VALIDATION OF A SOLID-PHASE MICROEXTRACTION/ GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF TRACE VOLATILE COMPONENTS IN CHARDONNAY AND PINOT GRIS WINES

by

KAREN L. HOWARD

Submitted in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In the

Chemistry

Program

YOUNGSTOWN STATE UNIVERSITY

May 2003

Validation of a Solid-Phase Microextraction/Gas Chromatographic Method for the Analysis of Trace Volatile Components in Chardonnay and Pinot Gris Wines

KAREN L. HOWARD

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Karen L. Howard — 4-29-03

Karen L. Howard, Student — Date

Approvals:

James H. Mike — 4/29/03

James H. Mike, Ph.D., Thesis Advisor — Date

Daryl W. Mincey — 4/29/03

Daryl W. Mincey, Ph.D., Committee Member — Date

S. M. Schildcrout — 4/29/03

Steven M. Schildcrout, Ph.D., Committee Member — Date

Peter J. Kasvinsky — 5/1/03

Peter J. Kasvinsky, Ph.D., Dean of Graduate Studies — Date

ABSTRACT

Volatile compounds, even in trace quantities, greatly influence the flavor and aroma of wine. It is therefore important to identify these compounds, their origins in either the grape or the fermentation and aging processes, and the minimum levels at which they become detectable to human senses. This research provides a simple, inexpensive, solvent-free and fully validated method to identify and quantitate a number of important aroma components covering several organic classes. Solid-phase microextraction (SPME) was combined with gas chromatography/mass spectrometry (GC/MS) and automated sample handling to produce excellent sensitivity and reproducibility. Several internal standards were utilized to target the extraction and chromatographic behavior of each class of analytes in the matrix, providing more reliable quantitation than the single internal standard used in many other studies. Limits of detection for the target aroma components were in parts per million to parts per trillion levels.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to and love for my husband, David, and my daughter, Rachel. They spent countless hours without me and completed many tasks that should have been my responsibility in order to free my time for research and study. Without their support and love, this research and the resulting degree would have been infinitely more difficult.

I also wish to thank my research advisor, Dr. Jim Mike, for his patience with my task-oriented, get-it-done-now mentality. Thank you for the opportunity to present at Pittcon 2003 – a great learning experience! The other members of my committee, Dr. Steven Schildcrout and Dr. Daryl Mincey, offered valuable suggestions on my research and this thesis. Bruce Levison and Ray Hoff provided technical help and training that were invaluable for keeping the research moving forward. Dr. Roland Riesen, Director of Research for the Lake Erie Enology Research Center, has been an encouraging presence, smoothing some of the roadblocks as well as managing to pass along to this novice a bit of his vast knowledge of and love for enology. I am also grateful for Tünde Meyers, my office mate and group member, who brightened many days with laughter and friendship.

My deepest and most profound debt is to Jesus Christ, who gave himself in my place and then, as if His gifts were not already enough, gave me this dream and the chance to fulfill it. He fills my world with color and breathes into it the joyful dance of life. May my life, a single thread in a vast and extraordinary tapestry, bring honor to Him.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

A	Asymmetry factor
amu	Atomic mass unit
C_s	Molar concentration in the stationary phase
C_m	Molar concentration in the mobile phase
d_f	Film thickness
DVB	Divinylbenzene
EtOH	Ethanol
F	Flow rate
FID	Flame Ionization Detector
GC	Gas Chromatography
i.d.	Internal diameter
k'	Capacity factor
K_D	Partition coefficient
l	Column length
LEERC	Lake Erie Enology Research Center
LOD	Limit of detection
LOQ	Limit of quantitation
mg	Milligram
min	Minute
mL	Milliliter
mol	Mole
MS	Mass Spectrometry

LIST OF ABBREVIATIONS (CONTINUED)

MSD	Mass Selective Detector
MW	Molecular weight
m/z	Mass-to-charge ratio
N	Number of theoretical plates
N^*	Modified number of theoretical plates
π	pi (~3.14)
PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
r_c	Column radius
R_s	Resolution
RSD	Relative Standard Deviation
sec	Second
SIM	Selected Ion Monitoring
SPME	Solid-Phase Microextraction
t_o	Dead time
t_r	Retention time
TCD	Thermal conductivity detector
$\bar{u}$	Linear velocity
µL	Microliter

LIST OF ABBREVIATIONS (CONTINUED)

μm	Micrometer
V_R	Retention volume
W or W_b	Peak width at the base
$W_{½}$	Peak width at half peak height

DEFINITIONS OF TERMS

Aroma components – compounds which contribute to the taste and aroma of wine; they may originate in the grape must or may form during fermentation or aging.

