1-1-2009

Development of a Headspace Solid-Phase Microextraction (HS-SPME) Procedure for the Determination of Short-Chain Fatty Acids (SCFAs) in Activated Sludge by GC-FID

Shiyan Cheng

Development of a Headspace Solid-Phase Microextraction (HS-SPME) Procedure for the Determination of Short-Chain Fatty Acids (SCFAs) in Activated Sludge by GC-FID

by

Cheng, Shiyan

Thesis

Submitted to the Department of Chemistry

Eastern Michigan University

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in Chemistry

Thesis Committee:

Heather Holmes, Ph. D.

Timothy Brewer, Ph. D.

Steven Pernecky, Ph. D.

September 1st, 2009

Ypsilanti, Michigan

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor, Dr. Heather Holmes, for her teaching and guidance and for giving me independence to learn and grow during the research. Her continuous support and encouragement helped me overcome lots of difficulties in my research. I would also like to give thanks to Dr. Timothy Brewer. He not only served as a committee member, but he also helped me in numerous difficulties over the past 2 years.

Also, I would like to thank Dr. Steven Pernecky for serving on my thesis committee and supporting my experiments. I would also like to thank Dr. Ruth Armitage for her help during my experiment.

Special thanks go to John Dawson and Chuck Hines from Ann Arbor Wastewater Treatment Plant. Their kindness and nice introduction on the plant inspired and encouraged me a lot for completing this research project. Without their help on the activated sludge sampling, none of my efforts on the fermentation experiments would be possible to advance.

I also feel indebted to my professors and classmates at EMU. First, I want to thank all of the professors who have been my instructors: Dr. Cory Emal, Dr. Harriet Lindsay, Dr. Dave Nickell, Dr. Ross Nord, Dr. Jose Vites, and Dr. Larry Kolopajlo. I also feel so indebted to my friends of my classes, especially Charles, Srikanth, Jolisa, Rohini, Vamsi, Ranadhear, Sudeer. I thank all of you for being my peers and friends while I've pursued my studies here at EMU.

Finally, I would like to thank my husband, Dongxuan, my daughter, Noelle, my parents, Bingzhe Cheng, Liu Xishun, and my mother-in-law, Okran Yang, for their continuous encouragement and support. Without their love and support, none of my work would be possible.

ABSTRACT

The process of wastewater treatment includes the removal of inorganic solids such as sand and gravel as well as organic materials, phosphorus, and nitrogen. Activated sludge, containing a variety of living organisms, is added into the wastewater treatment system. Short-chain fatty acids (SCFAs), an energy source of the bioorganisms, are produced during activated sludge digestion, and play a significant role in phosphorus and nitrogen removal as well as the removal of organic materials during the process of wastewater treatment. Extensive research has been carried out recently in search of optimized conditions to increase the level of fatty acids in the activated sludge. It has been suggested that pH, carbohydrate concentration, sodium dodecyl sulfate (SDS), and ultrasonic sample treatment have significant effects on hydrolysis and acidogenesis. However, investigations into the effects of these variables have until now been conducted individually. In the present work, a comprehensive study of the effects of these variables on the fermentation process was carried out, following the development of a feasible method for the detection of SCFAs in activated sludge using gas chromatography with flame ionization detection. Then, SCFA production was utilized as an indicator for the efficacy of wastewater treatment using samples from the Ann Arbor Wastewater Treatment Facilities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Introduction to Headspace Solid-phase Microextraction Coupling Gas Chromatography with Flame Ionization Detection (HS-SPME GC-FID)

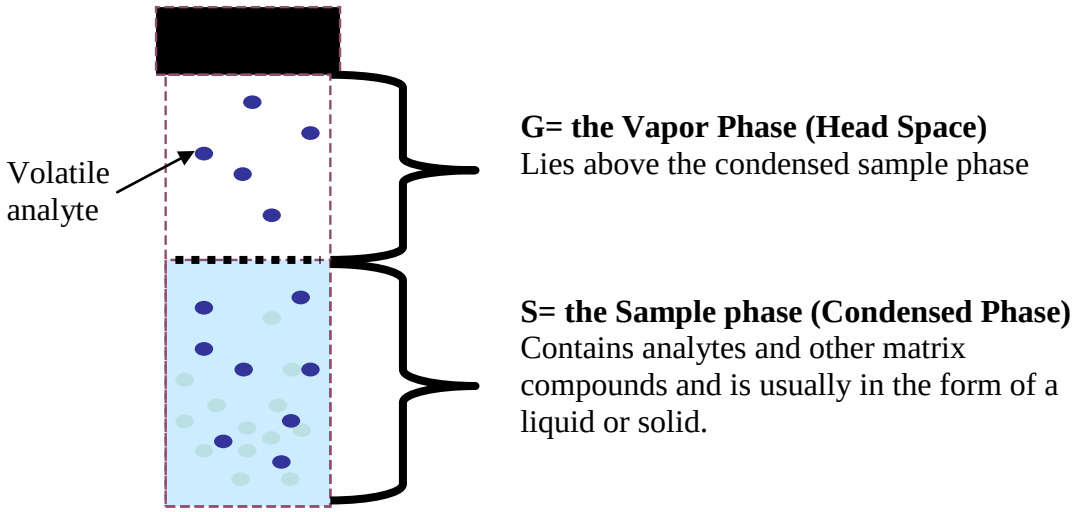
HS-SPME GC-FID is a method of analysis that combines two sampling techniques, headspace (HS) and solid-phase microextraction (SPME), interfacing with gas chromatography (GC) where flame ionization detection (FID) is employed as a universal detection method.

1.1a HS analysis

With the growing popularity of GC in the decades since its introduction, sampling procedures for injection have received worldwide attention.[1-3] Problems often arise when sampling volatile analytes from complicated sample matrices containing high molecular weight non-volatile compounds. Very often researchers introduce a liquid sample containing both volatile and non-volatile samples directly to the column. In such cases, the volatile sample passes through the column with the mobile phase; however the non-volatile samples can be retained in the column leading to column contamination. Moreover, when the volatile compound is present in a solid sample, the sampling procedure is further complicated.[4]

Headspace analysis not only solved the problem of column contamination from complex matrices, but simplified the elaborate sample preparation as well. A HS sample is prepared in a vial such that a liquid or solid matrix containing volatile analyte is in equilibrium with the vapor phase (Figure 1.1). The condensed phase contains many

compounds where volatile compounds are mixed with non-volatile ones. The gas phase, so called headspace, contains the volatile compounds evaporated from the complex liquid



Equation 1: Partition Coefficient

$K = [A]_{\text{Condensed Phase}} / [A]_{\text{Vapor Phase}}$

Equation 2: Phase Ratio

$\beta = V_{\text{Vapor Phase}} / V_{\text{Condensed Phase}}$

Figure 1.1: Phases of headspace in a vial with the equations for partition coefficient (K) and phase ratio (β).

sample mixture. The volatile compounds in the HS are then introduced to the GC for separation and analysis. In order to obtain high sensitivity, both sample preparation and instrumental parameters need to be optimized.[5, 6]

Each analyte has a unique partition coefficient (K), which is the equilibrium distribution of analyte between the condensed and vapor phases, shown in Equation 1.

$$K = \frac{[A]_{\text{Condensed Phase}}}{[A]_{\text{Vapor Phase}}} \qquad [1]$$

A compound with a lower K will evaporate more easily into the headspace from the liquid phase mixture leading to a large instrument response and low limits of detection. The value of K depends on the temperature of the environment, the composition of the sample phase, and on the phase ratio of sample to headspace. Optimization of K involves determining conditions under which lower K values are obtained. One common method is to increase the temperature of the sample. The analyte of interest diffuses to a greater extent into the headspace when the temperature of the sample is elevated to a certain degree. Each sample has its own optimum temperature for sampling. The addition of salt to a liquid matrix helps to promote the transfer of analytes to the headspace as well. The common salts used for this purpose include ammonium chloride, ammonium sulfate, sodium chloride, sodium citrate, sodium sulfate, and potassium carbonate. Among these salts, sodium chloride has demonstrated steady solubility with increasing temperatures and is the most commonly used. [8] The phase ratio (β) is another factor to be optimized for higher concentration of analyte in the headspace (Figure 1.1). It is defined as the ratio between the volume of headspace and the volume of the condensed sample in the vial. A lower value of phase ratio yields a lower value of K, and thus a higher response. Derivatization of the target analyte is also commonly employed for obtaining higher sensitivity and a lower limit of detection. Common derivatization techniques include esterification, alkylation, acetylation, and silylation. The final method to increase the yield is to increase the volume of headspace sampled and introduced into the instrument for analysis.[5, 6, 7] All of these conditions must be considered together to optimize K for a specific analysis.

1.1b Solid-phase microextraction (SPME)

SPME is a relatively new sample preparation technique developed by Pawliszyn and co-workers in 1990. [9, 10] Commercial SPME devices consist of 2 major parts: a thin piece of fiber and a fiber holder (Figure 1.2). The fiber part is comprised of a tensioning spring, sealing septum, septum-piercing needle, fiber attachment needle, and fused-silica fiber, as shown in Figure 1.2.

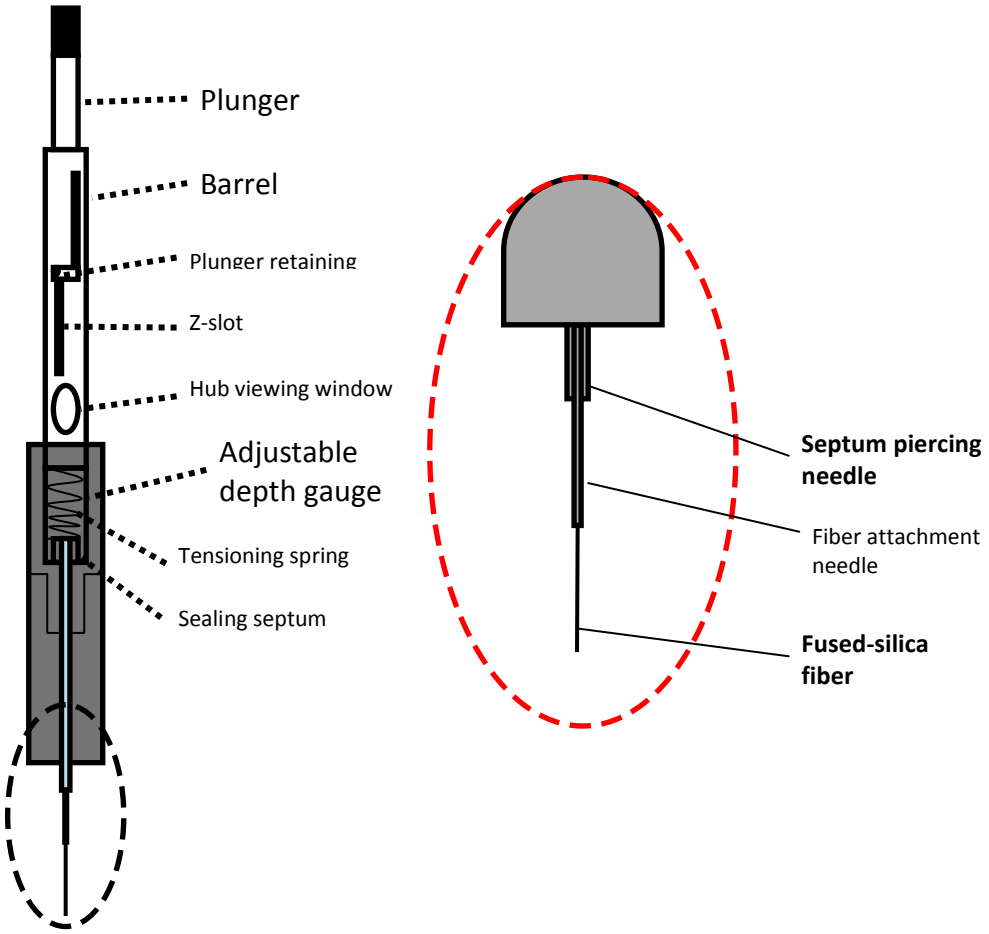


Figure 1.2 Commercial SPME device made by Supelco. Reproduced from Ref. [10].

The fused-silica fiber, coated with a thin film of polymeric extraction phase, is mounted on the stainless-steel fiber attachment needle. It is protected by the septum-

piercing needle. The septum-piercing needle is used for protecting the fiber during storage and carrying, and during the sampling procedure, the rigid stainless-steel material plays a dual rule by piercing the septum as well as protecting the fiber.

