from Leap Technologies. A polydimethylsiloxane-divinylbenzene (PDMS/DVB) fiber was used for the experiment. Prior to its use, the fiber was conditioned for 30 min at 50°C.

Each sample was heated and agitated for 15 minutes at 65°C. The needle holder was then inserted into the headspace for 60 minutes at 65°C. After extraction, the fiber was withdrawn from the sample vial and thermally desorbed into the gas chromatography system for two min.

D. Gas Chromatography/Mass Spectrometry

Gas chromatography analysis was accomplished using an Agilent 6890N Chem Station. The collected compounds were resolved onto a HP-5MS column (30-m x 0.25 mm i.d. x with 0.25 µm film). The temperature program used was as follows: the inlets were set at 260°C; oven at 35°C, hold 2 min, increase at 5°C/min to 280°C and hold for 5 min. The GC system runtime was 65 minutes.

The detector (Agilent 5973 MSD) was set either in full scan (40 to 340 m/z) or selective ion mode (SIM). The following ions were chosen: MIB-95 m/z; GSM-112 m/z; (-)-menthone 112 m/z). For a qualitative screening of new samples where the composition was unknown, the mass spectrometer (MS) detector was used. Otherwise, a selective detector such as flame ionization (FID) was employed to enhance sensitivity for detecting certain group of compounds.
III. Results

Products and ingredients that were identified as musty from product evaluations were analyzed. The identity of geosmin and 2-MIB in raw and processed beans was verified by matching the mass spectrum at the retention time at which the compounds were eluted from the column (Appendix Figures A.1-A.17).

Results from the SPME-GC analysis of baked beans are presented in Table 3.1. Geosmin was detected in musty level 1 and level 3 baked beans at mean concentrations of 10.2 and 35.2 ng/kg, respectively, per gram of sample collected from headspace. 2-MIB, was detected only in musty level 3 baked beans (29.1 ng/kg). Neither compound was detected in musty level 0 (control) baked beans.

Table 3.1. Concentration and recovery of geosmin and 2-MIB in baked beans.

<table>
<thead>
<tr>
<th>Musty Level</th>
<th>Concentration a (ng/kg) and % recovery b of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geosmin</td>
</tr>
<tr>
<td>level 0 (control)</td>
<td>- c</td>
</tr>
<tr>
<td>level 1</td>
<td>10.2 (17.0)</td>
</tr>
<tr>
<td>level 3</td>
<td>35.2 (12.0)</td>
</tr>
</tbody>
</table>

a Mean concentration of sample in headspace vial; n =3.
b Mean percent (%) recovery in parentheses; n=3. Recovery was calculated as: (Conc. detected in sample spiked with menthone at 72.8 ng/kg – conc. detected in non-spiked sample)/72.8 x 100%
c Not detected.
Geosmin was detected at a mean concentration of 4.4 ng/kg in 3% musty product with a mean relative recovery of 73.2%, while 2-MIB was detected in all of the brine products including the control (Table 3.2).

Table 3.2. Concentration and recovery of geosmin and 2-MIB in navy beans processed in brine.

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration a (ng/kg) and % recovery b of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geosmin</td>
</tr>
<tr>
<td>0% musty</td>
<td>-  c</td>
</tr>
<tr>
<td>1% musty</td>
<td>-</td>
</tr>
<tr>
<td>3% musty</td>
<td>4.4 (73.2)</td>
</tr>
</tbody>
</table>

a Mean concentration of sample in headspace vial; n =3.
b Mean percent (%) recovery in parentheses; n=3. Recovery was calculated as:
(Conc. detected in sample spiked with menthone at 72.8 ng/kg – conc. detected in non-spiked sample)/72.8 x 100%
c Not detected.

