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Title of Thesis: "Solid Phase Microextraction (SPME) as a Method to Detect the Presence of *Escherichia coli* in Water by Headspace Sampling and Gas Chromatography/Mass Spectrometry Analysis"

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## ABSTRACT

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# APPLICATION OF HEADSPACE SOLID PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY/MASS SPECTROMETRY FOR DETECTION OF *ESCHERICHIA COLI* IN WATER

BY

CPT TARA L. HALL

Thesis submitted to the Faculty of the Department of Preventive Medicine and Biometrics Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirement for the Degree of Master of Science in Public Health, 2002

# DEDICATION

To my husband, Frank, for the sacrifices you have made during my Army career and during the last two years of study. I dedicate this thesis to you and to our two wonderful children, Kurt and Shyanne, who are the light, joy and inspiration of my life.

To my mother Terry Glass, thank you for all your hard work and dedication to me throughout my life. You taught me the value of hard work and commitment, which has been paramount in completing this course of study.

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# Chapter 1
# Introduction

## Statement of the Problem

Safe water, in sufficient quantities, is essential to every human being. People around the world consume water in larger volumes than any other food or drink (1). Therefore, it comes as no surprise that the health risks associated with consumption of contaminated water are of great interest to public health professionals. An insufficient quantity or quality of water is not only debilitating to the individual, but for the military the lack of safe water can also have a major impact on unit operational readiness and the ability of the unit to accomplish its mission. When the military deploys around the world units are faced with the challenge of providing safe drinking water to their soldiers. Typically the theaters in which deployments occur have experienced significant destruction and public health infrastructure is in poor condition or is nonexistent. The shipment of potable water into the theater requires a large amount of scarce or nonexistent cargo space. Therefore, the military has developed the ability to produce potable water using the equipment available within the unit.

To determine potability, the military primarily uses the membrane filter method of detection for the presence of *Escherichia coli (E. coli)* as an indicator of fecal contamination. This traditional analytical method requires a significant amount of time to obtain results (typically 24 to 48 hours) (2). In today's rapid deployment scenarios, waiting 24 hours or more for information regarding the safety of water is too long. As a result, the membrane filter method has lost

some of its value because it does not provide timely results. The need exists for the military to rapidly detect the presence of fecal contamination in water.

According to *Standard Methods for the Examination of Water and Wastewater*, differentiation among the coliform group is considered of limited value in assessing drinking water quality because the presence of any coliform bacteria renders the water potentially unsatisfactory and unsafe (2). New methods have been developed which simultaneously detect coliforms, in general, and *E. coli*, specifically, in water (within 24 hours for Colilert and within 18 hours for Colilert-18) with sensitivities and specificities equivalent to or better than those of the standard multiple-tube fermentation method or the membrane filtration method (1). However, 18 to 24 hours is still a substantial amount of time considering that most methods to detect chemical contamination can be performed in several hours or less. Therefore, it seems reasonable to assume that by developing a chemical detection method to determine the presence of fecal coliforms, the time required for analysis could be lessened.

## Research Goal

This purpose of this study is to assist the U.S. military in identifying technologies which could be used for the rapid detection of microbial contamination in water as alternatives to the current standard methods. There is no current research being conducted exploring the use of solid phase microextraction (SPME) for this purpose. The results of this study will help to

determine the feasibility of pursuing SPME technology for the purpose of rapid detection of microbial contamination in potable water.

## Research Question and Specific Aims

**Research Question:** Can fecal contamination of drinking water be detected using SPME coupled with gas chromatograph/mass spectrometry (GC/MS) analysis?

**Specific Aims:**

1) Determine which metabolites of *Escherichia coli* (*E. coli*) can be detected by SPME when the organism is grown on agar.
2) Determine the instrument detection level (IDL), the lower level of detection (LLD), and the method detection level (MDL), as defined in *Standard Methods for the Examination of Water and Wastewater* (2), for the identification of E. coli using SPME.
3) Determine the statistical correlation of SPME with membrane filtration.

# Chapter 2
# Literature Review

## Organisms

Potable water, by definition, is water that does not contain disease-producing organisms, poisonous substances, chemical or biological agents, or radioactive contaminants at levels that can produce disease or injury (3). Water can be a carrier of many microscopic organisms, some of which can cause disease. An epidemic of one of these diseases among military members can be more devastating than enemy action and can cause great damage to morale as well as health (3). In addition to native-water bacteria, water sources may and often do contain a variety of bacteria as a result of contamination. Contamination can come from the air, soil, and human and animal excreta. Pathogens present in water typically originate from either the flesh of animals or persons who have died of infectious diseases or, more likely, the excreta of infected animals or persons (4). Bacteria are the major component of feces, comprising approximately 55% of solids (4). It has been estimated that one gram of large intestine contents contains about 150 times more bacteria than there are people on the planet at this time. These enteric bacteria play a key role in many processes in the large bowel including carbohydrate and protein fermentation, bile acid and steroid transformations, metabolism of xenobiotic substances, development of the immune system, as well as activation and destruction of potentially mutagenic metabolites (4).

One group of enteric bacteria, the coliform group, consists of several genera of closely related bacteria belonging to the family Enterobacteriaceae.

This group is defined as all facultative anaerobic (able to grow aerobically or anaerobically), Gram-negative, non-spore-forming, rod-shaped bacteria with simple growth requirements. These organisms are found mostly in the vertebrate intestine as normal flora or as pathogens. *Escherichia coli (E. coli)* are further defined as coliform bacteria that possess the enzyme ß-glucuronidase (2, 5, 6).

The presence of coliforms has been used to indicate fecal contamination of water samples for over 100 years (2, 6). As early as the 1900s, methods were developed to assess water quality by sampling for coliforms, with particular emphasis on *E. coli* (1). *E. coli* is the predominant facultative organism in feces, and its presence (along with that of other coliforms such as *Klebsiella*, *Enterobacter*, and *Citrobacter*) is used by public health departments as presumptive evidence for fecal water contamination (2, 5).

## Traditional Methods

Experience has established the significance of coliform group density as a criterion of the degree of pollution and thus the sanitary quality of the water. Consequently, the military also uses the presence of coliforms as the microbiological indicator for potability, with a standard of zero coliform forming units (cfu) per 100 milliliters (mL) of water (3).

There are two standard methods for the detection and enumeration of bacteria of the coliform group: the multiple tube fermentation method and the membrane filter method (2). The multiple-tube fermentation method reports results as a most probable number index. This index is based on probability

formulas and is an estimate of the mean density of coliforms in the sample; it is not an actual enumeration (2). By contrast, a direct plating method such as the use of a membrane filter, permits a direct count of viable coliform colonies (2). Both the multiple-tube fermentation assay and the membrane filter assay are based on lactose fermentation (2).

Multiple-tube fermentation is a 3-phase assay consisting of the presumptive, confirmed and completed phases. The presumptive phase tests for the production of an acidic reaction or gas within 48 hours in tubes containing the sample in lauryl tryptose broth. Tubes with positive presumptive reactions are then submitted to the confirmed phase where the bacterial growth is transferred to tubes containing brilliant green lactose bile broth. If gas and/or acidic growth are observed within 48 hours of incubation, the sample is considered positive and 10% of those samples are further submitted to the completed phase. In the completed phase, results are considered positive if there is gas formation in a second tube of lauryl tryptose broth that has been incubated an additional 48 hours. The completed phase also requires identification of gram-negative, nonspore-forming, rod-shaped bacteria from an agar culture incubated for 24 hours in order to confirm positive results (2).

The membrane filter technique is highly reproducible and yields quantitative results more rapidly than the multiple-tube fermentation method. This procedure calls for the sample to be filtered through a membrane filter and incubated on an Endo-type medium for 24 hours. All bacteria that produce a red colony with a metallic sheen within that time are considered members of the

coliform group (2). *E. coli* is detected with these same methods, using elevated temperatures, with different medium formulations, and a follow-on test for indole production (multiple-tube fermentation method) (1,2). These traditional methods for detecting coliforms and *E. coli*, however, require up to 96 hours and involve complicated procedures (2), thus making them difficult in a field setting and unappealing for military use.