The fiber holder consists of a plunger, a barrel, a plunger retaining screw, a Z-lot, a hub viewing window, and an adjustable depth gauge. When sampling, the fiber is assembled into the holder, and the insertion depth (the length of fiber exposed during sampling or injection) is adjusted appropriately.[11-23]

There are several types of thin films used as extraction phases (Table 1). These films, depending on their composition chemistry, are generally classified as bonding, non-bonding, partially cross-linked, and highly cross-linked. In addition, they are used in different film thicknesses which provide appropriate extraction for a variety of applications.[24, 25] In my investigation, detection of short chain fatty acids (SCFA), the relatively polar PA (polyacrylate) fiber was used because the polar SCFAs have higher affinity toward PA fiber.[26] The analytes in the sample are extracted and concentrated onto the coated polymeric fiber and desorbed into the GC or HPLC for further analysis. The most common method for GC injection is thermal desorption, while the solvent desorption method is found to be more common for HPLC applications. [25]

There are two modes for the sample extraction: direct immersion solid-phase microextraction (DI-SPME) and headspace solid-phase microextraction (HS-SPME). Both methods are shown schematically in Figure 1.3.[24,25] The sampling procedure consists of 6 general steps: piercing septum (septum piercing needle protects the fiber when penetrating through the septum), exposition of the fiber, extraction of analytes, retraction of septum piercing needle, retraction of the fiber, and desorption of analytes in

the GC. In DI-SPME, the fiber is directly immersed into a liquid sample phase so that the analytes are extracted and concentrated onto the fiber. To facilitate the efficiency of the extraction, physical techniques are applied during the extraction, such as stirring with a small stirring bar, rapid vial agitation, and sonication. After equilibration, the fiber is removed from the liquid sample and is coupled with a GC or HPLC for analysis. In HS-SPME, the fiber is inserted directly into the headspace of the vial for sample extraction. After some suitable time, the analytes in the solution phase diffuse into the headspace, and the fiber is exposed in the headspace. However, the extraction time, i.e. the equilibration of the extraction phase on the fiber, should be determined based upon the type of the fiber and target analytes accordingly. Some volatile samples reach equilibration faster than non-volatile samples. After an appropriate time, the fiber is retracted from the headspace of the vial and is transferred to a GC or HPLC injection port for analysis. [24,25]

1.1c Advantages of the HS-SPME

Conventional methods for detecting volatile compounds include liquid-liquid extraction, [27, 28] purge-and-trap for aqueous samples,[29,30] and porous polyurethane foam or multiple-adsorbent tube traps for air sampling.[31,32] In addition, method utilizing capillary electrophoresis and indirect UV detection or laser-induced fluorescence have been developed. [33]

The advantages of the HS-SPME, compared to conventional methods which require extensive sample preparation, are that the fiber is protected from the liquid sample contaminants such as proteins and organic solvents, and is therefore free of interference, and easy preparation for the next cycle of sampling. Also, the fiber is

protected from direct exposure to very acidic and basic conditions resulting from pH modification of the sample. Most of the commercial fibers need special care to use with a pH range from 2 to 11. Acidification and alkalization of the sample is carried out easily with the HS-SPME technique. In addition, the HS-SPME sample preparation technique is clean, simple, rapid, solvent-free, and less laborious, compared to traditional sample preparation methods for direct sample injection.[11-23] However, HS-SPME also has its drawbacks. It is limited to the detection of volatile and semi-volatile samples. Sample loss due to gas leaking from the vapor phase at higher temperatures is difficult to avoid. In addition, because of the fragile nature of the fiber, extra attention and calmness is required during sample handling.

1.1d Applications for HS-SPME Analysis

A wide variety of real samples can be detected using HS-SPME. Pharmaceutical companies use the HS-SPME technique to detect volatile impurities in the products.[34] In blood alcohol analysis, HS-SPME has been used for quantification of alcohol.[35,36] In food science, flavors, vegetables and fruits juices, soft drinks, alcoholic beverages, and diary products such as milk and cheese are tested.[25] In addition, in medical science, fecal samples[37] and meconium[14]are studied for disease determination using this technique. Furthermore, in environmental science, fatty acids in wastewater or activated sludge are studied for the improvement of wastewater treatment.[38,39]

Table 1: Commercially available SPME fibers (Supelco, Bellefonte, PA)

Fiber Core (Stationary Phase)	Thickness	Bond Type	pH range	Recommended Operation Temperature °C	Application	Recommended Use
Polydimethylsiloxane (PDMS)	100 µm	Non-bonded	2-10	200-280	Volatile	GC/HPLC
	30µm	Non-bonded	2-11	200-280	Non-polar semivolatile	GC/HPLC
	7µm	bonded	2-11	220-320	Moderately polar to non-polor semivolatiles	GC/HPLC
Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)	65µm	Partially crosslinked	2-11	200-270	Polar volatile	GC
Carboxen/ Polydimethylsiloxane (Carboxen/PDMS)	75µm	Partially crosslinked	2-11	250-310	Trace-level volatile	GC
Polyacrylate (PA)	85µm	Partially crosslinked	2-11	220-300	Polar semivolatile	GC/HPLC

Polyethylene glycol (PEG)	60µm	crosslinked	2-9	200-250	alcohols and polar compounds	GC
Divinylbenzene/Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS)	50/30µm	Highly crosslinked	2-11	230-270	analytes C3-C20 (trace compounds)	GC
Carbowax/Divinylbenzene (Carbowax/DVB)	70µm	Highly crosslinked	2-9	200-240	Polar analytes	GC
	65µm	Partially crosslinked	2-9	200-250	polar analytes, especially for alcohols	GC
Carbowax/Templated resin (CW/TPR)	50 µm	Partially crosslinked	-	-	Anionic surfactants	HPLC

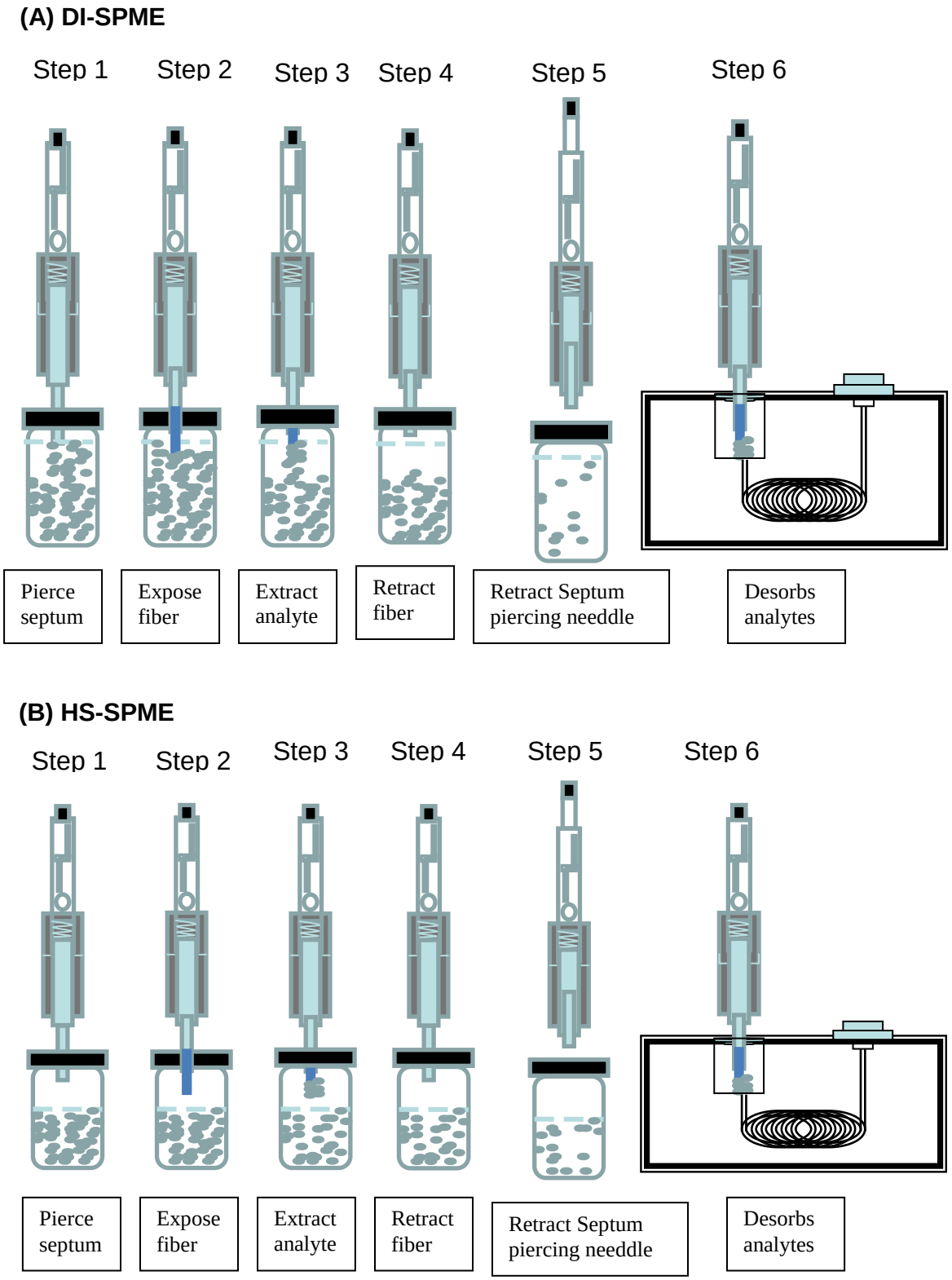


Figure 1.3 Two extraction mode: (A) Direct immersion solid-phase microextraction (DI-SPME); (B) Headspace solid-phase microextraction (HS-SPME).

1.2 Applications to Activated Sludge from Wastewater Plant

A typical wastewater treatment system consists of a conveyance system, preliminary treatment, primary treatment, secondary treatment, advanced treatment, final treatment, sludge digestion, and solid dewatering.[40,41] The overall wastewater treatment schematic is shown in **Figure 1.4.** Depending on the age and design of the wastewater treatment system, component 7 (Primary sludge fermentation) is optional for the integrated system.

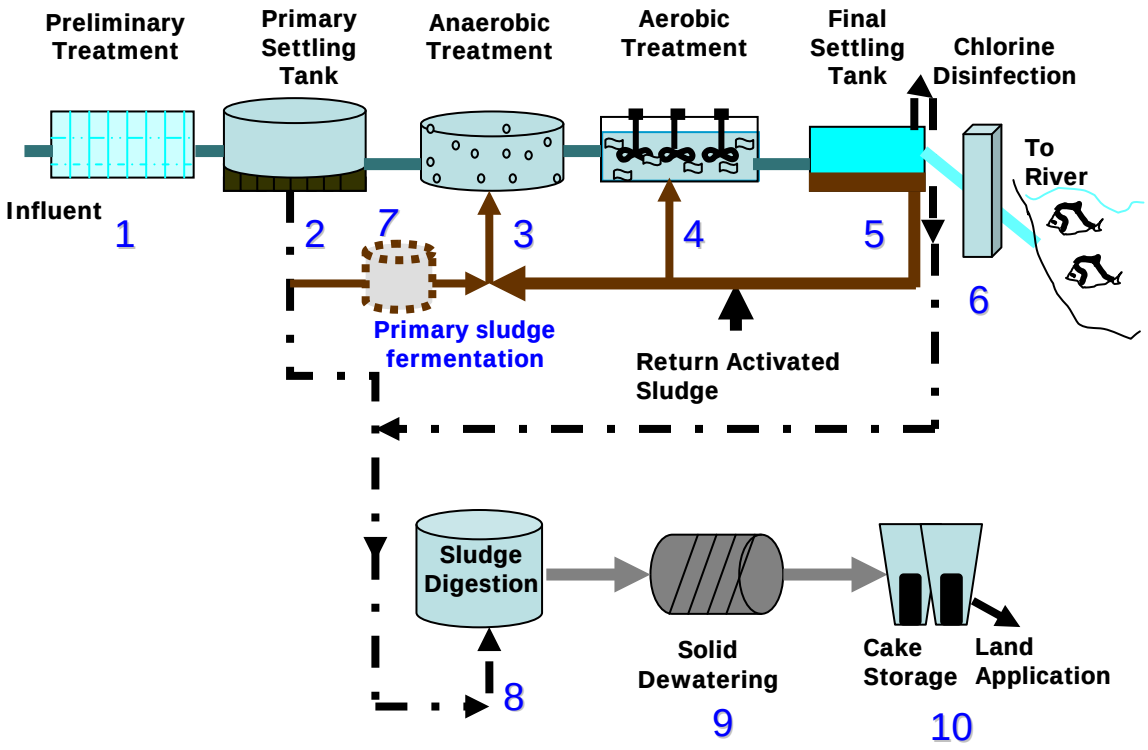


Figure 1.4 Schematic wastewater treatment process

1.2a The Biological Wastewater Treatment System [40,41]

(1) CONVEYANCE SYSTEM

The conveyance system consists of collection sewers and pumping systems. Each house has its own pipes that are connected to the underground pipe line network and the wastewater further flows into pump stations. Most of the sewer is designed to move downhill and at pumping stations the wastewater is pumped uphill by the special pumps.

(2) PRELIMINARY TREATMENT (Figure 1.4 part 1)

The basic rule of preliminary treatment is bar screening. Large pieces of trash in wastewater could damage the wastewater plant equipment. For the protection of the plant and efficiency of the treatment process, metal screens are placed every few inches in order to filter large trash such as plastic materials, sticks, papers, etc.

(3) PRIMARY TREATMENT (Figure 1.4 part 2)

Several settling tanks are used for removing most of the heavy materials as well as floating grease. After a few hours of settling, the heavy solids fall down to the bottom of the tank. When these solids become a thicker sludge, removal is accomplished by large mechanical scrapers. The sludge is then transferred to a digestion system. In addition, the floats are skimmed off the top of the settling tank and pumped to the sludge digestion system.