For ingredients (Table 3.3), 2-MIB was detected at a mean concentration of 28.4 ng/kg in navy beans and 106 ng/kg in moldy navy beans with a mean relative recovery of 10.8 and 25%, respectively. Geosmin was detected in moldy navy beans at a mean concentration of 249 ng/kg with a relative recovery of 60.4%. Neither geosmin nor 2-MIB was detected in smoked bacon or water.
Table 3.3. Concentration and recovery of geosmin and 2-MIB in ingredients.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>2-MIB</th>
<th>Geosmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacon</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Navy beans</td>
<td>-</td>
<td>28.4 (10.8)</td>
</tr>
<tr>
<td>Moldy navy beans</td>
<td>249.0 (60.4)</td>
<td>106.0 (25.0)</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Mean concentration of sample in headspace vial; n =3.
b Mean percent (%) recovery in parentheses; n=3. Recovery was calculated as: 
(Conc. detected in sample spiked with menthone at 72.8 ng/kg – conc. detected in non-spiked sample)/72.8 x 100%
c Not detected.

IV. Discussion and Conclusions

Menthone was chosen as the internal standard because it is known to separate well from compounds and achieve a relative recovery close to 100% (Lu et al., 2003). In preliminary studies performed, the efficiency of (-)-menthone as an internal standard was compared to that of biphenyl-d$_{10}$ (Sigma). (-)-Menthone proved to be the better internal standard, and was used for calculation of percent recovery of geosmin and 2-MIB from bean samples.

The sample size of 5.5 grams was chosen because preliminary research also showed that extraction of smaller samples yielded adequate signals resulting in a better recovery of desired compounds. The PDMS/DVB fiber was chosen because it has been proven to be a suitable selection for extraction/adsorption of compounds and analytes of interest with good recovery. The addition of salt (NaCl) also aided in the extraction of
desired compound along with the addition of heat and agitation (Harmon, 2002; Zierler, et al., 2004).

According to Jeleń and others (2003), PDMS/DVB fiber gave highest efficiency taking length into account. The GC oven program was optimized to allow sufficient time for the compounds to be eluted from the column and detected. The retention times for geosmin and 2-MIB were 40.96 minutes and 26.66 minutes, respectively (Appendix). In preliminary studies performed, it was determined that an increase in the program runtime for the GC program aided in the elution of the compounds from the column.

GC analysis of product and ingredients indicated that geosmin and 2-MIB are the compounds associated with musty, off-flavor in processed beans. Although 2-MIB was detected, geosmin is the major contributor of the musty off-flavor and odor, with navy beans being the ingredient source. Future studies will focus on identification of the microorganisms responsible for the production of the two compounds.
V. References


PART FOUR: *Aspergillus flavus* AND *Penicillium* SPECIES
ASSOCIATED WITH MUSTY OFF-FLAVORS IN NAVY BEANS
Abstract

A study was conducted to identify the organisms responsible for production of geosmin and 2-methylisoborneol (2-MIB) in moldy navy beans. Ten grams of moldy navy beans (n=3) were placed directly onto the surface of actinomycetes isolation agar, potato dextrose agar, dichloran rose bengal agar, and Czapeck agar and incubated at 25°C for 3 to 5 days. Samples of non-moldy navy beans were also examined. Molds were isolated and identified by an expert mycologist. A gas chromatography method employing solid phase micro-extraction (SPME-GC-MS) was employed to confirm the presence of geosmin and 2-MIB.

Aspergillus flavus, Penicillium expansum, and Penicillium commune were isolated from moldy beans. Using SPME analysis, geosmin was detected in beans inoculated with the respective molds; 2-MIB was not detected. These data suggest that geosmin is the source of mustiness in raw navy beans. A. flavus was the dominant producer of geosmin over a 30 day period. However, both P. expansum, and P. commune also produced geosmin.
I. Introduction

White navy beans are often used alone or as an additional ingredient in food items such as salads, soups, and most famously, baked beans (USA, 2005). Because they are bland in flavor, the presence of foreign odors or flavors may result in objectionable off-flavors and consumer rejection (Buttery et al., 1976). Geosmin and 2-methylisoborneol (2-MIB) have been identified as the cause of musty, off-flavors and/or odors in baked bean products, with moldy navy beans being the source.

Off-flavors and odors, such as geosmin and 2-MIB, are microbial metabolites produced during food degradation or spoilage. Growth of off-flavor producing microorganisms is affected by the environment and chemical composition of the food they infect. Physical properties affecting growth include water activity, pH, atmospheric condition, and temperature under which food is stored (Whitfield, 1998).