## Current Research

The U.S. Army Center for Environmental Health Research at Fort Detrick, Maryland, is currently administering a number of contracts that are investigating various rapid detection technologies for bacterial contamination of water. Most of these efforts are directed toward the use of polymerase chain reaction or similar methods. None of the proposed techniques include the use of chemical detection methods to monitor bacterial metabolites (7).

## Solid Phase Microextraction

Solid-phase microextraction (SPME) is a simple, fast, and relatively inexpensive sample extraction technique that can achieve trace level detection of organic compounds when coupled to an analysis method such as gas chromatography. It was first fielded to sample volatile compounds in the environment, with its use now extended to the sampling of a variety of matrices (gas, liquid, and solid) and to a wide range of analytes from volatile to nonvolatile compounds (8, 9, 10). SPME can be used in instances where purge and trap, liquid-liquid, and solid-phase extraction methods would be appropriate (10). It is

a field-friendly technology capable of analysis outside of the laboratory, making it potentially useful for monitoring the presence of organic water contaminants (8).

SPME extracts and concentrates organic compounds from gas, liquid or solid matrices without the use or expense of solvents (8, 11). It consists of two steps. The first step involves the partitioning (movement) of target analytes between the sample matrix and the SPME fiber coating (12, 13, 14). In the second step, when conducting GC analysis, the fiber bearing the concentrated analytes is transferred to the heated injection port and the analytes are transferred onto the head of a capillary column for subsequent separation, detection and quantitation (8, 9, 13).

A brief description of the SPME sampling equipment and procedures follows. The SPME sampler consists of a fused silica fiber, coated with a suitable absorbent or adsorbent material that is bound to the tip of a syringe plunger. The fiber can be retracted into the hollow needle of the attached syringe by a plunger assembly (Figure 2-1). The needle serves to protect the delicate fiber and is used to penetrate the septum of the sample vial. In the extraction step, the fiber is lowered from the needle into the sample by depressing the plunger. The fiber can be directly immersed into the liquid sample or placed in the headspace above. Analyte molecules are absorbed or adsorbed onto the


**Figure 2-1**
**Solid Phase Microextraction Fiber**

coating, depending on the type of fiber coating material used (8). These molecules are partitioned between the sample and the fiber coating until equilibrium is reached. Agitation is normally used to achieve faster equilibration because it enhances the diffusion of analytes toward the fiber (9). On completion of sampling, the plunger is withdrawn to retract the fiber into the needle, and the syringe needle is removed from the vial through the septum (9, 10).

A time-profile curve may be constructed, which shows the dependence of the amount of the analyte extracted as a function of time. This profile is obtained by preparing a set of vials containing a known amount of a standard and then extracting them for progressively longer periods of time (13). The equilibration time is then defined as the time after which the amount of extracted analyte remains constant and corresponds within experimental error to the amount extracted at an infinite extraction time. The extraction time must be very well controlled to ensure good reproducibility (9).

While this study used the headspace extraction mode, there are two other modes of SPME that could have been considered: direct extraction and membrane-protected SPME (13). In direct extraction, the coated fiber is directly immersed into the sample and the analytes diffuse from the sample matrix to the fiber coating. In this case, the fiber coating can be damaged from high-molecular-mass interferences such as proteins or humic matter, which are often present in surface raw water samples. In the membrane-protected SPME, which is used for the extraction of analytes in very polluted samples, the coating is protected from damage by a semi-permeable sheath that surrounds the fiber

coating allowing only small molecules to penetrate. In contrast, in the headspace mode, the analytes diffuse to the fiber through the headspace, i.e. the air present above a solid or liquid sample (9, 13).

The efficiency of the extraction process is dependent on the distribution constant. The distribution constant describes how an analyte will partition by quantifying its affinity for the sample matrix versus the fiber coating (13). Coating volume also determines method sensitivity (the ability of the fiber to detect low levels of analyte) and thicker coatings result in longer extraction times to attain equilibrium. Therefore, there is a trade-off between sensitivity and time (8, 13). If sensitivity is the prime consideration, longer extraction times will produce optimal results. If time is a consideration, shorter time periods for extraction should be chosen. Typically, the chemical nature of the target analyte determines the type of coating to be used. The general rule for selection of the proper fiber coating is “similar attracts similar” (13). Selectivity is based primarily on the polarity and volatility of the target analytes.

***Coatings:*** Polydimethylsiloxane (PDMS) coatings are the most commonly used and the most versatile of the available coatings. They are relatively rugged, liquid-like, nonpolar coatings that sample by absorption. Absorption is a non-competitive process where, at equilibrium, the amount of analyte extracted is dependent on 1) the distribution constants of the sample to the air and then from the air to the fiber, 2) the volumes of the fiber coating and sample, and 3) the initial concentration of the analyte in the sample (14). PDMS coatings are typically used to extract nonpolar analytes, but can also extract

more polar analytes once extraction conditions have been optimized (13). Polyacrylate (PA) coatings are absorptive, liquid, polar coatings that are generally more suitable for polar analytes. The distribution constants for PA are typically lower than for PDMS, resulting in longer extraction times (13).

There are also mixed-phase, solid coatings that use the principle of *ad*sorption instead of *ab*sorption (Figure 2-2). During the process of *ad*sorption, the analyte molecules are trapped by the active sites on the surface of the fiber instead of being diffused into the coating as occurs in *ab*sorption. The number of

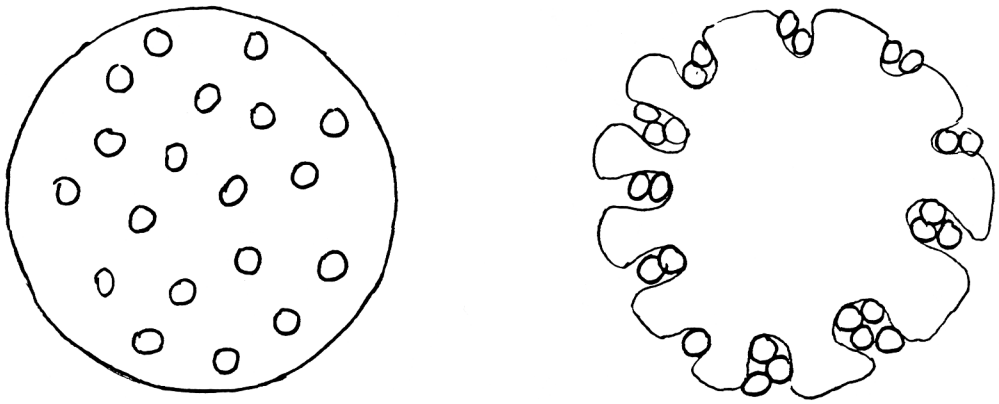


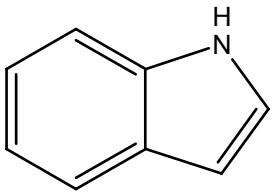

**Figure 2-2**
**Absorption vs. Adsorption**

active sites available on a coating is limited and, therefore, so is the number of analyte molecules that can be adsorbed. Adsorption is a competitive process in which molecules that have a higher affinity for the fiber coating can replace analyte molecules and reduce the amount extracted (14). Extraction times are typically shorter for these fiber coatings. Unfortunately, for quantitation they offer a smaller linear range (the range of analyte concentrations over which the amount recovered maintains a linear relationship with the amount present) in which the analyte can be accurately quantified (13).

Optimization studies are used to determine which fiber coating is best suited for a given analyte. Fiber optimization is performed by sampling a known concentration of a standard, extracting with different fibers for a given amount of time, and then comparing the results of the amount extracted by each (13).