(4) SECONDARY TREATMENT (Figure 1.4 part 3, 4, 5)

Secondary treatment consists of 3 steps: anaerobic treatment, aerobic treatment, and a final settling. Some wastewater plants combined both anaerobic and aerobic treatment, and others only employ aerobic treatment. However, research has shown that the combination of aerobic and anaerobic procedures increased the efficiency of the

treatment.[42] The activated sludge, containing lots of microorganisms to biodegrade organic materials, is used in both aerobic and anaerobic tanks. The purpose of anaerobic treatment is to remove phosphorus and nitrogen from the wastewater, while aerobic treatment effectively removes the organic material such as protein, fats, and carbohydrates through aerobic respiration.

(5) ADVANCED TREATMENT

When water is used for the irrigation of golf courses or parks, a higher degree of treatment is needed. In this case, filtration of the water using sand or crushed coal is applied for removing of small solid pieces.

(6) FINAL TREATMENT (Figure 1.4 part 6)

After secondary treatment, many bacteria and viruses are still alive in the wastewater. The final disinfection process is required to remove these disease-causing bacteria. Some post-process treatments can be employed such as chlorine disinfection, ultraviolet disinfection, and other chemical methods. In chlorine disinfection, the effluent water from the secondary treatment is held in a tank to which chlorine is added. In ultraviolet disinfection, the water is exposed to the high levels of ultraviolet radiation. After this, the process water is either reused for irrigation or discharged to the ocean, a river, or a lake.

(7) PREFERMENTATION TANK (Figure 1.4 part 7)

Based on the age and design of the wastewater treatment system, this part is an optional component for the integrated system. However, based on a recent study [60-63], it is indicated that short chain fatty acids play a crucial rules in denitrification, sulfate reduction, and phosphorus reduction in wastewater. In addition, by controlling

fermentation properly, the hydrolysis and acidogenesis (the process for the production of short chain fatty acid from organic material) can be accelerated. On the other hand, the methanogenesis, the process for the production of methane, can be slowed down by the same operation.

(8) SLUDGE DIGESTION (Figure 1.4 part 8)

Sludge digestion is a separate line of the wastewater treatment system designed for removing the excess sludge collected from the primary and the secondary treatment procedures. The typical treatment in this process is anaerobic digestion during which anaerobic bacteria are employed to biodegrade the organic materials. The temperature of the digester is controlled at 37 to 38 °C with continuous mixing. After a period of 10 to 20 days, the bacteria decompose the organic material in the sludge and produce byproducts such as methane and carbon dioxide.

(9) SOLID DEWATERING (Figure 1.4 part 9)

The digested liquid sludge is then sent to the dewatering system to squeeze water from the biosolids to reduce volume. A general method includes a filtering process and centrifugation. After dewatering, the cake-like biosolid is sent to the cake storage bin.

1.2b The Goal of Wastewater Treatment System

The major goal of the secondary treatment system using activated sludge is to biologically remove excess phosphorus and nitrogen as well as organic material in wastewater through cellular respiration [40, 41] (Figure 1.5).

The living organisms in the wastewater tank utilize organic materials such as proteins, polysaccharides, and fats as nutrients for their growth.

Although chemical precipitation can be used for the removal of phosphorus, the biological phosphorus removal process possesses several advantages such as a relatively lower cost (economical) and reduced sludge production for the treatment (environmentally friendly).

Polyphosphate-accumulating organisms (PAOs) contain: β-2 *Proteobacteria*, *Actinobacteria Rhodocyclus*, *Propionibacter*, *Candidatus Accumulibacter phosphatis* , *Pseudomonas* and *Tetrasphaera*, etc.[43,44] These PAOs, contained in the activated sludge, take up the phosphorus from the wastewater and are eventually removed with waste sludge. Under anaerobic condition, PAOs assimilate short chain fatty acids (SCFAs), such as acetic acid, propionic acid, and butyric acid, which are the fermentation products.[44,45] Then, the PAOs utilize polyphosphate as an energy source to convert SCFAs to intracellular Polyhydroxyalkanoates (PHAs) and simultaneously release orthophosphate to the environment. Under aerobic conditions, the PHA is metabolized by the PAOs as an energy source for cell growth. At the same time the PAOs incorporate orthophosphates, also existing in wastewater, into stored polyphosphates. Finally, the PAOs containing the stored polyphosphates are removed from the wastewater treatment system as waste sludge.[44,46,47] The PAOs are taken to agricultural land, along with the cake-like waste sludge, where both used as a valuable

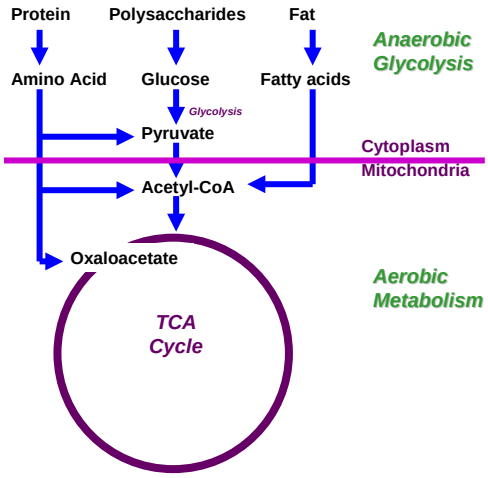


Figure 1.5 Cellular respiration

fertilizer. Then the phosphorus moves back to the phosphorus cycle where the PAOs release phosphorus back to the soil, ready for the plants to absorb.

Denitrification ($2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$)

in the wastewater treatment system is conducted biologically by the denitrifying organisms. In an anaerobic environment, the nitrate ions are utilized as electron acceptors and are converted to nitrogen gas by the denitrifying organisms such as *Pseudomonas*, *Bacillus* and *Paracoccus sp.*, etc.[48-50] Then, the nitrogen gas diffuses into the atmosphere following the nitrogen fixation in the nitrogen cycle **(Figure 1.6 step 1).**

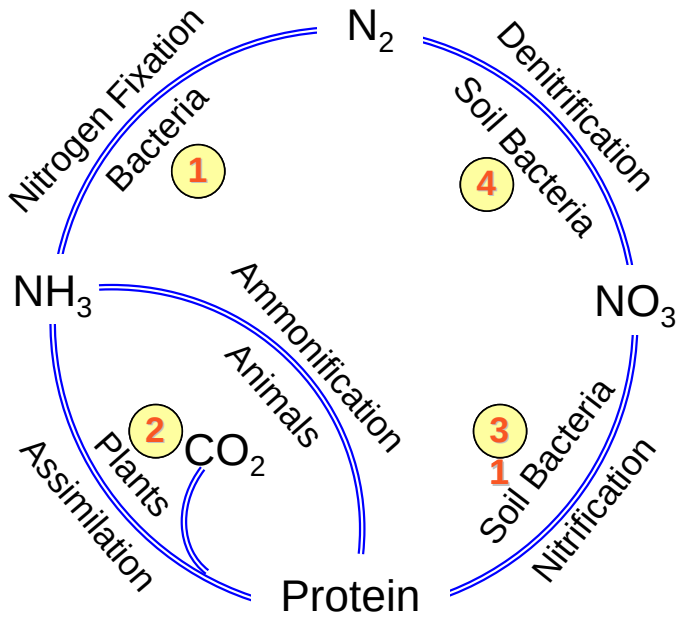


Figure 1.6 Nitrogen cycle

1.3 Significance of Project

Short-chain fatty acids (SCFAs) are low molecular weight organic acids such as acetate, lactate, propionate and butyrate, which have volatile and hydrophilic properties.[51]

They are produced from biodegradation of fats, proteins and carbohydrates under aerobic and anaerobic conditions.[51] (Figure 1.7)

SCFAs have been found in various samples such as activated sludge[52-55], landfill leachates[56-59], dairy products, and cecal samples. Recently, SCFAs have drawn widespread interest due to the involvement of SCFAs in environmental chemistry. The goal of the wastewater treatment plants is to remove excess inorganic solids such as sand and gravel, organic materials, phosphorus, nitrogen from wastewater before releasing to natural waters. After treatment, about 90-95 percent of the organic materials are removed biologically. The SCFAs, an energy source of the bioorganisms, can dramatically improve the efficiency of the biological nutrient removal, phosphorus removal, and denitrification in wastewater treatment.[60-63] In a wastewater treatment system, the SCFAs are produced in the activated sludge and then the activated sludge is fed to the Secondary Treatment System for the biological nutrient removal, biological phosphorus removal, and biological denitrification. Therefore, it is necessary to establish a suitable detection method for SCFAs in activated sludge. The research goal is to develop a quantitative HS-SPME coupled with GC-FID method in determining SCFAs in activated sludge.

In addition, much research effort has been carried on for increasing the SCFAs yields in activated sludge during anaerobic digestion for in order to increase the efficiency of wastewater treatment. [64-71] Under the anaerobic digestion, the organic materials undergo three steps: hydrolysis, acidogenesis, and methanogenesis.[67,68,71] (Figure 1.7) The microbial cells, contained in waste activated sludge, consist of cell membranes and cell walls. First, these cells need to be hydrolyzed in order to liberate the intracellular organic substances from the cell. Then these organic materials undergo

acidogenesis, which is a process converting organic substances to short chain fatty acids (SCFAs) or low molecular weight carbon sources. Further, these SCFAs are converted to methane and carbon dioxide through methanogenesis. Thus, for increasing the SCFA production, it is important to control the anaerobic digestion by inhibiting the rate of methanogenesis while accelerating the rate of the hydrolysis and acidogenesis.

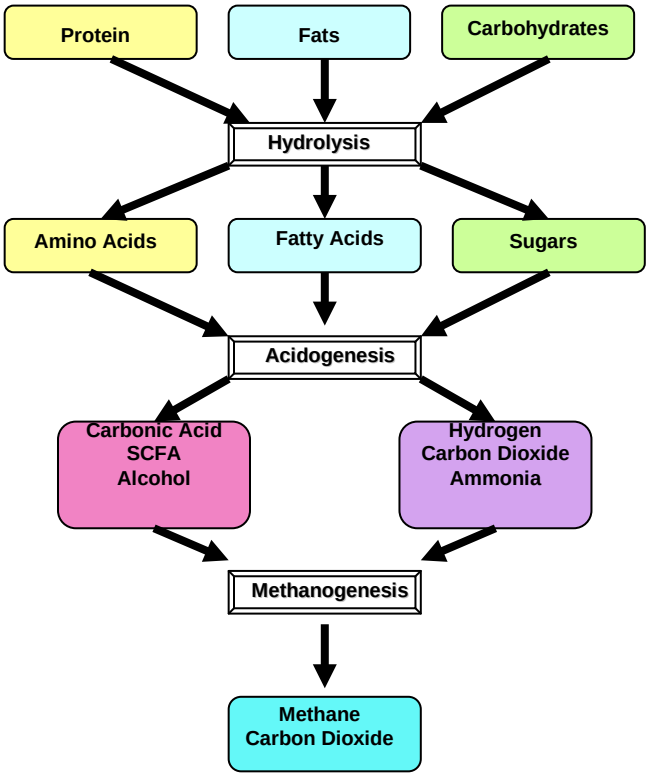


Figure 1.7 Anaerobic Digestion

1.4 Research objectives

Extensive research has been carried out recently in search of optimized conditions to increase the level of fatty acids in the activated sludge. It has been suggested that pH[70,71], carbohydrate concentration[66], sodium dodecyl sulfate (SDS)[68], and ultrasonic sample treatment[64,65] have significant effects on hydrolysis and acidogenesis. However,

investigations into the effects of these variables were conducted individually. In the present work, a comprehensive study of the effects of these variables on the fermentation process is carried out.

The goals of the research:

1. To optimize the HS-SPME sampling procedure for the detection of SCFAs.
2. To develop a HS-SPME procedure for the determination of SCFAs in the activated sludge utilizing GC-FID.
3. To investigate an optimum fermentation method for increasing SCFA production from the activated sludge combining variables including pH, carbohydrate, sodium dodecyl sulfate, and ultrasonication.

CHAPTER 2

EXPERIMENTAL

2.1 Instrumentation and experimental parameters

A SHIMADZU GC-14A gas chromatography coupled with flame ionization detection was used for this investigation. The SPME fiber holder and fibers were obtained from Supelco (Bellefonte, PA, USA). The parameters for this experiment are listed in Table 2. For the protection of the SPME fiber, a glass inlet without glass wool was used. A water bath shaker held at 37 °C was employed for the fermentation (model: 406015).

Table 2 Experimental parameters for HS-SPME GC-FID

GC	Column type	DB-5	WAX
	Column dimension	3m long, 0.25mm (ID), 1μm film thickness	12m long, 0.25mm (ID), 0.5μm film thickness
	Carrier gas	99.999% Helium	
	Injector Temperature	250 °C	
	Column temperature	130 °C	
	Column Head Pressure	150 kPa	
SPME	Fiber type	Polyacrylate/85μm film thickness	
FID	FID detection temperature	250 °C	

2.2 Materials

The activated sludge sample was collected from the Ann Arbor Wastewater Treatment Plant. Propionic acid, butyric acid, sodium dodecylsulphate (SDS), sulfuric

acid, sodium chloride, and sodium hydroxide were purchased from Sigma-Aldrich. The septa and vials used for headspace analysis were purchased from Restek (Bellefonte, PA).