Extraction – removal of the desired analytes from the aqueous wine sample for analysis.

Limit of detection – the lowest analyte concentration that produces a response detectable above the noise level of the system; typically, three times the standard deviation of the noise level.

Limit of quantitation – the lowest level of an analyte that can be accurately and precisely measured; typically giving a response that is at least ten times the standard deviation of the noise level.

Linearity – determination of a concentration range where analyte response plotted against concentration shows a linear relationship.

Must – a mixture of crushed grapes including the juice, pulp, seeds and skin; once the juice is separated from the solids and begins fermentation it may be called must, juice, or young wine.

Precision – the amount of scatter in the results obtained from multiple analyses of a homogeneous sample.

Racking – siphoning the clear juice or wine to separate it from accumulated sediment.

Range – the concentration interval over which acceptable accuracy, linearity and precision are obtained.

Recovery – determining, via standard additions or spiked samples, that the analyte response is consistent with the known concentration.

Separation – resolution of the chromatographic peaks to a level that allows quantitation.

Specificity – the ability of the method to accurately measure the analyte response in the presence of other sample components.

Stability – consistency of the response over a specified period.

Validation – the process of proving that an analytical method is acceptable for its intended purpose.

CHAPTER I

Introduction

A. Gas Chromatography

Chromatography is the name given to several powerful separation techniques that find application in all branches of chemistry. These techniques are designed to physically separate the components of a mixture based upon the interactions of each analyte with a mobile phase and a stationary phase. The mobile phase may be gaseous (as is the case with gas chromatography), liquid (as in liquid chromatography and thin-layer chromatography) or a supercritical fluid. The mobile phase is forced through an immiscible stationary phase commonly consisting of a solid or liquid polymer coated onto a solid support. The polymeric stationary phase may be held to the solid support by relatively weak intermolecular forces or limited crosslinking, or it may be chemically bound to the support. Gas chromatography is the separation method of choice for gaseous or volatile liquid analytes.

B. Component Retention and Separation

In liquid chromatography, the composition of the mobile phase greatly influences the separation of a mixture of analytes. In gas chromatography (GC), the mobile phase is a flowing, inert gas such as helium, hydrogen or nitrogen. The flowing gas serves only as a carrier to sweep a mixture of analyte solutes through the column toward the detector and has little or no impact on the separation of the analytes. The separation of the components of the mixture is accomplished primarily through interactions between the analytes and the stationary phase. The chromatographer exerts some control over the

separation by adjusting the temperature at which the separation is carried out and through selection of a stationary phase with a given set of chemical and physical properties.

Early GC experiments employed a wide bore column similar to the columns used in liquid chromatography. These were typically packed with spherical particles coated with stationary phase. However, these early columns required high gas pressures to force the carrier gas through the field of tightly packed stationary phase; this disadvantage inhibited the use of longer columns that might achieve greater resolution. Capillary columns were developed in response. These allow much greater resolution by utilizing thin stationary phase films coated on the inside walls while a narrow open core allows gas flow even at low carrier gas pressure. A typical capillary GC column is less than 1 millimeter in diameter and ranges from a few meters to more than 60 meters in length.

Each analyte exhibits characteristic partitioning into and out of the stationary phase based upon molecular weight, intermolecular forces and vapor pressure. When an analyte exhibits a high degree of partitioning into the stationary phase, it is said to be 'retained' by the column more effectively. Transport through the column to the detector takes longer, thus producing a longer retention time. This partitioning between the mobile phase and the stationary phase approximates an equilibrium process, as shown below:

$$A_{mobile} \leftrightarrows A_{stationary}$$

where A_{mobile} represents the analyte presence in the mobile phase and $A_{stationary}$ represents the analyte presence in the stationary phase. The equilibrium constant for this process is described by the following equation:

$$K_D = \frac{C_s}{C_m}$$

where C_s is the molar concentration of the analyte in the stationary phase and C_m is the molar concentration in the mobile phase. This equilibrium constant can also be called the distribution constant, the partition ratio or the partition coefficient.