## Why SPME?

SPME has been used extensively in the identification of chemicals in food, water, air and soil, and it has had limited use in the area of bacterial detection in those media (8, 9, 10, 11, 13). It is known that bacteria undergo many different biochemical reactions during growth and metabolism and that many of these reactions produce volatile or semivolatile chemicals. Therefore, it is rational to assume that the detection of these chemicals would be achievable with SPME sampling. Indole was thought to be a good candidate to allow for detection of coliform water contamination through the use of SPME sampling. Indole is semi-volatile, water soluble, and is detectable by its odor at higher concentrations (15). Table 1 shows the chemical characteristics of indole which are important in deriving distribution constants and predicting analyte behavior in the studied system (16).




Indole

| | |
|---|---|
| Indole | $C_8H_7N$ |
| Molecular Weight | 117.15 |
| Boiling Point | 253$^{o}$ C |
| Melting Point | 52$^{o}$ C |
| Density | 1.22 g/cu m |
| pKa | -2.4 basic |
| Log Kow | 2.14 |
| Solubility in Water | 3560 mg/L |
| Vapor Pressure | 0.0122 mm Hg |
| Henry's Law Estimate | 5.3 x $10^{-7}$ atm-cu m/mol |

Among the *Enterobacteriaceae*, indole is produced by *E. coli* and certain members of the Subfamily *Proteeae*, such as *Proteus vulgaris*, *Providencia* sp., and *Morganella* sp (5, 17, 18). It is formed from the metabolism of tryptophan by the tryptophanase enzyme (17). Indole production is a common diagnostic marker for identification of *E. coli* (5, 17, 18, 19, 20) and it has been shown that 99% of all strains will produce indole (18).

# Chapter 3
# Materials and Methods

## Overview

This study was designed in three phases, which correspond with the three specific aims established in Chapter 1. Phase I was conducted to determine which metabolites of *Escherichia coli* (*E. coli*) can be detected by solid phase microextraction (SPME) when the organism is grown on agar. Phase II was performed to determine the instrument detection level (IDL), the lower level of detection (LLD), and the method detection level (MDL), as defined in *Standard Methods* (2), for the identification of *E. coli* using SPME. Phase III used the data collected from phase II to determine the statistical agreement of SPME with membrane filtration.

## Materials

*E. coli* strain DH5α was obtained from Gibco BRL (Rockville, MD). Indole standard and granular sodium chloride (NaCl) were purchased from Aldrich (Milwaukee, WI). The indole was dissolved in methanol for all analyses of standard material. Luria bertani (LB) broth was purchased from DIFCO Laboratories (Detroit, MI). M-endo broth, petri dishes (47 mm), and cellulose ester filters (45 mm) were purchased from Millipore Corporation (Bedford, MA).

All SPME fibers and holders used in this study were obtained from Supelco (Bellefonte, PA). The following fiber coatings were utilized (film thickness as indicated): polydimethylsiloxane (PDMS, 100 µm), polyacrylate (PA, 85 µm), carbowax/divinylbenzene (CW/DVB, 65 µm), carboxen/

polydimethylsiloxane (CAR/PDMS, 65 µm), and polydimethylsiloxane/ divinylbenzene (PDMS/DVB, 65 µm). Prior to use, each fiber was conditioned following the manufacturer's recommendations.

### Gas Chromatograph/Mass Spectrometry (GC/MS) Methods

The SPME samples were analyzed immediately after collection using a field portable Viking Spectra Trak 573 (GC/MS) system (Figure 3-1). The MS section of this instrument is based on an Agilent Technologies 5973 ion source and monolithic quadrupole mass filter. The injection port as used for SPME samples


**Figure 3-1**
**Viking GC/MS**

was equipped with a deactivated injection port liner designed for thermal desorption of analytes from a SPME fiber (0.75 mm I.D., Supelco). The GC was fitted with a 30 m x 0.250 mm I.D. DB-1 column (x 0.25 µm film thickness, J&W Scientific). The carrier gas was helium with an initial velocity of 35 cm/s. The

injection port and injector transfer line were maintained at 250 °C throughout the analysis.

For Phase I, two temperature programs were used to increase the probability of detecting the most metabolites. In the first, the GC oven temperature began at 35 $^{o}$C for one minute and then increased at a rate of 15 $^{o}$C/minute to 270 $^{o}$C. The program ran splitless for the first 0.8 minutes. In the second program, the GC oven temperature began at 50 $^{o}$C for one minute and then increased at a rate of 10 $^{o}$C/minute to 260 $^{o}$C. The program ran splitless the entire run. For phases II and III, the GC oven temperature began and was held at 40 °C for 2 min, and then increased at 20 °C/min to 180 °C and then increased at 30 $^{o}$C/min to 280 $^{o}$C. These analyses were performed in splitless injection mode, with split flow (30 mL/min) started at 2.0 min. The MS transfer line was maintained at 290 °C. Electron ionization (EI, 70 eV) was used and mass spectra were collected over the range 35-350 *m/z* operating with quadrupole and ion source temperatures of 106 and 230 °C respectively. For selected ion monitoring (SIM) analyses, 117 and 90 *m/z* ions were used.

**Phase I**

In the preliminary stages of the research, the goal was to resolve or separate chromatogram peaks and identify the analytes present using a mass spectrometer (MS), the National Institutes of Science and Technology (NIST) mass spectral library (21), and chemical standards. The headspace above cultures of *E. coli,* K12 strain, grown on LB agar in standard petri dishes was

sampled. The Uniformed Services University of the Health Sciences (USUHS) Microbiology department provided these sample organisms. During this phase, sampling involved the use of both 85 µm PA and 100 µm PDMS fiber coatings for 15 minutes at two extraction temperatures (37 $^{o}$C and 50$^{o}$C) in order to maximize the probability that most volatile metabolites were identified. Blank samples were run simultaneously on empty agar plates, on agar plates with media, and on the non-exposed fibers in order to identify background analytes. Once the resulting GC/MS peaks were presumptively identified and verified as being consistent with known microbiological pathways, standards were run to confirm each metabolite identified.

## Phase II

### *Quantitative Analysis of Indole in Solvent by Direct Injection GC/MS.*

Once it was shown that certain metabolites were present and were detectable in the gas phase headspace above the viable organism by SPME, phase II focused on determining the different levels of detection, as defined in *Standard Methods* (2), for the SPME method in identifying *E. coli* in broth. First the instrument detection level (IDL) for the metabolites identified was determined. The IDL is defined as "the constituent concentration that produces a signal greater than five times the signal/noise ratio of the instrument" (2). In order to estimate the mass of indole loaded onto a SPME fiber, splitless injection analyses of indole in solvent were completed to obtain a curve with mass of analyte injected plotted against SIM GC/MS peak area for indole. Five samples were analyzed at indole concentrations ranging from 9.5 pg to 9.5 µg. The same instrument and

conditions as for SPME samples were used, except a split/splitless injection port liner (Supelco) was used in place of the narrow bore liner used for SPME samples. Sample introduction was with a 10 µL syringe (Hamilton, NV).

***Initial Fiber Selection.*** SPME fiber selection from among those tested was accomplished by obtaining duplicate samples taken in the headspace of 15 mL vials having screw top closures fitted with polytetrafluoroethane (PTFE)-lined silicone septa and containing 3 ml LB broth. The vials were spiked with indole (1.9 mg/mL in methanol) by piercing the PTFE-lined silicone septum with a 10 µL syringe (Hamilton, Reno, NV) and injecting 5.0 µL of the solution into each vial. A solvent chase method was used in which 1 µL of methanol was drawn into the syringe, followed by 0.5 µL of air, and then the measured aliquot of the indole solution. The samples were allowed to equilibrate for 15 minutes at 45 $^{o}$C before the SPME fiber assembly outer sheath pierced the vial septum. The temperature, 45 $^{o}$C, was chosen to reflect a temperature that was elevated, but would still allow for growth of *E. coli*. Immediately following this equilibration period, sampling began by lowering the SPME sampling fiber through the outer needle into the headspace of the vial. The vial was maintained at 45 $^{o}$C throughout the sampling period. The vial temperature was maintained by placing the vial to be sampled in a digitally controlled hot-block heater (Barnstead/Thermolyne, Dubuque, IA)(Figure 3-2).