2.3 Sample collection and treatment

The activated sludge sample was collected in a 1-L plastic bottle with a tight cap and transferred to the lab on ice. The sample was processed for fermentation within a 2-hour period of collection. The activated sludge samples were fermented for 14 days to enhance the production of the SCFA. The fermentation culture sample was then subjected to the HS-SPME treatment prior to the GC-FID analysis.

2.3a Fermentation method

The activated sludge was divided into 10 equal volumes of 200 mL (each) and transferred to 10 brown glass bottles fitted with air-tight caps before fermentation (Figure 2.1). The effects of various combinations of experimental parameters, as listed in Table 3, were studied. After adjusting the parameters of the fermentation bottles (200 mL), 20 mL of the activated sludge, collected from the pipe of return activated sludge, were added into each of the 200 mL fermentation bottles as seed for the fermentation, and then all 10 bottles were placed in the incubator and held at 37°C. In bottle # 1, the pH of the sample was adjusted to 10 and rice was added together with SDS after ultrasonic treatment of the sample was carried out. Bottle # 6 contained half of the SDS when compare to bottle #1. Among bottles # 2 to 5, only three of the four parameters were varied. In bottle # 2, pH, rice, and SDS were controlled. In bottle # 3, rice was absent. In bottle # 4, SDS was absent. In bottle # 5, the pH was adjusted to neutral. Among bottles numbered from 7 to 9, only one of the four parameters was employed for fermentation. In bottle # 7, only ultrasonic treatment was conducted. In bottle # 8, the pH was raised to 10, and in # 9,

SDS was added. # 10 was not controlled by any variables (blank). The pH was adjusted by titrating samples with a solution of NaOH (4M).

Figure 2.1 Fermentation of activated sludge. Activated sludge in the incubator (Left). Fermented activated sludge (Right).

Table 3: Fermentation conditions

Sample No.	pH	Rice (g)	SDS (g)	Ultrasonic
1	10	10.1	0.702	Y
2	10	10.11	0.701	***N***
3	10	***0***	0.705	Y
4	10	10.26	***0***	Y
5	***7***	10.18	0.703	Y
6	10	10.1	***0.351***	Y
7	7	0	0	***Y***
8	***10***	0	0	N
9	7	0	***0.83***	N
Blank	7	0	0	N

2.3b Extraction procedure

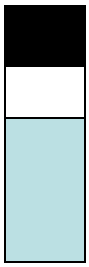
An aliquot of 1-mL from each fermentation bottle was placed in a 5-mL HS-SPME vial, containing 0.4 g of NaCl. The vials were equipped with airtight septa caps,

and were kept in a 70°C oven for about 30 min to reach full equilibration. Before the extraction was performed, the fiber was preconditioned in the GC injector for 30 minutes at 250 °C, followed by 8 minutes equilibration in the vial under the HS-SPME mode **(Figure 1.3)**. Then, the SPME fiber was introduced to the GC injector and the analytes were desorbed at 250 °C.

2.4 Standard addition method

To accurately quantify the levels of fatty acids in the samples, standard addition was utilized in this study. The standard addition method, often referred to as spiking, is a method used to determine analyte concentration in complex sample matrices such as biological fluids or soil samples. The main purpose of the standard addition method is to avoid the interference of other components contained in the sample matrix and to obtain the most accurate instrumental response toward the analytes.

In the experiment, the sample solutions were spiked with 5 µL, 10 µL, 15 µL, and 20 µL of standard butyric acid solution respectively. As demonstrated in Figure 2.2 and Figure 2.3, the concentration of unknown sample can be determined. In step 1, the total volume after spiking, Vs+Vstd, was treated to be equal to Vs by approximation, because the relative ratio of V_{std} and V_s was less than 0.2 %. After spiking, the instrumental response (R) increased with the increasing concentrations of the sample solution (C_{sa}). Then the standard addition curve was generated according to the instrumental response. Finally, the actual sample concentration was determined by extrapolation of the standard addition plot (Figures 2.2 and 2.3).



C_{unk}= concentration after spiking
V_s= volume of the sample
C_s= concentration of the sample
C_{std}= concentration of the standard solution
V_{std}= volume of the standard solution

$$(1)\quad C_{unk} = \frac{C_s V_s + C_{std} V_{std}}{V_s + V_{std}} \qquad \text{Since } \frac{V_{std}}{V_s} < 0.2\%,$$

$$\approx \frac{C_s V_s + C_{std} V_{std}}{V_s}$$

Instrumental response to the analyte is R= K * concentration, where K is instrumental sensitivity.

$$(2)\quad R = \frac{K C_s \cancel{V_s}}{\cancel{V_s}} + \frac{K C_{std} V_{std}}{V_s}$$

$$(3)\quad \text{Now set } C_{sa} = \frac{C_{std} V_{std}}{V_s}$$

$$(4)\quad R = K C_s + K C_{sa} \qquad (y = b + mx)$$

$$(5)\quad 0 = K C_s + K C_{sa}$$

$$(6)\quad C_s = -C_{sa}$$

Figure 2.2 Mathematical derivation of standard addition method.

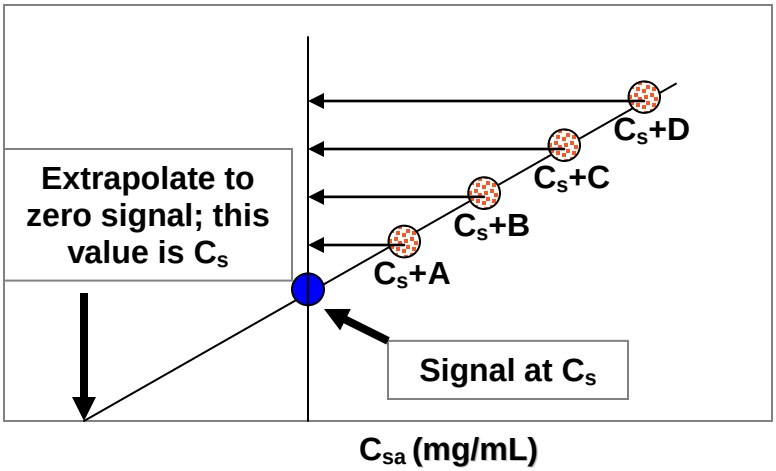


Figure 2.3 Standard addition curve

2.5 Reproducibility evaluation

In order to investigate the reproducibility of the method, experiments were carried out using the fermented activated sludge sample from bottle # 5. First, ultrasonic treatment (50/60 Hz) was employed for 1.5 hours to obtain a homogeneous sample solution. Then 10 mL of the sample was transferred to each of the 5 vials (20 mL) equipped with air-tight caps. Then 10 mL of sample from each vial was diluted to 20 mL. Following spiking with 5 µL, 10 µL, 15 µL, 20 µL of standard pure butyric acid solutions, the vials were labeled as 2, 3, 4, and 5. After that, 1mL of solution was taken from each of the 20-mL vials and was transferred into individual 5-mL HS-SPME vials containing 0.4 g of NaCl. Finally, the butyric acid was extracted after equilibration in an oven under 70 °C for about 30 minutes. The same procedure was repeated 2 times.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of the HS-SPME procedure

Extensive studies have been carried out for obtaining an optimum condition for HS-SPME sampling. As described in Chapter 1 various factors including NaCl concentration, oven temperature, acidification, and extraction time play significant roles in increasing the efficiency of the sample extraction and pre-concentration. These experiments not only used a simplified sample analysis procedure, but also enabled reproducible results.

3.1a NaCl

As discussed in the literature [72], salting-out leads to a better detection limit. NaCl is the salt typically used, as it shows nearly constant solubility over a relatively wide temperature range. As shown in Figure 3.1, peak areas for propionic and butyric acids in standards of (0.992 g/mL, 0.964 g/mL) increased linearly with the increasing of concentration of NaCl. Thus a saturation concentration of NaCl was employed throughout this investigation.

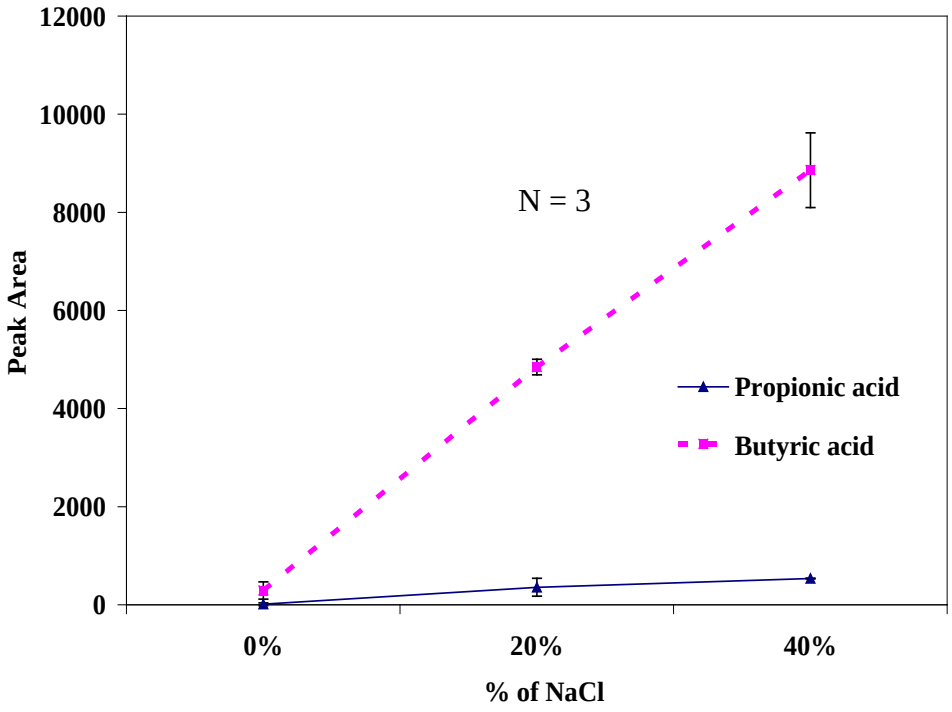


Figure 3.1 Effect of NaCl concentration in the liquid phase. (Error bar: 2 times standard deviation) (The error bar is the range from the average value minus standard deviation to the average value plus standard deviation and the error bars in the following sections have the same definition.)

3.1b Oven temperature

Temperature is another important parameter that can improve the instrumental response[73]. Before the extraction, the vials containing sample were placed in an oven, and allowed to equilibrate prior to extraction. As illustrated in Figure 3.2, the peak areas for propionic acid and butyric acid increased accordingly with the rising oven temperature. However, when temperature reached over 75°C, the plastic HS-SPME vial cap became loose, and leakage occured. Thus, the best temperature for the experiment was seen to be at 75°C.

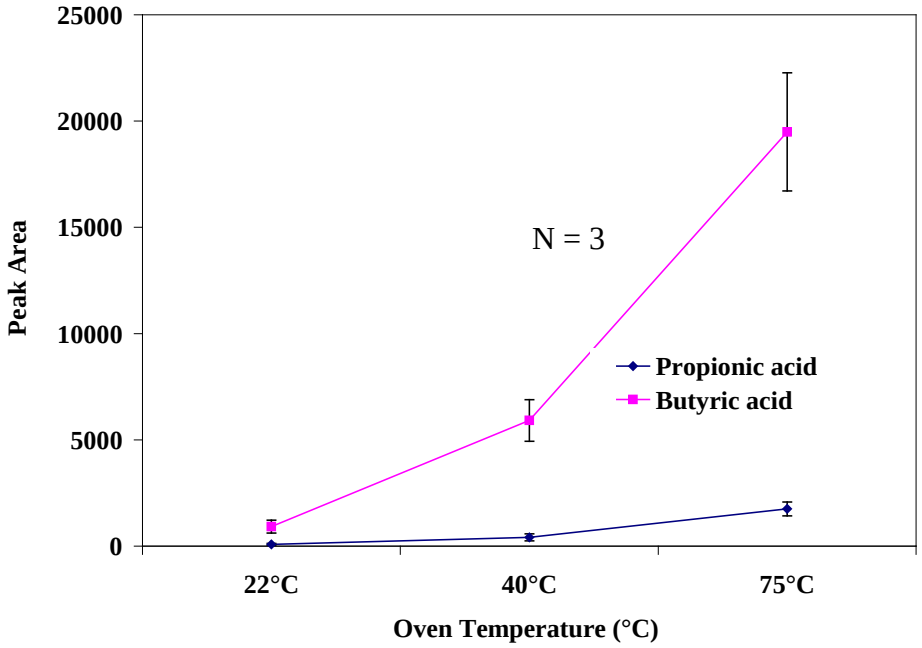


Figure 3.2 Temperature effect on the extraction of fatty acids

3.1c Acid effect

Acid effect is another important factor needed for optimization. Extensive studies have shown that lowering pH can improve the limit of detection (LOD).[73] For samples from Bottle # 1 and # 5, the response increased around 3-fold when 1 drop of 12 M HCl was added to 1 mL of the samples containing 40% NaCl (Figure 3.3). Figure 3.4 shows the result of a similar experiment using H_2SO_4. Here, NaCl was added to standards that were already acidified. The results were opposite of those shown in Figure 3.4; the standards containing both acid and salt gave lower peak areas than those in acid alone This is thought to be caused by the order in which the experimental steps were performed. Under high acidic conditions (20% of H_2SO_4 in H_2O), the solubility of NaCl became problematic. Thus the initial addition of sulfuric acid hampered the salting-out effect. To

avoid the incompatibility between acidification and salting-out effect, NaCl needs to be added first and then appropriate amount of acid needs to be added in the sample.