The actual partitioning of each analyte is a complex process involving chemical and physical interactions as well as the volumes of the mobile and stationary phases. These volumes, particularly of the stationary phase, are difficult to know with certainty in gas chromatography. Therefore, peak retention is normally calculated based upon the capacity factor, k', which is defined as follows:

$$k' = \frac{t_r - t_o}{t_o}$$

where t_r is the retention time of a particular analyte and t_o is the 'dead time' or the time required for an unretained component to elute from the column. Small values for k' indicate little interaction between the analyte and the stationary phase, resulting in short retention times similar to those for the unretained reference peak. Higher values for k' correspond to longer retention times and indicate greater interaction between the analyte and the stationary phase.

The dead time is a common reference point that is dependent upon the head pressure and thus the linear velocity of the carrier gas. The relationship between linear velocity and dead time is:

$$\bar{u} = \frac{l}{t_o}$$

where $\bar{u}$ is the linear velocity of the carrier gas (cm/min), l is the column length (cm) and t_o is the dead time. In gas chromatography, volumes are often used to reference elution

points rather than time. The retention volume V_R of a component is defined as the volume of gas required to carry a component maximum through the column. It is related to retention time by the following equation:

$$V_R = t_r F$$

with the flow F described by:

$$F = \frac{\pi\left(r_c^2\right)l}{t_o}$$

The radius of the column (r_c) and the column length (l) are expressed in centimeters while the dead time (t_o) is expressed in minutes.

Column efficiency is the ability of a column to interact with a particular analyte in a way that allows elution of a sharp, clearly defined peak. There are two methods for calculating column efficiency based upon the historically used unit of N, or 'number of theoretical plates.' For peaks that exhibit a symmetrical Gaussian shape, as in the center peak of Figure 1.1 (1), the equation describing theoretical plates is:

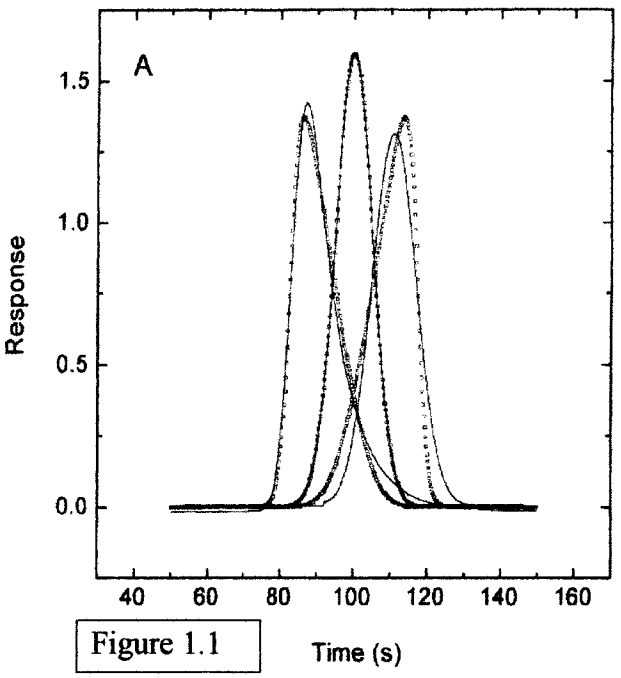


Figure 1.1

$$N = 16\left(\frac{t_r}{W_b}\right)^2$$

with N as the number of theoretical plates, t_r representing the retention time of the peak and W_b as the peak width at the base, in minutes. If a peak is non-Gaussian in shape as in the first (tailing) peak in Figure 1.1 or the third (fronting)

peak, the asymmetrical edge causes a larger baseline width than is truly representative. A modified equation that helps compensate for the irregular peak shape is:

$$N^* = 5.54\left(\frac{t_r}{W_{½}}\right)^2$$

where N^* is the modified number of theoretical plates, t_r is the peak retention time, and $W_{½}$ is the width of the peak at half the peak height. Moving halfway up the peak to measure the width lessens the effect of a fronting or tailing edge.