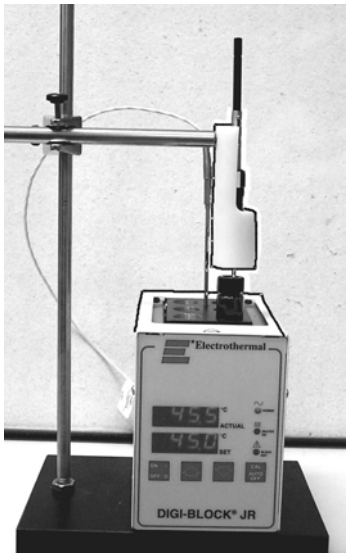



**Figure 3-2**
**Sampling Set-Up**

After a 30 minute extraction (sampling) period, the SPME fiber was retracted back into the needle and was then removed from the vial and immediately introduced into the heated GC injection port. The sampling fiber was then lowered into the midrange region of the heated injection port liner and GC/MS analysis began. The fibers giving the highest GC/MS peak areas for the indole peak were selected for further review to determine the most optimum fiber. Experimental data resulting from the fiber selection were examined for differences between SIM GC/MS indole peak areas. The statistical test used was the one-way analysis of variance (ANOVA). As appropriate, this was followed by Tukey's *post hoc* (22) comparison method to evaluate the source of differences observed. Based on the results of this analysis and a literature review, the PA fiber coating was used in all subsequent sampling.

***Effect of Sampling Time.*** Another set of vials was spiked using the same method. Following the 15 minute equilibration period, the PA fiber was exposed at 45 $^{o}$C over an increasing sampling time period (5.0, 10.0, 30.0 60.0, and 120.0 minutes) to determine when equilibrium was reached, i.e. no additional net analyte loading occurred. Two replicate samples were collected at each sampling time.

Experimental data resulting from the sampling time analyses (uptake curve) were examined for differences between SIM GC/MS indole peak areas. The same statistical tests were used as in the preceding paragraph. Tukey's *post hoc* multiple comparison test has very strict criteria for signifance and may be too likely not to find a statistically significant difference between values. Therefore, a Student's T-test, which has looser criteria for significance, was performed to determine if the differences between the means of the 60 and 120 minute sampling times were statistically different from each other. It is understood, however, that the Student's T-test is inappropriate for making multiple comparisons.

***Broth Headspace SPME.*** Once the effect of SPME sampling time was studied, the optimal conditions for extracting indole from the headspace of LB broth were studied. First, duplicate vials were sampled using the PA fiber at 45$^{o}$C and at room temperature (25$^{o}$C) to determine the effect of temperature on extraction. Based on these analyses, further experiments were conducted at 45 $^{o}$C. Next, duplicate vials were sampled at the optimal temperature, 45$^{o}$C, either

using a 7 mm stir bar and magnetic stirring or static conditions to determine the effect of stirring on extraction. Based on the results of these analyses, further experiments were conducted without stirring. Finally, duplicate vials were sampled at 45$^{o}$C with 3 mL of saturated salt water (distilled/deionized water saturated with NaCl) or with no salt, to determine the effect of salting on extraction. Student T-tests were completed on all three data sets for comparison.

***Broth Uptake.*** To study the effect of sampling time on the system that was to be used for the remainder of the experiments, spiked broth sample replicates (n=2, 1.9 mg/ml indole in 3 mL LB broth) were collected using a PA fiber coating at 45$^{o}$C with 3 mL of NaCl saturated water added as per above, at SPME extraction times of 5.0, 10.0, 20.0, 60.0, and 120.0 minutes. The extraction time analyses were examined for differences between SIM GC/MS indole peak areas using the statistical tests used in previous experiments. Again, recognizing that Tukey's multiple comparison may be less likely to find a statistically significant difference, a Student's T-test was performed to determine if the differences between the means of the 60 and 120 minute sampling times were statistically different from each other.

***Lower Level of Detection.*** Once the method was optimized (among the parameters studied), the lower level of detection (LLD) was determined. The LLD is defined as the constituent concentration of indole produced from *E. coli*

that produces a signal sufficiently large that 99 percent of the trials with that amount will produce a detectable GC/MS SIM signal for indole, that is two standard deviations above the mean of the blank analyses (2). Because the amount of indole at low levels of contamination is more influenced by the incubation time than the level of contamination, the LLD looked to define the incubation period required for a sample containing 1 coliform forming unit (cfu) after which enough indole was produced to meet the quantitative requirements described above. This was accomplished by starting with a sample of *E. coli* that had been incubated for 24 hours, approximately 2 x $10^9$ coliform forming units per milliliter (cfu/ml). That sample underwent eight ten-fold dilutions in sterile, distilled water. From those dilutions two 100 ul aliquots from $10^{-6}$, $10^{-7}$, and $10^{-8}$ dilutions were plated on LB agar and incubated 24 hours to enumerate the colonies that were present in the dilution. The average of the two plates from each dilution was used as the approximate number of original colonies for comparison with the samples that were prepared for SPME analysis. A second 100 ul aliquot from the same dilutions was added to 15 ml vials with septa containing 3 ml of LB broth. These samples were incubated and sampled at varying times to determine the shortest incubation time required to produce the metabolite in the lowest detectable concentration, the LLD.

***Method Detection Level.*** Finally, the method detection level (MDL) was determined. The method detection level is defined as the constituent concentration of indole produced from *E. coli* that, when processed through the

complete method, produces a signal with a 99 percent probability that it is different from the blank. For seven replicates of the sample, the mean must be 3.14*s* above the blank where *s* is the standard deviation of the seven replicates (2). To prepare the dilution aliquots of *E.* coli to be used, an overnight culture of *E. coli* DH5α in 3 mL LB broth underwent eight ten-fold dilutions in sterile, distilled water. From those dilutions 100 µL aliquots from $10^{-6}$, $10^{-7}$, and $10^{-8}$ dilutions were plated on LB agar and incubated 24 hours to enumerate the colonies that were present in the dilution. Once optimal extraction parameters and equilibration were identified (from among those studied), forty 200 mL samples of distilled, deionized, sterile water were randomly inoculated with aliquots of varying amounts of *E. coli* (1-10 cfu/100 mL, 11-100 cfu/100 mL, 101-1000 cfu/100 mL or 0 cfu/100 mL). This was a randomized, block design and inoculation was performed by an individual other than the person performing the SPME sampling and GC/MS analyses (the analyst). The sample identities were not known to the analyst. Although the definition established in *Standard Methods* required only seven replicates, ten replicates were made at each of the four specified contamination levels. Ten replicates were chosen because the data collected for the method detection level was subsequently used to complete phase III of the study.

These samples were split after inoculation (two 100 mL samples) and filtered through a cellulose ester filter using a Millipore filter assembly, Figure 3-3. One of the filters was placed aseptically in a 15 mL vial, as described previously, containing 3 mL LB broth. The other filter was placed aseptically in a 47 mm

diameter petri dish containing 2 mL M-endo agar. M-Endo agar is used by the Environmental Protection Agency to detect and enumerate total coliforms in water (2, 23). The membrane filtration samples were incubated for 24 hours at 37 $^{o}$C and then the analyst enumerated colonies. The samples containing the 3 mL of LB broth were incubated for 14 hours at 37 $^{o}$C and then sampled by headspace SPME under the extraction parameters identified as optimal (from among those studied) and analyzed with GC/MS. The 14 hour incubation time used versus the 12 hour incubation time was selected because of logistical constraints related to lab access and travel.

The data were then stratified by contamination categories (1-10 cfu, 11-100 cfu, 101-1000 cfu and blank) based on the number of colonies recovered using the membrane filtration technique. The mean of the replicates was compared to 3.14 times the standard deviation added to the mean of the blank. If the mean of the replicates was above this number, that contamination level was determined to be the MDL.

**Phase III**

The purpose of this last phase was to compare the reliability of this method with the established/traditional membrane filtration method discussed earlier. Data collected during the determination of the method detection level were used to accomplish phase III. The results of the two methods, SPME and membrane filtration were compared. To determine the sample size required for this phase of the study, the total sample size required when using the correlation

coefficient table was used (22). According to this table, at least 29 samples were required.