The acidification did increase the LOD when used after the addition of salt, however, the working pH range for the fiber is from pH 2 to pH 9 according to the manufacturer. Very often, the coating of the fiber is stripped off the fiber after several uses under the severe acidic conditions. As a result, the fiber can no longer be used for accurate analytical applications, because of poor reproducibility. Considering pros and cons, the optimization through acidification was not considered further in the following studies.

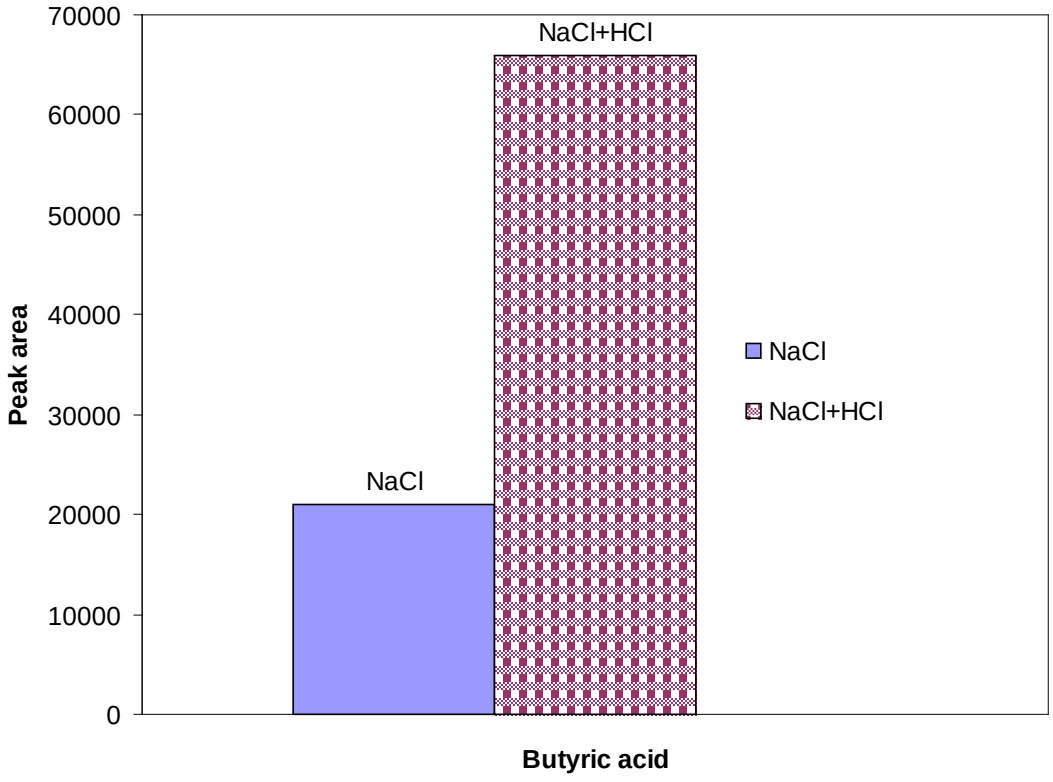


Figure 3.3 Acidification test with HCl. One drop of 4 M HCl was added to 1 mL of samples 1 and 5 that already contained NaCl.

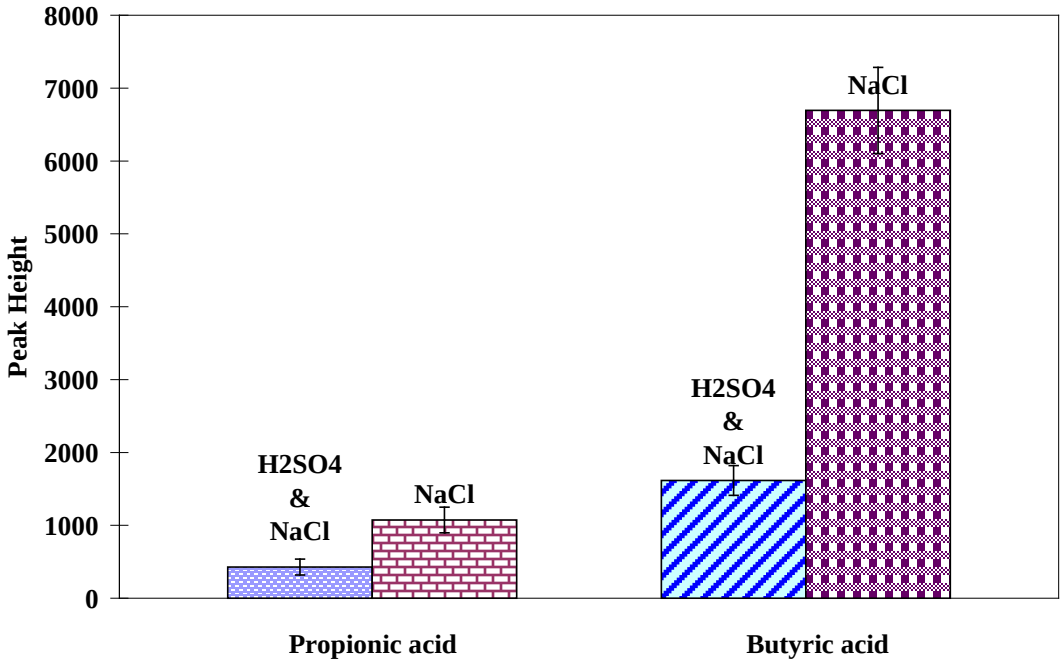


Figure 3.4 Acidification test with H_2SO_4. H_2SO_4 added 1st and NaCl. (20% of H_2SO_4 in sample)

3.1d Extraction time

During HS-SPME sample extraction, the analytes first evaporate from the liquid sample phase into the headspace of the vial and arrive at an equilibrium between the two phases. The analytes in the headspace equilibrate with the SPME coating as well, and are then desorbed into the GC inlet at a high temperature. Each analyte has two unique partition coefficients (K) which influence the equilibria of the analyte at both interfaces: the liquid phase and the headspace, the headspace and the stationary phase of the fiber coating. Thus, the optimum adsorption time (extraction time) varies depending on the chemical natures of the analytes under fixed incubation conditions such as temperature, concentration, and sample volume. Studies were carried out to obtain the best analyte

extraction time for butyric acid and propionic acids. Figure 3.5 shows the effect of extraction time on the peak areas of propionic and butyric acids. The peak area for propionic acid remains constant over the range of 2 to 14 minutes, while that of butyric acid increases to a maximum at 8 minutes, and then remains fairly constant. Figure 3.6 shows similar data, but data points were collected in shorter time intervals. In Figure 3.6, peak areas for both propionic and butyric acids increase up to an extraction time of 8 minutes. Here it appears that the peak areas for butyric acid actually decrease somewhat for extraction times longer than 8 minutes. The data in Figures 3.5 and 3.6 indicate that the best extraction time is 8 minutes, and was therefore used for the remainder of the experiments.

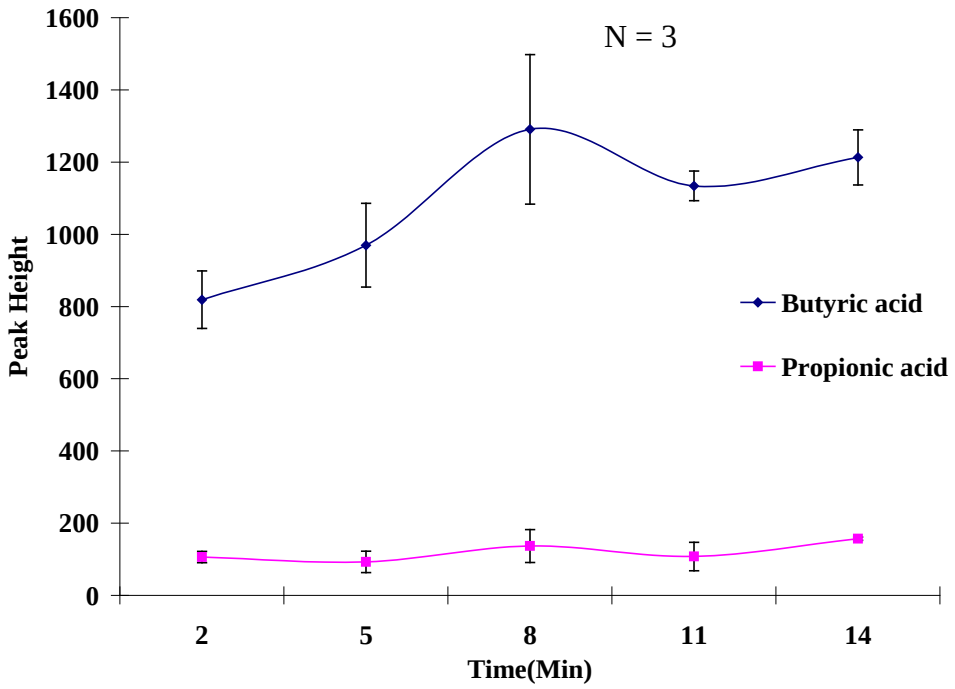


Figure 3.5 SPME sample extraction time (Detection in longer time interval)

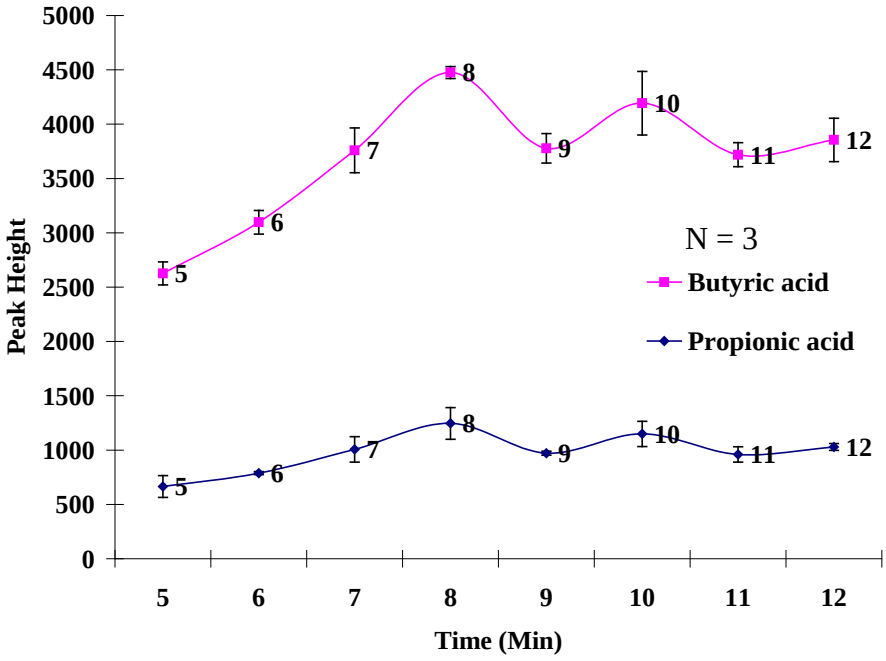


Figure 3.6 SPME sample extraction time (Detection in shorter time interval)

3.2 Fermentation result

The fermented samples were subjected to GC analysis using the HS-SPME technique after 5 days and 9 days of fermentation. Combined results for propionic and butyric acid after fermentation for 5 days are shown in Figure 3.7. In this experiment, 1 drop of HCl was added to acidify the sample. Among the 10 sample bottles, butyric acid was produced only in Bottles 1-6. Samples 3 and 5 resulted in the lowest production, while 4 and 6 resulted in the highest production. Propionic acid was found in Bottles 2, 4 and 6, with Bottle 2 yielding better overall production. Figures 3.8 and 3.9 show the data for butyric (Figure 3.8) and propionic (Figure 3.9) acids individually to better emphasize the scale.

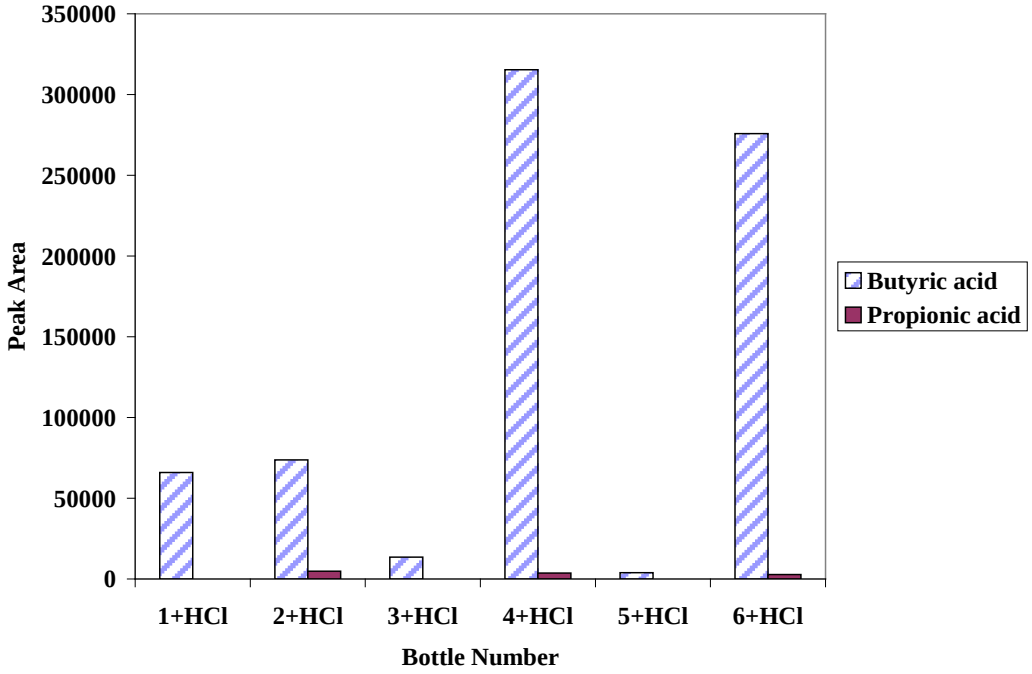


Figure 3.7 Production of propionic acid and butyric acid after 5 days of fermentation. 40% of NaCl and 1 drop/mL of HCl were used before sampling.