Microorganisms capable of producing geosmin and 2-MIB include actinomycetes (Buttery et al., 1976; Schöller et al., 2002; Suguira et al., 1998; Zaitlin and Watson, 2006), cyanobacteria (Izaquirre and Taylor, 2004; Watson, 2003), and some species of filamentous fungi, including Aspergillus, Penicillium and Fusarium (Whitfield, 1998; Zaitlin and Watson, 2006).

Geosmin and 2-MIB have been identified as the causes of mustiness in moldy navy beans. However, the organism(s) responsible for their production has not been identified. Therefore, the objective of this study was to identify bacteria and/or fungi that are responsible for the production of geosmin and 2-MIB in navy beans.
II. Materials and Methods

A. Chemical, Fibers, and Samples

Geosmin (minimum 98% purity) and 2-methylisoborneol were obtained from Sigma. (-)-Menthone (90% purity) was purchased from Aldrich. Methanol was purchased from Fisher Scientific. Sodium chloride reagent (crystals; 99+% purity) was obtained from ACROS. Polydimethylsiloxane-divinylbenzene (PDMS/DVB) fibers were purchased from Supelco.

Five pound containers of navy beans (moldy and navy) were obtained from bean processor plant A. Samples were stored at -20°C until used.

B. Microbiology

1. Enumeration and Identification of Microorganisms

Ten grams of the respective bean samples (non-moldy navy beans and moldy navy beans) were placed directly onto triplicate plates of actinomycetes isolation agar (AIG), potato dextrose agar (PDA), dichloran rose-bengal chloramphenicol agar (DRBC), and Czapeck agar. Media were prepared according to the manufacturer’s instructions.

Mold samples were collected from media and submitted to a private laboratory for identification by an expert mycologist; molds were identified to the species level. Pure cultures of the isolated molds were transferred to DRBC plates and incubated at 25°C for 3-5 days.
2. Monitoring of Geosmin and 2-MIB Production by Isolated Organisms

Pure cultures of the isolated organisms were used. Fifteen ml of sterile water was added to the pure culture, and the plates were gently agitated to loosen spores. From the plate suspension, 1 ml was transferred into 9 ml of sterile water. Serial dilutions were made and surface plated (0.1ml) onto DRBC agar to determine the initial spore concentration. Plates were incubated at 25°C for 5 days.

An additional 10 ml of the plate spore suspension was added to 300 grams (n=3) of non-moldy navy beans in a sterile bag, which were mixed thoroughly. The 300-g composite sample was divided aseptically into 30-g aliquots, which were placed into sterile Petri dishes and incubated at 25°C for 10 days; during incubation, beans were kept moist by regular addition of sterile water. Samples were analyzed every two days for the presence of mold and production of geosmin and 2-MIB. Mold counts were obtained by adding 5 g of beans to 10 ml sterile water, blending in a stomacher, and surface plating serially diluted samples onto DRBC. DRBC plates were incubated at 25°C for 5 days before counting mold colonies.

C. Solid Phase Micro-extraction

Whole beans were finely ground with a Black & Decker® Smart Grinder. One gram of beans were combined into a 10 ml headspace vial with 1.5 grams of NaCl, 3 ml of water, and 20ul of (-)-menthone. Three samples were prepared for each isolated mold.

To determine the relative recovery of geosmin and 2-MIB, triplicates subsamples of the same sample were prepared in the manner described above, but with
the addition of 40 μl of geosmin and 2-MIB, respectively, to yield concentrations of about 235ng/kg.

The SPME extraction process was achieved using an automated sampling system (Combi-PAL) from Leap Technologies. For the SPME extraction process, samples were heated for 15 min at 65°C. The needle holder was then exposed to the headspace for 20 minutes at 65°C. After adsorption, the fiber was withdrawn from the vial and thermally desorbed into the gas chromatograph for 2 minutes. A polydimethylsiloxane-divinylbenzene (PDMS/DVB) fiber was used.

D. Gas Chromatography/Mass Spectrometry

GC-MS analysis was accomplished using an Agilent 6890N ChemStation. The temperature program was as follows: the inlets were set at 260°C; oven, 35°C, hold 2 min, increase at 5°C/min to 280°C and hold for 5 min. The compounds were resolved on an HP-5MS column (30-m x 0.25 mm i.d. x with 0.25 μm film). The program runtime was 72.5 minutes. The detector (Agilent 5973 MSD) was set either in full scan (40 to 340 m/z) and/or selective ion mode (SIM). The following ions were chosen: MIB-95 m/z; GSM-112 m/z; (-)-menthone 112 m/z.