# Chapter 4
# Results and Discussion

## Phase I

*E. coli* is known to produce indole as a product during metabolism (5, 17, 18). This compound was the only metabolite consistently identified using SPME sampling. Although other metabolites were undoubtedly produced, none were unequivocally detected using this method. Indole was detectable using both fiber coatings, polyacrylate (PA) and polydimethylsiloxane, at both extraction temperatures studied. A gas chromatograph/mass spectrometer (GC/MS) chromatogram is shown in Figure 4-1. Initial identification of indole was made by conducting a mass spectrum search and library match. The analysis performed on the authentic indole standard confirmed the identification by matching retention time and mass spectrum.

## Phase II

### *Quantitative Analysis of Indole in Solvent by Direct Injection GC/MS.*

The curve completed by the direct, on-column injection of indole is shown in Figure 4-2. The results indicate that the instrument maintains good linearity ($R^2$=0.985) between 9.5 ng to 9.5 µg. The samples that were below 9.5 ng were not within the instrument's linear range. The generation of this curve provided an external means to determine the mass of indole absorbed by the SPME fiber during the data collection by comparing indole peak area in an unknown sample to the resulting regression curve.

***Initial Fiber Selection.*** Table 2 shows the data obtained during fiber selection experiments. The results of the one-way analysis of variance (ANOVA) showed that differences existed between selected ion monitoring (SIM) GC/MS peak areas for indole that were fiber dependent ($F_{(4,5)}$= 273.222, P<0.001). Tukey's *post hoc* comparison showed that the PA and PDMS/DVB fiber coatings gave a statistically indistinguishable response under the conditions tested (P = 0.066), although it was nearing significance. The PA fiber also gave a statistically indistinguishable response with the CW/DVB fiber (P = 0.535). The two other fibers, PDMS and CAR/PDMS were statistically indistinguishable from each other (P = 1.00) but differed from the other fibers with significance (P < 0.001). Although the PA and the PDMS/DVB fiber coatings were statistically indistinguishable in their response, the PA fiber was chosen to perform further analyses based on a review of the literature and its use in sampling for indole and like substances (15).

***Effect of Sampling Time.*** The solid phase microextraction (SPME) uptake curve completed with the simple system using the PA fiber coating is presented in Figure 4-3. There was a rapid increase in the SIM GC/MS area response with increasing sampling time up to 60 minutes where the response leveled out. Equilibrium was statistically established at 60 minutes. The results of the ANOVA test showed that differences existed between SIM GC/MS peak areas for indole ($F_{(4,5)}$=53.162, P<0.001). Tukey's *post hoc* analysis showed that there was a statistically indistinguishable response between 60 and 120 minutes

(P = 0.203) and that there was a meaningful difference between 60 and 30 minutes (P = 0.041). Furthermore, the more powerful Student's T-test, which was performed to compare the means of the 60 and 120 minute analyses, also showed a statistically indistinguishable response (two-tailed, t= -3.187, P = 0.090).

***Broth Headspace Sampling.*** Table 3 shows the data obtained during temperature, salting and stirring experiments with the PA fiber coating. The results of the Student's T-tests showed that heating (45 $^{o}$C) was favorable over room temperature extraction (25 $^{o}$C) (two-tailed, t= -67.600, P <0.001); salting (3 mL NaCl saturated water) was favorable over not salting (two-tailed, t= 17.828, P = 0.003); and not stirring was favorable over stirring (two-tailed, t= -4.621, P = 0.043). The results of the stirring experiment were unexpected and I hypothesize that the stir bar was too small and did not provide consistent agitation throughout the vial; therefore, the indole was not driven into the headspace, but was simply driven to the area of the vial where agitation was not occurring.

***Broth Uptake.*** The SPME uptake curve completed with the broth system using the PA fiber coating is presented in Figure 4-4. The rapid uptake again occurs until approximately 60 minutes, where equilibrium was statistically determined. The results of the ANOVA test showed that differences existed between SIM GC/MS peak areas for indole ($F_{(4,5)}$=114.868, P<0.001). Tukey's *post hoc* analysis showed that there was no difference in response between 60

and 120 minutes (P = 0.07) and that there was a meaningful difference between 60 and 20 minutes (P = 0.005). Furthermore, a Student's T-test also showed a statistically indistinguishable response between the 60 and 120 minute sampling times (two-tailed, t= -3.218, P = 0.152).

***Lower Level of Detection.*** The purpose of this part of the study was to verify that the method was capable of meeting the EPA standard of detecting as low as 1 coliform forming unit (cfu)/100 mL of drinking water. Using the method established, it was shown that the mean of two samples containing as low as 1 cfu/100 mL water, incubated for 12 hours at 37 $^{o}$C was greater than two standard deviations above the mean of the blank analyses (see Table 4). Although, the lower level of detection was determined to be 1 cfu/100 mL after 12 hours of incubation, as noted previously, the remainder of the study was conducted using a 14 hour incubation time due to logistical considerations.

***Method Detection Level.*** Appendix 1 contains the data obtained from the 40 split samples of inoculated water. Of the 40 samples listed in Appendix 1 (Raw Data) 39 were analyzed in Table 5 after being stratified according to the number of colonies present on the membrane filter. One sample was rejected due to a positive SPME response in a known blank sample. I hypothesize that this was caused by contamination of the sample, and therefore, the results were not included in the analysis of the method detection level.

As described in the methods section, the method detection level is defined as requiring the mean of the samples to be 3.14 times the standard deviation above the mean of the blank analyses. Each of the dilutions from which aliquots were taken for inoculation was assumed to be a homogeneous mixture. This assumption becomes tenuous at the lower dilutions. Because of this relative uncertainty of the precise number of cfu's in the dilution aliquot inoculated into a sample, the results were stratified by orders of magnitude of contamination (1-10 cfu's, 11-100 cfu's, and 101-1000 cfu's). This became problematic in the determination of the method detection level due to the variance in the GC/MS area response that was seen at varying contamination levels. As a result, the method detection level for this study was determined to be 101-1000 cfu's. It was the only contamination level that met the statistical definition for method detection level established *Standard Methods* (2). However, it was shown that SPME gave positive results when membrane filtration detected a positive sample 90.9% of the time at the 11-100 cfu level and 54.5% of the time at the 1-10 cfu level.

## Phase III

Using the data from all 40 samples that were collected in the method detection level determination in Phase II, the results of the SPME analyses were compared to the membrane filtration analyses. Both Pearson correlation and Kappa statistical analyses were performed on the data. Traditionally, Pearson correlation would be used on a continuous data set that could be assumed to

have come from a population of normally distributed data (24). After reviewing this data set, however, it was not apparent that these data were normally distributed, and they could not be assumed to come from a population of normally distributed data. For this reason, the Kappa statistic was used to determine the agreement between the two methods in determining if a sample was contaminated with *E. coli*. The data were transformed into a binomial variable, positive or negative for contamination, and analyzed using SPSS (22). The resulting Kappa statistic of 0.571 showed that the results agreed 57.1% more frequently than what would have been expected by chance. This infers a high level of agreement ($P<0.001$). As stated, the Pearson correlation was also performed and it showed a high correlation between the two methods (Pearson's $R = 0.603$, $P<0.001$).

The overall ability of the SPME method to correctly identify a contaminated sample was 75.9%, and to correctly identify a non-contaminated sample was 90.9%. The overall ability of the membrane filtration method to correctly identify a contaminated sample was 97.5%, and 100% to correctly identify a non-contaminated sample. Stratification of the data provided a clearer picture of the value of this method at the different contamination levels. The ability of the SPME method to correctly identify a contaminated sample at the 1-10 cfu, 11-100 cfu and 101-100 cfu levels was 54.5%, 90.9% and 100%, respectively.