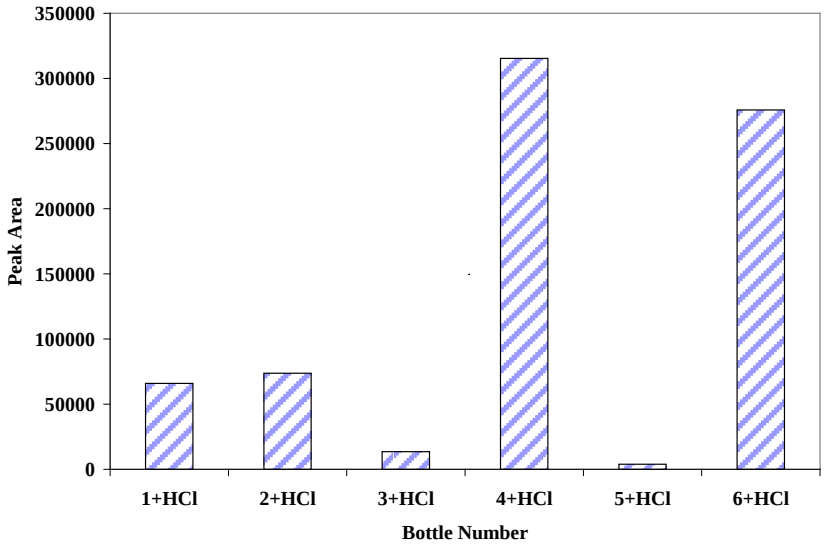


Figure 3.8 Production of butyric acid after 5 days of fermentation. 40% of NaCl and 1 drop/mL of HCl were used before sampling.

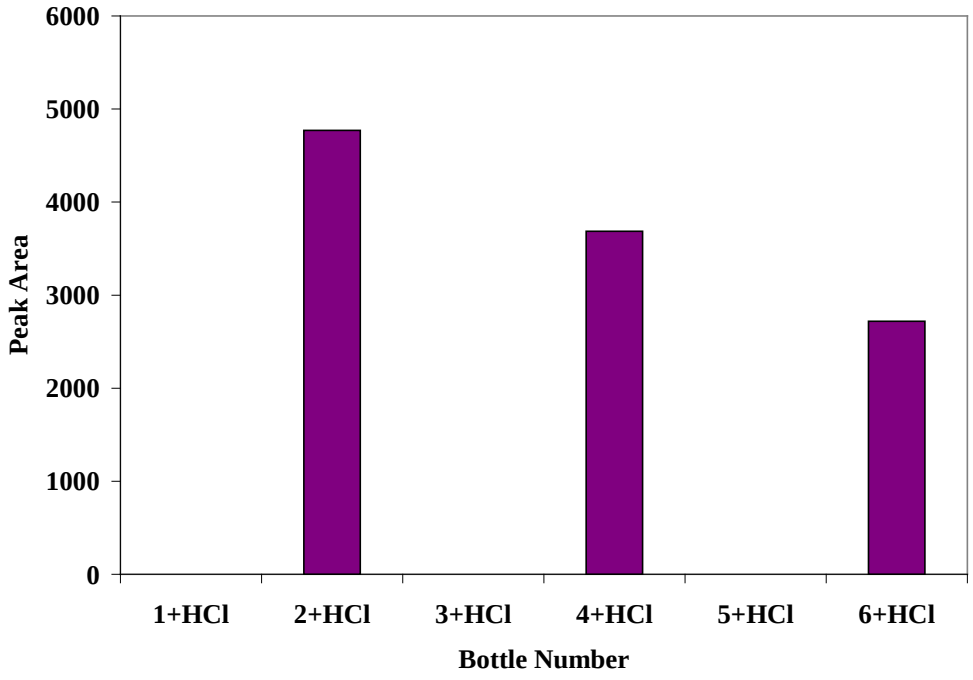


Figure 3.9 Production of propionic acid after 5 days of fermentation. 40% of NaCl and 1 drop/mL of HCl were used before sampling.

Figure 3.10 details the results of fatty acid production after 9 days of fermentation. In this experiment, I did not add any acid in the sample. Butyric acid was produced in Bottles 1, 2, and 4-6, with the highest amounts again in samples 4 and 6. Propionic acid was again found in Bottles 2, 4, and 6. All 3 samples yielded concentrations less than those after 5 days of fermentation. In addition, acetic acid was found in Bottles 2, 4, 6, and 9. Among the samples containing acetic acid, Bottle 6 showed the highest yield. Figures 3.11-3.13 show the data for each acid individually to emphasize the scale. Of the three fatty acids observed, butyric acid showed the highest yield overall for both 5 and 9 days of fermentation.

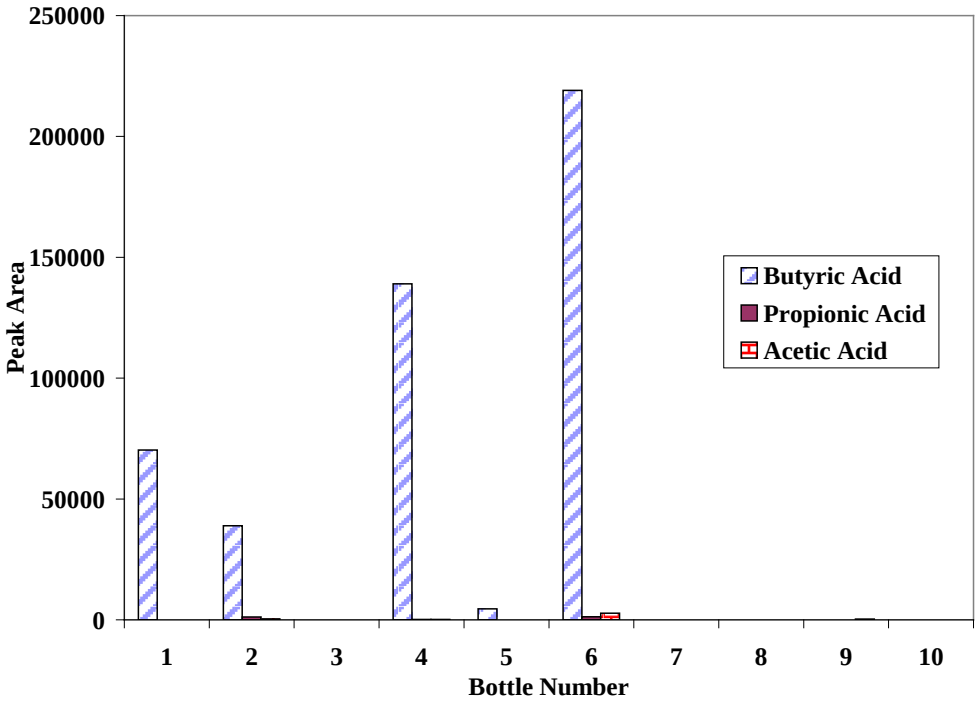


Figure 3.10 Production of the Acetic acids, propionic acid and butyric acid after 9 days of fermentation. Only 40% of NaCl was used.

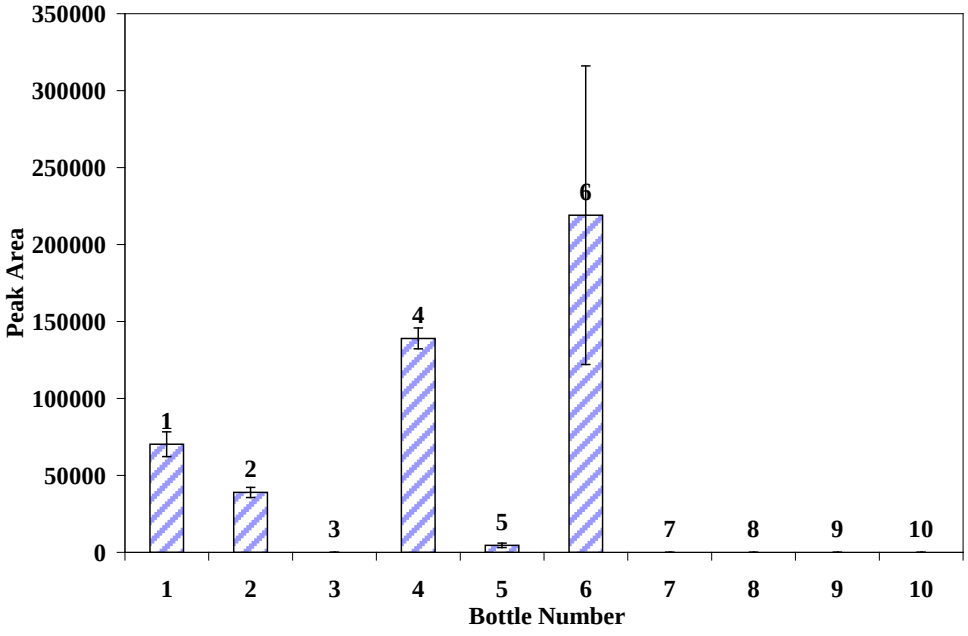


Figure 3.11 Production of butyric acid after 9 days of fermentation. Only NaCl was used.

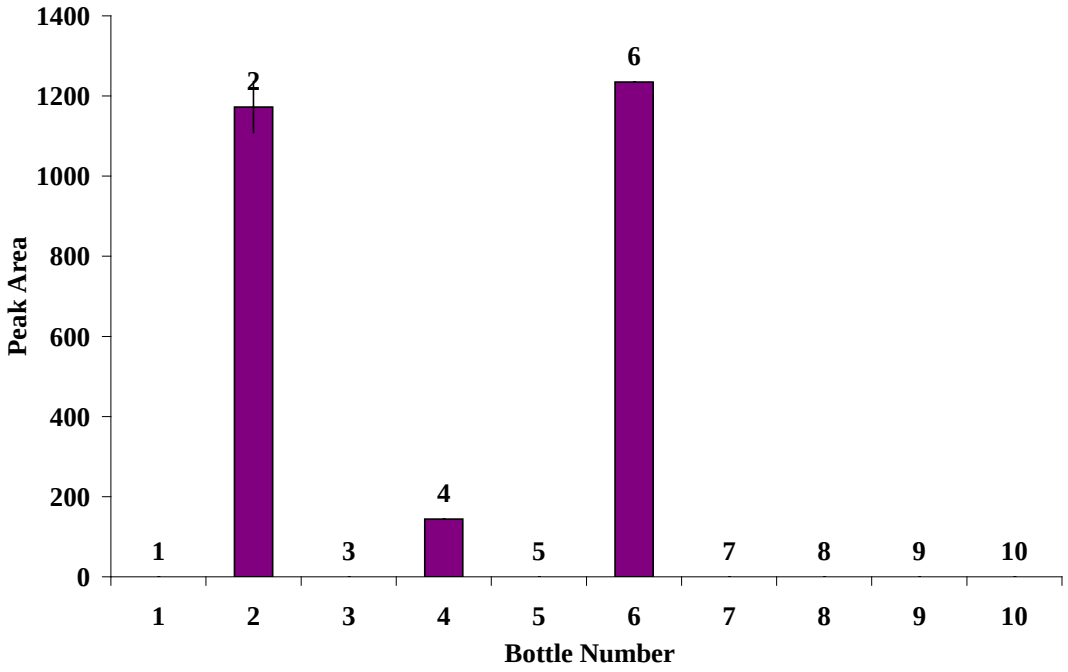


Figure 3.12 Propionic acid production after 9 days of fermentation. Only NaCl was used.