For qualitative screening of new samples where the composition was unknown, the MS detector was used. Otherwise, a selective detector such as flame ionization (FID), was employed to enhance sensitivity for detecting certain groups of compounds even in the presence of other compounds of higher concentrations (Miller, 2005). The parameters used are standard for GC-MS analysis.
III. Results

*A. flavus, P. commune* and *P. expansum* molds were isolated from moldy navy beans. The molds were identified to the species level by an expert mycologist. No other organisms were isolated from contaminated beans.

Geosmin was produced in samples by *A. flavus, P. commune,* and *P. expansum.* The initial population of *A. flavus* was about 7.2 log CFU/g (Figure 4.1). After an initial decline in population, *A. flavus* populations increased to about 9.2 log CFU/g after 9 days of incubation. As predicted, on day 0, geosmin was not detected in navy beans. Geosmin was detected on day 3, and the concentration increased throughout the 9-day incubation as mold growth increased to a final concentration of 216 ng/kg. Geosmin was also produced in navy beans inoculated with *P. commune* at an initial concentration of about 7 log CFU/g of beans (Figure 4.2), although at level substantially lower than that produced by *A. flavus.* Geosmin concentration increased during the 9-day incubation to a final level of 144 ng/kg. Interestingly, *P. commune* populations initially declined to about 5 log CFU/g and did not increase throughout incubation.

*P. expansum* was the slowest growing mold out of the three isolated with an initial population of about 4 log CFU/g (Figure 4.3). Geosmin was produced, but at concentrations well below those produced by *A. flavus* and *P. commune.* After 9 days of incubation, the maximum geosmin concentration was 19.5 ng/kg.
Figure 4.1. Mean concentration of geosmin produced by *A. flavus* in navy bean samples (n=3), and population of *A. flavus* enumerated in these samples.

Figure 4.2. Mean concentration of geosmin produced by *P. commune* in navy bean samples (n=3), and population of *P. commune* enumerated in these samples.
IV. Discussion and Conclusions

*Aspergillus flavus*, *P. commune*, and *P. expansum* were isolated from moldy navy beans. Using SPME-GC-MS analysis, geosmin was detected in beans inoculated with the respective molds. However, 2-methylisoborneol (2-MIB) was not detected. Of the three molds isolated, *A. flavus* was dominant producer of the geosmin, producing the highest concentration of the compound over time. This may indicate that *A. flavus* is the major contributor the off-flavor and odor problem in navy beans.

Bacteria or fungi that produce off-flavors on dry bean surfaces may be present but lack the right temperature and moisture levels for growth. Therefore, it may not be necessary to test for causative agents (Buttery et al., 1976). This may also be the reason
why a producer of 2-MIB was not identified. The levels of 2-MIB detected in navy beans in the previous study performed may have been due to other microorganisms that were not isolated during microbiological testing.

Dry beans are not sterile and may naturally harbor bacteria or mold flora. The dominant species at any one time is determined by the original inoculum, chemical composition of the produce, and temperature conditions of storage (Mathews, 1992). If poor storage conditions are maintained for navy beans, spoilage organisms, such as the molds isolated in this study, can grow and contribute to the presence musty odor compounds such as geosmin. Poor air ventilation and fluctuating temperatures may enable opportunistic microorganisms such as molds to grow (Mathews, 1989; Graham and Ranalli, 1997; Maiti, 1997).