## Chapter 5
## Conclusions

Indole was sampled by solid phase microextraction (SPME) in a simple system, and as a metabolite produced by *Escherichia coli (E. coli),* with the analysis by selected ion monitoring (SIM) gas chromatography/mass spectrometry (GC/MS). On examination of commercially available SPME fiber coatings using a system without broth, polyacrylate (PA) and polydimethyl siloxane/divinyl benzene (PDMS/DVB) fiber coatings were shown to give similar results and gave larger indole SIM GC/MS indole peak areas compared to the other fibers tested. After a detailed review of the literature, the PA fiber coating was chosen to conduct further analyses. For headspace SPME sampling in a system containing broth, the addition of heat (45 $^{o}$C) and salt (3 mL NaCl saturated water) with sampling times of at least 60 minutes resulted in the best sensitivity.

The lowest instrument detection level in the GC/MS linear response range was determined to be 9.5 ng of indole. The lower level of detection was determined to be 1 coliform forming unit (cfu)/100 mL water after an incubation period of 12 hours. The method detection level was determined to be 101-1000 cfu/100 mL water, however, the SPME method correctly identified contaminated samples in the 11-100 cfu/100 mL level 90.9% of the time.

The overall agreement/correlation between the two methods was high and statistically significant. Upon stratification, the true value of the SPME method in detecting specified levels of contamination after 14 hours of incubation could be examined. The method was much better at correctly identifying contaminated

samples with higher levels of bacteria and became much less reliable as the number of cfu decreased. This was likely due to the error involved in the inoculation at the lower dilutions resulting from a non-homogeneous mixture. The use of a 14 hour incubation period instead of a 12 hour incubation period for the determination of the method detection level and the correlation could have resulted in results that were slightly more reliable than might have be achieved after only 12 hours of incubation. Therefore, even though the lower level of detection was determined to be 1 cfu/100 mL water after 12 hours of incubation, the results of the correlation/agreement cannot be generalized to include samples that are analyzed after only 12 hours of incubation.

## Limitations

There are several limitations of this study. One significant limitation is that it only evaluated one organism that is considered to be an indicator for fecal contamination of water. There are additional organisms that could be present in water that would make it nonpotable and unsafe for human consumption. The study of those organisms, specifically those that are not part of the coliform group, is an area that requires further research. It should be noted; however, that this is also a limitation of other methods for detecting fecal contamination in water.

Additionally, this study was limited because it was performed in an artificial environment with only one type of organism present. It did not account for the potential effects on the outcome by other bacteria that could have produced

metabolites that might have resulted in false positives or false negatives. This did not create problems with the internal validity of this study because no other organisms were studied; however, the generalizability of the results must be explored further.

Another potential limitation could be an inadequate lower level of detection. It is possible that colony counts may be able to identify the same numbers of organisms in slightly more time than SPME with less equipment and required expertise. Analyzing the samples after 14 hours of incubation may not provide enough time savings to justify the requirement for a GC/MS system. Other types of GC detectors are available which are cheaper and potentially more sensitive.

A final limitation noted in the conduct of this study was the relative uncertainty that existed with inoculation of samples from $10^{-7}$ and $10^{-8}$ dilutions. Because the dilutions cannot be assured to be a homogeneous mixture, the aliquots taken from these dilutions have a certain probability of containing zero cfu. The stratification of the data probably lessened the overall impact of this bias, but it still probably had an impact on the outcome, particularly at the 1-10 cfu level.

## Recommendations

Based on the results of this study, replacement of the membrane filter method for detecting coliforms with the SPME method is not recommended. Under the parameters optimized with this study, the method is neither sensitive

nor fast enough to be considered superior over existing methods. Although the method, as it was studied, is not adequate for implementation, further research into its development to continue to optimize other parameters is warranted. It may be possible to reduce the lag time by optimizing the growth conditions for *E. coli*. This might be accomplished by using more enriched growth media that is selective for *E. coli* and that contains more tryptophan. Additionally by using more selective temperatures and agitation, it might be possible to enhance the growth of *E. coli* while suppressing the growth of other organisms that could be competing for nutrition. By optimizing these parameters, indole may be produced faster thereby increasing the speed of the method. Additionally, there are other more sensitive detectors, such as the nitrogen-phosphorous detector that could lower the instrument level of detection, thus requiring less indole to be produced in order to detect the presence of *E. coli*.

**Figure 4-1**

Chromatogram of Headspace Sampling and GC/MS Analysis of Indole Produced by *E. coli*

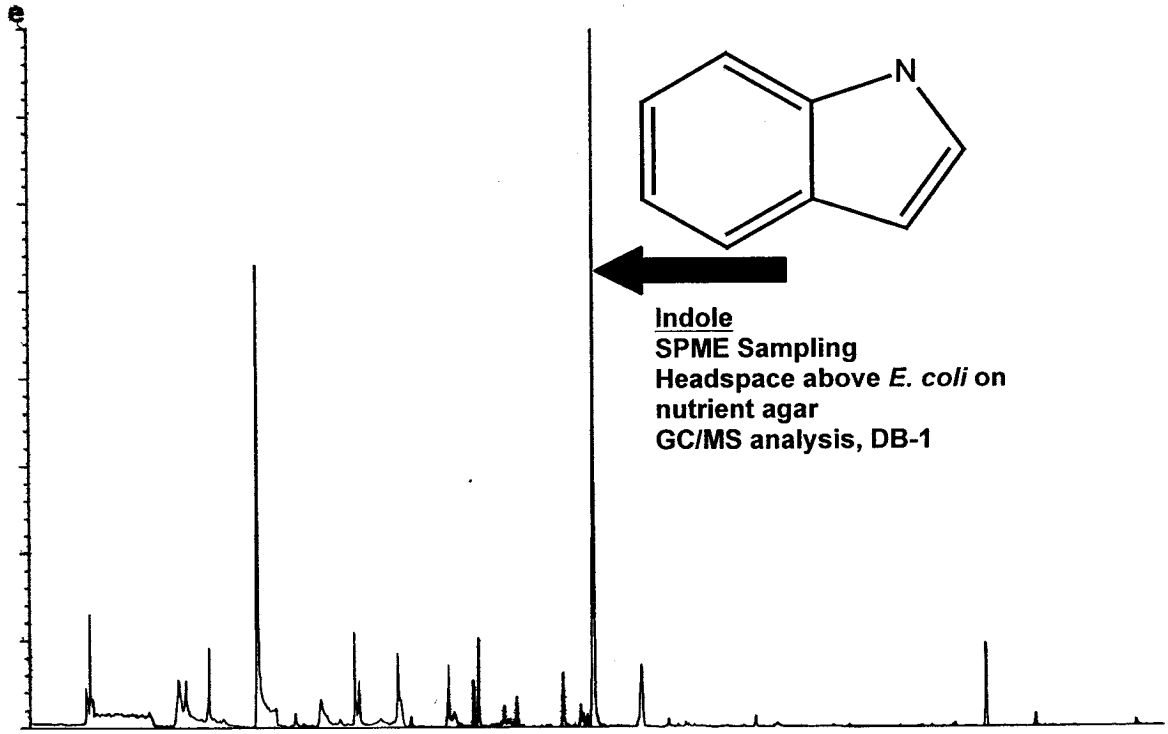
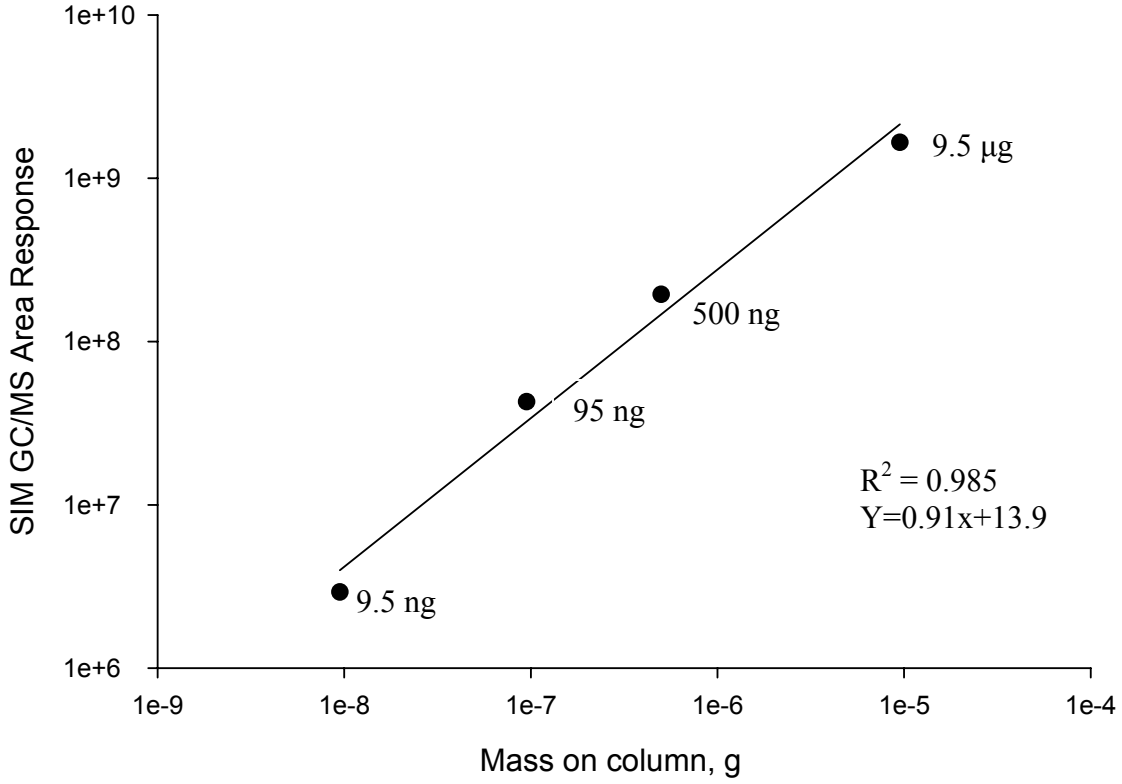