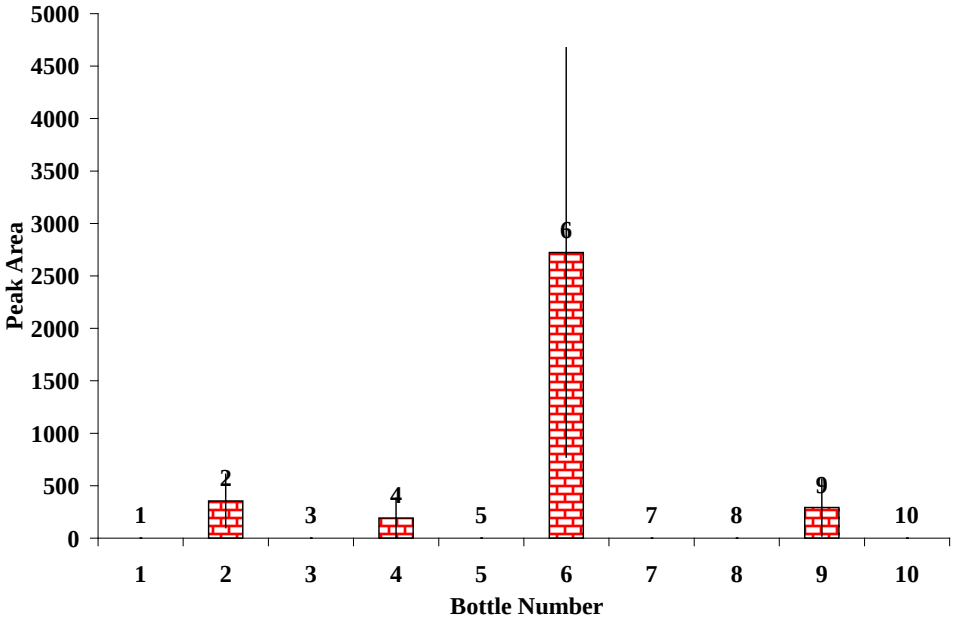


Figure 3.13 Acetic acid production after 9 days of fermentation. Only NaCl was used.

Table 4: Comparison of fatty acid production under varying fermentation conditions. (Total volume 220 mL) Samples 2, 4, and 6 are emphasized because they resulted in the highest production of fatty acids. Underlined numbers indicate the change of variables. See text for details.

Sample No.	pH	Rice	SDS(g)	Ultrasonication (1.5 hr), 55 ^{o}C)	Butyric Acid production after 5 days	Butyric Acid production after 9 days	Propionic acid production after 5 days	Propionic acid production after 9 days	Acetic acid production after 9 days
1	10	10.1	0.702	Y	4	3	ND	ND	ND
2	**10**	**10.11**	**0.701**	**N**	**3**	**4**	**1**	**2**	**2**
3	10	0	0.705	Y	5	ND	ND	ND	ND
4	**10**	**10.26**	**0**	**Y**	**1**	**2**	**2**	**3**	**4**
5	7	10.18	0.703	Y	6	5	ND	ND	ND
6	**10**	**10.1**	**0.351**	**Y**	**2**	**1**	**3**	**1**	**1**
7	7	0	0	Y	ND	ND	ND	ND	ND
8	10	0	0	N	ND	ND	ND	ND	ND
9	7	0	0.83	N	ND	ND	ND	ND	3
10 (Blank)	7	0	0	N	ND	ND	ND	ND	ND

(For comparison of fatty acid production: 1 represents the highest production and 6 the lowest under a given set of conditions)

Table 4 contains a numerical comparison of fatty acid production from the 10 different fermentation bottles. The four variables considered were pH, the addition of rice, concentration of SDS, and whether or not the samples were subjected to ultrasonication. Initial values for the variables were drawn from the literature, in which optimum values were reported individually; Sample 1 was fermented considering the optimum conditions as a group. The remainders of the samples were fermented under various combinations of the four variables in order to determine the optimum conditions in combination. The numbers in each column designate the rank of the samples in terms of acid production.

Effect of SDS: After 5 days of fermentation, butyric acid was observed in six of the ten samples (1-6). The highest amount was produced in Sample 4, followed by Sample 6, then Sample 2. Propionic acid was also observed in Samples 2, 4, and 6, in decreasing order. After 9 days of fermentation, butyric acid was observed in samples 1,2, and 4-6, and propionic in 2, 4, and 6. Sample 1, which was fermented under the optimum conditions for all four variables, resulted in poor production of butyric acid (ranked fourth after 5 days and third after 9 days). There was no observable propionic acid at either time. Removing the SDS altogether gave better results, as shown by Sample 4 in which butyric acid production ranked first after 5 days and second after 9. Propionic acid production in Sample 4 was ranked second after 5 days and third after 9. Overall, the best production of these acids occurred in Sample 6, for which the SDS concentration was half of that in Sample 1. Here, butyric acid production was ranked second after 5 days and first after 9, with propionic acid ranked third after 5 days and 1 first after 9. Acetic acid was observed as well, ranking first in production after both 5 and 9 days.

Effect of Ultrasonication: A comparison of Samples 1 and 2 highlights the effect of ultrasonication. Sample 2 was fermented under the same conditions of pH, rice, and SDS as Sample 1, but was not subjected to sonication. Production of butyric acid remained low (rank 3 and 4 at 5 and 9 days). Propionic acid was observed at both 5 and 9 days, ranked 1 and 2 respectively, and acetic acid was observed after 9 days (ranked 2). The increase in production without sonication likely results from the excess living bacteria in the fermentation bottle, because sonication will kill the bacteria in the samples.

Effect of Rice Addition: A carbohydrate, rice in this study, showed an important effect in production of fatty acids when comparing 1 and 3. Removing the rice altogether in bottle 3 decreased the SCFA production. Among the 3 SCFAs, only butyric acid was observable (ranked 5 in 5 days), while bottle 1 gave better result (ranked 4 and 3 at 5 and 9 days, respectively).

Effect of pH: When comparing samples 1 and 5, the pH of fermentation also showed a significant effect on the production of butyric acid. Although in both cases, propionic acid and acetic acid were not detectable, the results in Figures 3.8 and 3.11 (5 days, 9 days) indicated that sample 1 at pH 10 obtained a higher butyric acid concentration than Sample 5 at pH 7. Therefore, pH adjustment to 10 also is considered to be significant.

Combined Parameters: A comparison of Samples 1-6 (combined with multiple parameters) with Samples 7-10 (employed individual parameter) indicates that better fermentation resulted using a combination of multiple parameters. In Sample 7-10, the SCFAs were not detectable except for the acetic acid in Sample 9 at 9 days of fermentation.

3.3 Retention time of fatty acids

Retention times for each acid in the standard solution were obtained before the sample analysis. A sample chromatogram of propionic acid and butyric acid is shown in Figure 3.14.

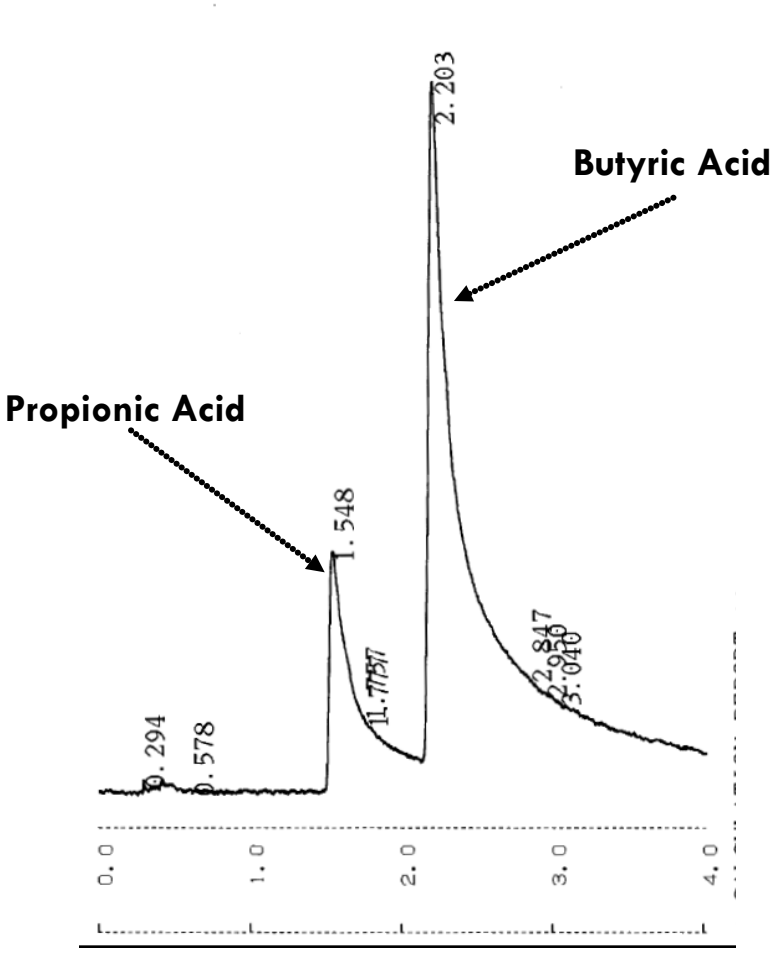


Figure 3.14 Chromatogram of GC for propionic acid and butyric acid.

3.4 Fatty acid quantification using standard addition analysis

Quantification of the fatty acids in Sample 1 was carried out using the standard addition method. First, the standard addition curve (Figure 3.15) was generated according to instrumental responses at various butyric acid concentrations. The slope value and y-

intercept values were determined to be 63209 min^{-1}, and 9347.7, respectively. Then, the x-intercept value (Csa) was was determined to be -0.14789. Thus, the concentration of diluted sample (C_o) is 0.14789 mg/mL. Since the original sample in the fermentation bottle was diluted 2 fold**,** the actual concentration was calculated as 2 times 0.14789 mg/mL (0.2958 mg/mL).

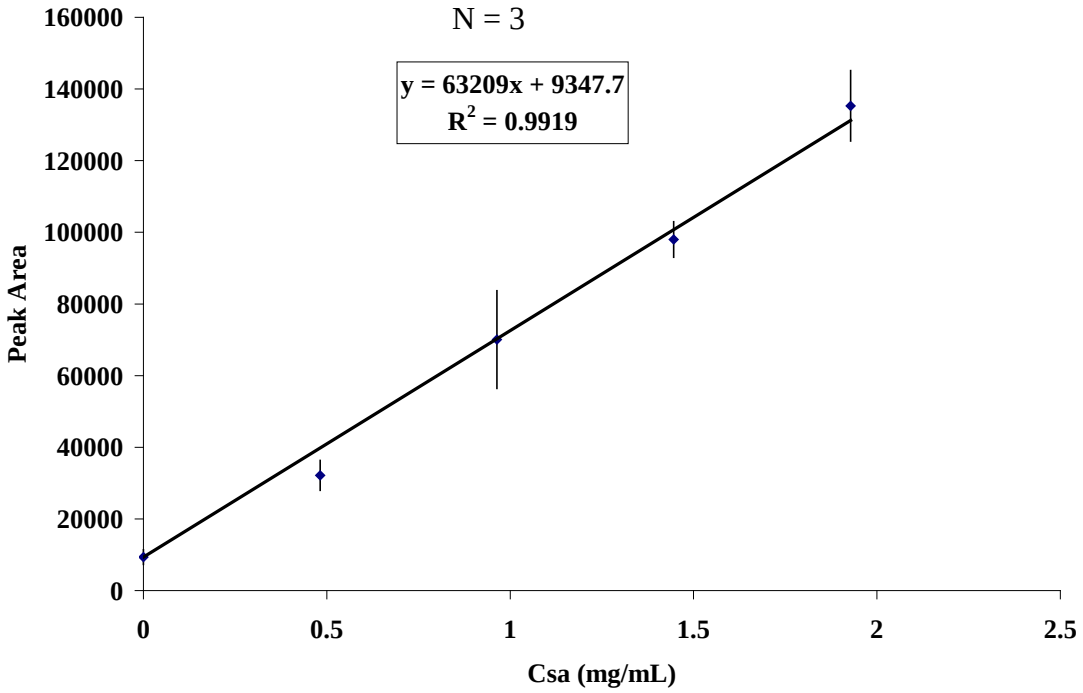


Figure 3.15 Standard addition curve for butyric acid in diluted sample of Bottle number 1.

3.5 Reproducibility

The reproducibility of the HS-SPME coupling with GC-FID analysis was evaluated using the same procedure and data analysis method. This time, Sample 5 was used. For three replicate injections, the average concentration of butyric acid was determined to be 0.080 mg/mL with a standard deviation of 0.007.

CHAPTER 4

CONCLUSIONS

4.1 Conclusions

The major advantage of the HS-SPME technique is that the process is free from the potential interferences from the compounds in the complicated sample matrices of materials such as biochemicals, proteins and organic solvents. Therefore, the analysis is free of contamination and easy to regenerate for the next round of use. In addition, the HS-SPME sample preparation technique possesses other advantages including minimization of sample preparation, clean extraction, and it is simple, rapid, solvent-free, and less laborious, when compared to traditional sample preparation methods developed for direct sample injection.[14]

A wide variety of real samples can be detected using HS-SPME such as pharmaceutical, biological, and food samples. The current study was undertaken to develop a feasible method for the environmental analysis based on the detection of short chain fatty acids (SCFA) in activated sludge. The SCFA plays a significant role in phosphorus removal, nitrogen removal, and the removal of organic materials during wastewater treatment. Therefore, the study of SCFA production can be utilized in the wastewater treatment as an important indicator for facilitating the efficiency of the treatment.

The results of the investigation suggested the optimum sampling conditions for the SCFA detection based on the HS-SPME coupled GC-FID technique. These conditions are summarized as follows: 40 % of NaCl (saturation concentration), 75°C

oven temperature, acidification, and 8 minutes of extraction time. However, for the fermentation samples, no acidification was used because of the potential damage to the fiber caused by the concentrated acid.