Growth of *A. flavus*, *P. commune*, and *P. expansum* is favored by warm dry soil conditions, and it can be particularly damaging in the warm arid regions of the tropics (Pitt and Hocking, 1997). *A. flavus* grows well between 17 and 42°C and in the declining soil moisture regime, providing relative humidity is maintained between 85 and 95%. Optimum temperature for aflatoxin production is 25-35°C. *Aspergillus* species such as *A. niger* and *A. flavus* are tolerant of mercury, low soil moisture and high temperature (Pitt and Hocking, 1997; Hocking, 2001). *Penicillium* species are present in soils and on surfaces of decayed fruits or vegetables. Most can grow at a temperature range from 0-32°C, but grow best at about 21°C. Each genus varies somewhat in temperature requirements, but all grow better when air, plant tissues, or seeds are damp (Pitt and Hocking, 1997).
The production of geosmin in stored navy beans by the isolated molds (or any microorganism for that manner) is dependent on water content or water availability of the crop, aeration, initial inoculum of organisms, and temperature (Mathews, 1992; Jeleń and Wasowicz, 1998). When the right conditions are met, synthesis of flavor and aroma compounds may occur. This study demonstrates that common soil fungi are capable of growing on dried navy beans when optimal storage conditions are not met. This growth can lead to production of the musty compound, geosmin, which results in products of unacceptable quality.
V. References


Summary

A major benefit of this project to the bean industry is that the source of musty off-flavors and/or odors was identified. The issue of musty product has been an on-going problem for the bean industry. Moldy navy beans have been implicated in the past, but were never confirmed scientifically, as the cause of the musty off-flavor and odor problem. From this study, the identity of the musty odor source was confirmed using instrumental methods employing solid phase microextraction with gas chromatography. Fungal producers of the compounds were also isolated from the beans.

These research findings should help to raise awareness of the problems associated with moldy navy beans. The presence of off-flavors and/or odors in raw and processed beans is considered to be a quality effect. Prompt removal of moldy navy beans from storage areas will reduce the occurrence of musty product, thereby increasing the quantity and quality of beans distributed. In return economic stability and consumer acceptance can be maintained.
Figure A.1. GC chromatogram of (-) menthone at a retention time of 13.53 minutes.

Figure A.2. GC chromatogram of 2-MIB at a retention time of 14.27 minutes.
Figure A.3: GC chromatogram of geosmin at retention time of 20.20 minutes.

Figure A.4. GC-MS chromatogram of geosmin in musty level 1 product at a retention time of 20.25.
Figure A.5: GC-MS chromatogram of musty level 1 product spiked with geosmin.

Figure A.6: GC-MS chromatogram of 2-MIB in musty level 3 product at a retention time of 14.27.
Figure A.7: GC-MS chromatogram of musty level 3 product spiked with 2-MIB.

Figure A.8. GC-MS chromatogram of geosmin in musty level 3 product at a retention time of 20.25.
Figure A.9. GC-MS chromatogram of musty level 3 product spiked with geosmin.

Figure A.10: GC-MS chromatograms of (-) menthone analyzed with modified GC program.
Figure A.11: GC-MS chromatogram of 2-MIB analyzed with modified GC program.

Figure A.12. GC-MS chromatogram of geosmin analyzed at a retention time of 40.96 with modified program.
Figure A.13. GC-MS chromatogram of 2-MIB in dry, navy beans at a retention time of 26.66 minutes.

Figure A.14. GC-MS chromatogram of 2-MIB in dry, navy beans at a retention time of 26.64 minutes.
Figure A.15. GC-MS chromatogram of geosmin in dry, moldy navy beans at a retention time of 40.96.

Figure A.16: GC-MS chromatogram of 2-MIB in moldy navy bean mixture at a retention time of 26.62 minutes.
Figure A.17: GC-MS chromatogram of geosmin in moldy navy bean mixture at a retention time of 40.95 minutes.
Vita

Coesha Ancoineet Fairley was born in Laurel, Mississippi on January 21, 1978 but was raised in the rural area of the small city of Collins, Mississippi. Coesha attended both the Hopewell Elementary and the Collins Elementary Schools. In addition, she attended the Collins Middle School and the Collins High School. Coesha graduated from high school in 1996. From there, she began studies at the Jones County Community College, later transferring to Mississippi State University at the start of her junior year. Coesha received a B.S. in Microbiology in 2000 and an M.S. in Genetics in 2002. After graduating, she worked as a Quality Assurance Analyst for Boar’s Head Provisions from 2002-2004.

Coesha earned the doctorate degree in Food Science and Technology (Food Microbiology) at the University of Tennessee, Knoxville, in August, 2007. She is a member of the International Association of Food Protection and the Institute of Food Technologists.