**Figure 4-2**

Quantitative Analysis of Indole: Direct Injection Onto GC Column




Raw Data

| Mass (ng) | Sample #1 | Sample #2 | Sample #3 |
|---|---|---|---|
| 9.5 | 1,435,893 | 4,634,374 | 2,694,110 |
| 95 | 47,536,608 | 50,187,147 | 45,844,698 |
| 500 | 183,434,635 | 188,519,680 | 211,313,334 |
| 9500 | 1,787,939,095 | 1,697,723,215 | 1,473,775,360 |

Samples reflect SIM GC/MS area count

## Table 2

Simple System Sampling: SPME Fiber Selection, SIM GC/MS Area Count for Indole for 30-minute Extraction at 45 ºC

| Fiber | Mean (x$10^8$) | Std Dev (x$10^6$) | Tukey's Multiple Comparison P values | | | | |
|---|---|---|---|---|---|---|---|
| | | | PDMS-DVB | PA | CW-DVB | PDMS | CAR-PDMS |
| PDMS-DVB | 1.69 | 8.49 | | 0.066 | 0.016 | <0.001 | <0.001 |
| PA | 1.4 | 1.74 | 0.066 | | 0.535 | <0.001 | <0.001 |
| CW-DVB | 1.49 | 7.29 | 0.016 | 0.535 | | <0.001 | <0.001 |
| PDMS | 0.38 | 4.32 | <0.001 | <0.001 | <0.001 | | 1 |
| CAR-PDMS | 0.39 | 0.17 | <0.001 | <0.001 | <0.001 | 1 | |

One-way ANOVA: $F_{(4,5)}$=273.222, P<0.001

Statistically indistinguishable (P>0.05)

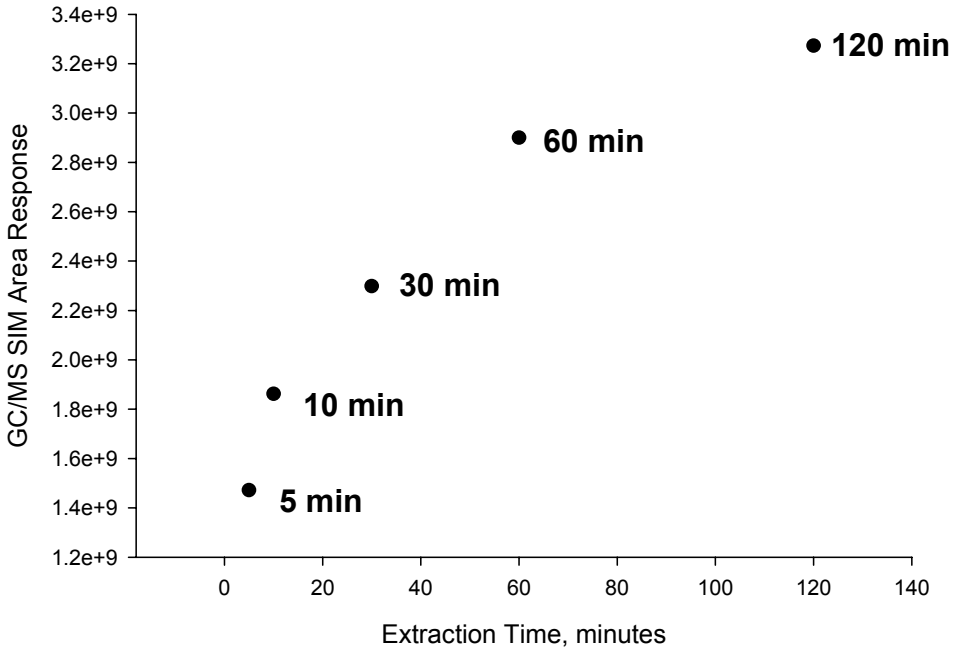
Raw Data

| Fiber | Sample #1 | Sample#2 |
|---|---|---|
| PDMS-DVB | 163,475,019 | 175,478,475 |
| PA | 154,431,269 | 144,114,218 |
| CW-DVB | 139,151,217 | 141,619,076 |
| PDMS | 41,489,552 | 35,385,702 |
| CAR-PDMS | 39,614,565 | 39,379,668 |

Samples reflect SIM GC/MS area count

**Figure 4-3**

Simple System Sampling: Optimal Extraction Time, SIM GC/MS Area Count for Indole at 45 $^{o}$C




*One-Way ANOVA: $F_{(4,5)}$=53.162, P<0.001
*60 min vs. 120 min: P=0.203
*30 min vs 60 min : P=0.041
*Significance determined at P<0.05

Raw Data

| Time (min) | Sample #1 | Sample #2 |
|---|---|---|
| 5 | 1,580,820,469 | 1,363,355,821 |
| 10 | 1,770,370,377 | 1,954,170,658 |
| 30 | 2,428,670,426 | 2,168,174,633 |
| 60 | 2,989,762,137 | 2,810,293,234 |
| 120 | 3,347,277,038 | 3,197,619,769 |