It has been suggested that pH[70,71], carbohydrate concentration[66], sodium dodecyl sulfate (SDS)[68], and ultrasonic sample treatment[64,65]can significantly improve the hydrolysis and acidogenesis process when employed individually. For the fermentation study, combination of 4 parameters including pH, carbohydrate, SDS, and ultrasonic treatment were investigated. The results indicated that the highest SCFA production was obtained with this combination of the 4 parameters: pH adjusted to 10, 10.1 g rice, 0.351 g SDS, and ultrasonic treatment. In addition, under these experimental conditions, the activated sludge showed the highest yield for butyric acid among the three fatty acids: butyric acid, propionic acid, acetic acid. Furthermore, quantification using standard addition analysis demonstrated relatively high reproducibility with a low standard deviation value less than 10%.

4.2 Future Work

As demonstrated in this study, the HS-SPME technique is suitable for the analysis of SCFA in activated sludge. However, the limit of detection was relatively high due to the instrumental insensitivity.

Recently more and more research effort has been directed toward developing novel derivatization methods. The derivatization methods normally employ the derivatizing reagents including diazomethane, 2,3,4,5,6-pentafluorobenzyl bromide (PFB Br), 1-(pentafluorophenyl) diazoethane (PFPDE), 1-pyrenyl-diazomethane (PDAM), tetramethylammonium hydrogen sulfate (TMA-HSO_4) and tetramethylammonium

hydroxide (TMA-OH). Based on different conjugation chemistry, various derivatization techniques have been developed including in-vial derivatization of vapor sample with PFPDE, in-solution derivatization with PFPDE, in-solution derivatizaton with PFB-Br, in-fiber derivatization with PDAM, in-fiber derivatization of analytes in aqueous samples using Diazomethane, and GC injector port derivatization using ion-pair reagents.[74] Derivatization converts the polar SCFA to less polar derivatives. Utilizing the in-fiber derivatization with PDAM method, for example, the fiber coating was first saturated with PDAM by soaking it in PDAM for 60 minutes, and the derivatized fiber was then inserted into the headspace for sample extraction. The SCFA partitioned into the fiber coating and reacted with the PDAM to form a pyrenylmethyl ester. And then the pyrenylmethyl ester, produced on the fiber coating, was introduced to the GC injection port.[26] The combination of the derivatization and HS-SPME can further enhance the partitioning of analytes from the HS into the fiber coating, thus improving the instrumental sensitivity, efficiency of the HS-SPME extraction, and chromatographic properties.[74,75]

References

(1) David, F.; Sandra, P. *J. Chromatogr., A* **2007**, *1152*, 54-69.

(2) Song, SM.; Marriott, P.; Ryan, D.; Wynne, P. *LCGC North Am.* **2006**, *24*, 1012.

(3) de la Guardia, M.; Garrigues, S. *Trends Anal. Chem*. **1998**, *17*, 263-272.

(4) Ettre, L. S. *LCGC North Am.* **2002**, *20*, 1120-1129.

(5) Kolb, B.; Ettre, L. S. *Static headspace-gas chromatography: theory and practice*; John wiley & Sons, Inc., Hoboken, 2006.

(6) Ioffe, B. V.; Veniaminovich B.; Vitenberg, A. G. *Head-space analysis and related methods in gas chromatography*; Wiley: New York, 1984.

(7) Restek chromatography products (PA), Restek Technical Guides, Lit. Cat. # 59895A.

(8) http://www.kayelaby.npl.co.uk/chemistry/3_2/3_2.html, 08, 05, 2009.

(9) Arthur, C. L.; Pawliszyn, J. *Anal. Chem.* **1990**, *62*, 2145.

(10) Zhang, Z. Yang, M. J.; Pawliszyn, J. *Anal. Chem.* **1994**, *66*, 844A.

(11) Pawliszyn, J. *Solid phase microextraction : theory and practice*; Wiley-VCH: New York, 1997.

(12) Ann, S.; Wercinski, S. *Solid phase microextraction : a practical guide*; Marcel Dekker: New York, 1999.

(13) Coleman III, W. M.; Dube, M. F. *J. Sci. Food Agric.* **2005**, *85*, 2645–2654.

(14) Hutson, J. R.; Aleksa, K.; Pragst, F.; Koren, G. *J. Chromatogr., B* **2009**, *877*, 8-12.

(15) Ibáñez, C. *J. Chromatogr., A* **2003**, *1017*, 161–166.

(16) Ioffe, B. V.; Veniaminovich B.; Vitenberg, A. G. *Head-space analysis and related methods in gas chromatography*; Wiley: New York, 1984.

(17) Jackson J.F., Linskens H.F., Eds. *Analysis of taste and aroma*; Springer: New York, 2002.

(18) Kim, H.; Nochetto, C.; McConnell, L. L. *Anal. Chem.* **2002**, *74*, 1054-1060.

(19) Lee, J.; Kim, D.; Chang, P.; Lee, J. *Food Chem.* **2007,** *105*, 414-420.

(20) Millsa, G. A.; Walkerb, V; Mughalb, H. *J. Chromatogr., B* **1999,** *730*, 113–122.

(21) Pacenti, M.; Dugheri, S.; Villanelli, F.; Bartolucci,G.; Calamai, L.; Boccalon, P.; Arcangeli, G.; Vecchione, F.; Alessi, P.; Kikic, I.; Cupelli, V. *Biomed. Chromatogr*. **2008**, *22*, 1155–1163.

(22) Plutowska, B.; Wardencki, W. *Anal. Chim. Acta.* **2008**, *613*, 64–73.

(23) Rouseff R. L., Cadwallader K. R., Eds. *Headspace analysis of foods and flavors : theory and practice Kluwer*; Academic/Plenum Publishers: New York, 2001.

(24) Mester, Z.; Sturgeon, R.; Pawliszyn, J. *Spectrochim. Acta, Part B* **2001**, *56*, 233-260.

(25) Kataoka, H.; Lord, H. L.; Pawliszyn, J. *J. Chromatogr., A* **2000**, *880*, 35-62.

(26) Pan, L.; Adams, M.; Pawliszyn, J. *Anal. Chem.* **1995**, *67*, 4396-4403.

(27) Misselbrook, T. H.: Clarkson, C. R.; Pain, B. F. *J. Agric. Eng. Res.* **1993**, *55*, 163-169.

(28) Van Langenhove, H.; Roelstraete, K.; Schamp, N.; Houtmeyers, J. *Water Res.* **1985**, *19*, 597-693

(29) Houser, B.; Popp, P. *J. Chromatogr. A* **2001**, *909(1)*, 3-12.

(30) Koe, L. C. C.; Tan, Y. G. *Int. J. Environ. Stud.* **1987**, *30*, 37-44.

(31) Islam, A. K. M.; HanaKi, K.; Matsuo, T. *Water Sci. Technol*. **1998,** *38*, 337-344.

(32) Ma, C. Y.; Skeen, J. T.; Dindal, A. B.; Bayne, C. K.; Jenkins, R. A. *Environ. Sci. Technol.* **1997**, *31*, 853-859

(33) Zuriguel, V.; Causse, E.; Bounery, J.D.; Nouadje, G.; Simeon, N.; Nertz, M.; Salvayre, R.; Couderc, F. *J. Chromatogr. A.* **1997**, *781*, 233.

(34) Kuwayama, K.; Tsujikawa, K.; Miyaguchi, H.; Kanamori, T.; Iwata, Y.; Inoue, H.; Saitoh, S.; Kishi, T. *Forensic Sci. Int.* **2006**, *160*, 44-52.

(35) Schulz, K.; Bertau, M.; Schlenz, K.; Malt, S.; Dressler, J.; Lachenmeier, D. W.; *Anal. Chim. Acta.* **2009**, *646*, 128-140.

(36) Schulz, K.; Schlenz, K.; Malt, S.; Metasch, R.; Romhild, W.; Dressler, J.; Lachenmeier, D. W. *J. Chromatogr., A* **2008,** *1211*, 113-119.

(37) Mills, G. A.; Walker, V.; Mughal, H. *J. Chromatogr., B* **1999**, *730*, 113-122.

(38) Abaloa, M.; Bayona, J. M.; Pawliszyn, J. *J. Chromatogr. A* **2000**, *873,* 107-115.

(39) Huang, Y.; Ortiz, L.; Garcia, J.; Aguirre, P.; Mujeriego, R.; Bayona, J. M. *Water Sci. Technol.* **2004**, *49*, 89-98.

(40) Kang, S. J.; Olmstead, K.; Takacs, K.; Collins, J. *EPA's Municipal Nutrient Removal Technologies Reference Document*, US Environmental Protection Agency: 2008.

(41) Water Environment Federation, *Biological Nutrient Removal (BNR) Operation in Wastewater Treatment Plants*, McGraw-Hill Professional: 2005.

(42) Lerner, M.; Stahl, N.; Galil, N. *Environ. Eng. Sci.* **2007**, *24*, 277-285.

(43) Kawaharasaki, M.; Tanaka, H.; Kanagawa, T.; Nakamura, K. *Water Res.* **1999,** *33*, 257-265.

(44) Oehmen, A.; Zeng, R. J.; Yuan, Z. G.; Keller, J. *Biotechnol. Bioeng.* **2005**, *91*, 43-53.

(45) Li, H. J.; Chen, Y. G.; Gu, G. W. *Bioresour. Technol.* **2008,** *99*, 4400-4407.

(46) Pijuan, M.; Casas, C.; Baeza, J. A. *Process Biochem. (Amsterdam, Neth.)* **2009,** *44*,

97-105.

(47) Zhou, Y.; Pijuan, M.; Zeng, R. J.; Yuan, Z. G. *Water Res.* **2009,** *43*, 1330-1340.

(48) Van Rijn, J.; Tal, Y.; Schreier, H. J. *Aquacult. Eng.* **2006**, *34*, 364-376.

(49) Carlson, C. A.; Ingraham, J. L. *Appl Environ Microbiol*. **1983**, *45(4)*, 1247-1253.

(50) DENARIAZ, G.; PAYNE, W. J.; GALL, J. L. *Int. J. Syst. Bacteriol.* **1989**, *39*, 145-151.

(51) Louis, P.; Duncan, S.H.; McCrae, S.I.; Jackson, M.S.; Flint, H.J. *J. Bacteriol*. **2004,** *186 (7*), 2099–2106.

(52) Narkis, N.; Henfeld-Furie, S. *Water Res.* **1978**, *12*, 437.

(53) Narkis, N.; Henefeld-Fourrier, S.; Rebhun, M. *Water Res.* **1980,** *14*, 1215.

(54) Lie, E.; Welander, T. *Water Res.* **1997**, *31*, 1269.

(55) Sans, C.; Mata Alvarez, J.; Cecchi, F.; Pavan, P. *Abstr. Pap. Am. Chem. Soc.* **1993**, *206*, 38.

(56) Jen, J.F.; Lin, C.W.; Lin, C.J.; Yan, C.T. *J. Chromatogr.* **1993,** *629*, 394.

(57) Manni, G.; Caron, F. *J. Chromatogr. A* **1995,** *690*, 37.

(58) Yan, C.T.; Jen. J.F. *Anal. Chim. Acta* **1992,** *259*, 259.

(59) Albaiges, J.; Casado, F.; Ventura, F. *Water Res.* **1986,** *20*, 1153.

(60) Wentzel, M.C.; Ekama, G.A.; Marais, G. *Water Sci. Technol.* **1991,** *23*, 555.

(61) Randall, A.A.; Benefield, L.D.; Hill, W.E.; Nicol, J.P.; Boman, G.K.; Jing, S.R. *Water Sci. Technol.* **1997,** *35*, 153.

(62) Eilersen, A.M.; Henze, M.; Kloft, L.*Water Res.* **1994**, *28*, 1329.

(63) Eilersen, A.M.; Henze, M.; Kloft, L. *Water Res.* **1995,** *29*, 1259.

(64) Liu, X. L.; Liu, H.; Du, G. C. *Water Environ. Res.* **2009**, 81, 13-20.

(65) Liu, X. L.; Liu, H.; Chen, J. H.; Du, G. C.; Chen, J. *Waste Manage.* **2008**, *28*, 2614-2622.

(66) Feng, L.; Chen, Y.; Zheng, X. *Environ. Sci. Technol.* **2009**, *43*, 4373–4380.

(67) Jiang, S.; Chen, Y.; Zhou, Q.; Gu, G. *Water Res.* **2007**, *41*, 3112-3120.

(68) Jiang, S.; Chen, Y.; Zhou, Q.; *Chem. Eng. J.* **2007**, *132*, 311-317.

(69) Zhang, P.; Chen, Y.; Huang, T.; Zhou, Q. Chem. Eng. J. **2009**, 148, 348-353.

(70) Chen, Y.; Jiang, S.; Yuan H.; Zhou, Q.; Gu, G. Water Res. **2007**, 41, 683-689.

(71) Yu, G. H.; He, P. J.; Shao, L. M.; He, P. P. *Water Res*. **2008**, *42*, 4637-4644.

(72) Al-Modhaf, H. F.; Hegazi, M. F.; Amer, S. A.; Abu-Shady, A. I. *Sep. Purif. Technol*. **2001**, *24*, 329-335.

(73) Lou, D. W.; Lee, X.; Pawliszyn, J. *J. Chromatogr. A* **2008**, *1201*, 228-234.

(74) Pan, L.; Pawliszyn, *J. Anal. Chem*. **1997**, *69*, 196-205.

(75) Mills, G. A.; Walker, V.; Mughal, H. *J. Chromatogr., B* **1999**, *730*, 113-122.