Samples reflect SIM GC/MS area count

**Table 3**

Broth Headspace Sampling: Effects of Salting, Stirring and Heating

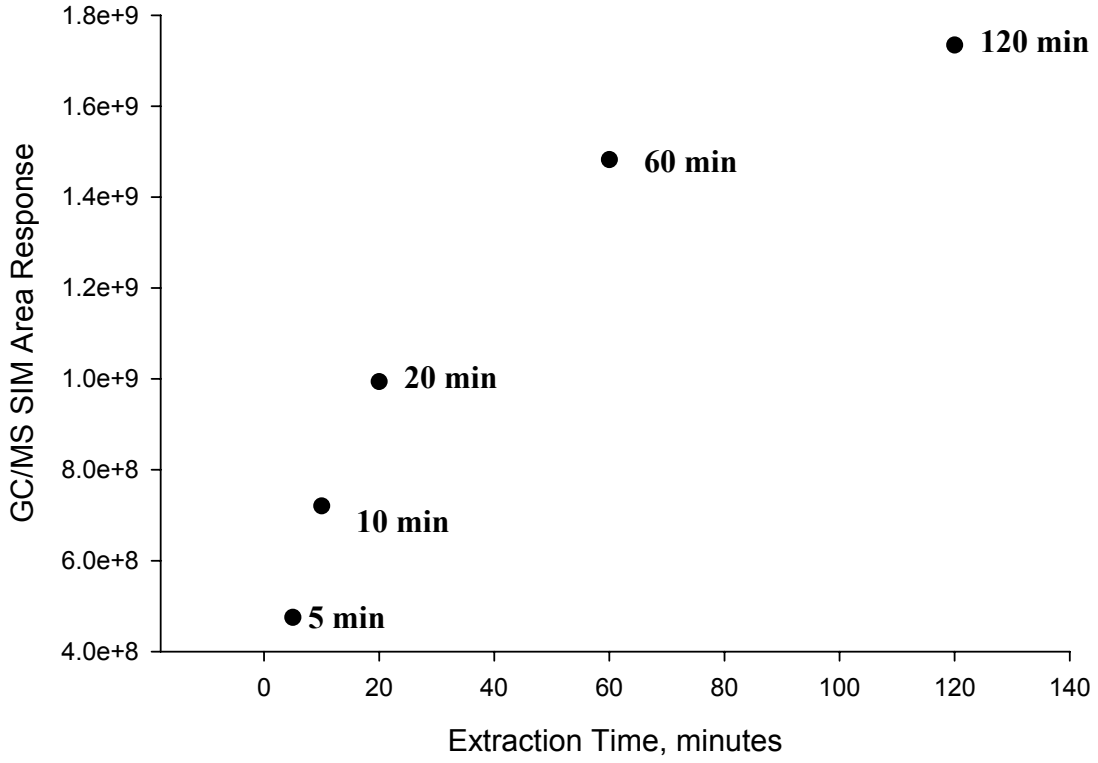
| Stir vs. No Stir | | |
|---|---|---|
| Time (min) | 20 | 20 |
| Stir | Yes | **NO** |
| Sample #1 | 53,246,511 | 272,145,503 |
| Sample #2 | 53,539,603 | 194,319,266 |
| **Mean ($x10^6$)** | **53.39** | **233.23** |
| Std Dev ($x10^6$) | 0.21 | 55.03 |
| Student's t (2-tailed) | t=-4.6215 | P=0.044 |

| Salt vs No Salt | | |
|---|---|---|
| Time (min) | 10 | 10 |
| Salt | **YES** | No |
| Sample #1 | 685,455,691 | 99,261,581 |
| Sample #2 | 755,211,781 | 97,251,625 |
| **Mean ($x10^6$)** | **720.33** | **98.25** |
| Std Dev ($x10^6$) | 49.32 | 1.42 |
| Student's t (2-tailed) | t=17.8284 | P=0.003 |

| Heat vs No Heat | | |
|---|---|---|
| Time (min) | 10 | 10 |
| Heat | **YES** | No |
| Sample #1 | 99,261,581 | 26,046,181 |
| Sample #2 | 97,251,625 | 25,289,739 |
| **Mean ($x10^6$)** | **98.25** | **25.66** |
| Std Dev ($x10^6$) | 1.42 | 0.53 |
| Student's t (2-tailed) | t=-67.6002 | P<0.001 |

**Figure 4-4**

Broth System Headspace SPME Uptake: Optimal Extraction Time, GC/MS SIM Area Count for Indole at 45 $^{o}$C with 3 mL NaCl Saturated Water




*One-Way ANOVA: $F_{(4,5)}$=114.868, P<0.001
*60 min vs. 120 min: P=0.07
*20 min vs 60 min : P=0.005
*Significance determined at P<0.05

Raw Data

| Time (min) | Sample #1 | Sample #2 |
|---|---|---|
| 5 | 510,574,683 | 439,196,844 |
| 10 | 685,455,691 | 755,211,781 |
| 20 | 1,050,869,730 | 936,388,945 |
| 60 | 1,555,986,163 | 1,408,175,216 |
| 120 | 1,759,931,042 | 1,708,488,768 |

Samples reflect SIM GC/MS area count

**Table 4**

Lower Level of Detection

| | |
|---|---|
| Average Original Colonies | Approximately 1cfu |
| Incubation Time, hours | 12 |
| Sample #1, SIM GC/MS area count | 846,092 |
| Sample #2, SIM GC/MS area count | 935,520 |
| **Mean** | **890,806** |
| Standard Deviation | 63,235.15 |
| LLD[a] | **640,897.79** |
| **Yes/No[c]** | **Yes** |

[a] LLD = (2 x Std Dev) + blank mean[b]
[b] blank mean = 514,427.5 (SIM GC/MS area count)
[c] Yes/No determined by comparing LLD to Mean. If LLD < Mean then YES, that incubation time/colony count combination can be considered the lower level of detection

**Table 5**

Method Detection Level

| cfu | n | Mean[a] ($x10^6$) | Std Dev ($x10^6$) | RSD % | MDL[b] | Pass/Fail[d] |
|---|---|---|---|---|---|---|
| 1-10 | 11 | 44.57 | 146.54 | 329 | 460.17 | **FAIL** |
| 11-100 | 11 | 133.91 | 222.84 | 166 | 699.75 | **FAIL** |
| 101-1000 | 7 | 437.73 | 138.89 | 32 | 436.16 | **PASS** |

[a]Mean SIM GC/MS area counts for samples

[b] MDL=(3.14 x Std Dev) + blank mean[c]

[c] blank mean = 41,672.2

[d] Pass/Fail determined by comparing MDL to mean.
If MDL < Mean, then PASS.

## Appendix

Method Detection Level: Raw Data

| Sample # | SPME area | SPME pos(3xbaseline) | MF colony | Average original colonies |
|---|---|---|---|---|
| 1 | 217,600 | yes | 4 | 5 |
| 2 | 178,043 | yes | 8 | 5 |
| 3 | 284,995,571 | yes | 30 | 26 |
| 4 | 55,239,207 | yes | 27 | 26 |
| 5 | 367,214,441 | yes | 143 | 266 |
| 6 | 379,455,145 | yes | 121 | 160 |
| 7 | 524,070 | yes | 0 | 0 |
| 8 | 8,234,854 | yes | 19 | 16 |
| 9 | 372,254,079 | yes | 145 | 160 |
| 10 | 402,946,810 | yes | 150 | 160 |
| 11 | 1,807,639 | yes | 4 | 15 |
| 12 | 13,591 | no | 1 | 2 |
| 13 | 73,453,517 | yes | 50 | 108 |
| 14 | 88,867 | no | 60 | 108 |
| 15 | 6,006 | no | 0 | 0 |
| 16 | 71,785 | no | 0 | 0 |
| 17 | 2,404,712 | yes | 13 | 13 |
| 18 | 32,826 | no | 0 | 0 |
| 19 | 31,515 | no | 0 | 2 |
| 20 | 607 | no | 2 | 2 |
| 21 | 34,513 | no | 0 | 0 |
| 22 | 1,016 | no | 0 | 0 |
| 23 | 150,287 | no | 2 | 2 |
| 24 | 297,939,909 | yes | 128 | 134 |
| 25 | 185,307,286 | yes | 12 | 15 |
| 26 | 167,482 | no | 0 | 0 |
| 27 | 147,534 | no | 2 | 2 |
| 28 | 19,665 | no | 3 | 2 |
| 29 | 43,075 | no | 0 | 0 |
| 30 | 4,036,545 | yes | 19 | 15 |
| 31 | 112,973,319 | yes | 17 | 68 |
| 32 | 314,840 | yes | 1 | 6 |
| 33 | 10,187 | no | 11 | 68 |
| 34 | 7,670 | no | 0 | 0 |
| 35 | 20,834 | no | 0 | 0 |
| 36 | 746,295,188 | yes | 85 | 117 |
| 37 | 537,520,712 | yes | 112 | 117 |
| 38 | 706,761,427 | yes | 101 | 117 |
| 39 | 486,401,135 | yes | 6 | 11 |
| 40 | 1,070,983 | yes | 3 | 2 |

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