Peak resolution is defined as the degree of separation between two adjacent peaks on a chromatogram. The resolution factor, R_s, is calculated according to the following equation:

$$R_s = \frac{2\left[(t_r)_B - (t_r)_A\right]}{W_A + W_B}$$

where t_r is the retention time of the adjacent peaks for components A and B, and W is the width of each component peak at the base. A resolution of $R_s < 1$ indicates unresolved peaks while $R_s = 1$ indicates peaks which are adequately resolved for quantitation but do not exhibit baseline resolution. Baseline resolution occurs when the signal returns completely down to the background signal before rising for the start of the next peak. When $R_s \geq 1.5$, baseline resolution has been achieved.

Peak resolution is strongly affected by peak symmetry. A peak that exhibits a strong fronting or tailing edge may partially merge with an adjacent peak even though symmetric peaks of the same spacing would show full baseline resolution. Some types of interactions between the stationary phase and the analyte can lead to peak asymmetry. Often one class of analytes such as organic acids will exhibit asymmetry while all other

components produce symmetric peaks. Asymmetry can also be caused by various extra-column interactions that occur in the injector, detector or column connections.

To measure asymmetry, a perpendicular bisecting line must be drawn from the peak apex to the baseline. The peak width before and after the peak apex line is measured at 10% of the total peak height. Asymmetry is calculated as follows:

$$A_s = \frac{b}{a}$$

where b is the peak width after the apex line and a is the width before the line. A value of $A_s = 1$ indicates a mathematically symmetrical peak, $A_s < 1$ indicates a fronting peak and $A_s > 1$ indicates a tailing peak.

C. Detection of Solutes in Gas Chromatography

After the gas chromatograph separates the components of a mixture, they must be detected as they elute from the column. A number of GC detectors are available, depending upon the sensitivity and selectivity required. Flame ionization detectors (FID) are among the most common in gas chromatography. A flame ionization detector employs a hydrogen/air flame to combust organic analytes as they exit the column. As the analyte molecules combust, they decompose into ions. These ions are detected by an electrode, producing a signal. The FID is extremely sensitive and is relatively non-specific – it will combust and detect virtually all organic molecules. This non-specificity is both an advantage and a disadvantage because it allows detection of a wide range of analytes but cannot identify the organic classes to which they belong.

The same characteristics of high sensitivity and non-specificity apply to the mass selective detector (MSD). Mass selective detectors are rapidly gaining in popularity as

their increased sensitivity is brought to bear on a wide range of trace analyses. As the components elute from the column, they enter an ionization source that ionizes the molecules. The extra energy imparted by the ionization process often causes the molecules to fragment; this fragmentation can give information about the chemical identity and structure of the analyte. The MSD separates the analytes from each other based upon mass-to-charge ratio (m/z) using magnets, electrical fields or long flight tubes.

Both the FID and the MSD are classified as destructive detectors; each must chemically alter the analyte molecules in order to detect them. The MSD gives more information than the FID due to the fragmentation of the molecules and has greater sensitivity, particularly when used in the selected ion monitoring (SIM) mode. In this mode, target m/z ratios are selected for the analytes, and the MSD is programmed to scan rapidly from one target mass to the others, ignoring the other m/z ratios that may be present. In this way, more scans can be conducted within the elution window of a target analyte. This increased number of scans allows much greater sensitivity and is especially helpful in trace analysis.

D. Wine Aroma

The relationship between the chemical composition of wines and sensory evaluation is complex. Several hundred compounds have been identified as important contributors to wine aroma; most are present only in trace amounts. These aroma components comprise several classes of organic compounds including esters, alcohols,

organic acids, ketones, aldehydes and monoterpene alcohols. These trace aroma components are generally volatile and thus well-suited to gas chromatographic analysis.

Wine is a complex aqueous matrix containing ethanol, glycerol, varying levels of fructose and glucose depending on the wine type and fermentation stage, and high levels of non-volatile acids such as lactic acid and tartaric acid. Each of these components affects the extraction of the volatile aroma compounds for analysis.

E. Solid-Phase Microextraction

Before the aroma components can be separated and analyzed, they must be extracted from the wine matrix. Extraction techniques often used for this purpose are dynamic headspace, static headspace, purge-and-trap, solid-phase extraction and solvent extraction. Most of these techniques suffer from several disadvantages including extensive equipment requirements, the use of significant quantities of expensive and environmentally unfriendly solvents, multiple handling steps that may increase error and a need for concentration of the target analytes to detectable levels.

Development of a new extraction technique called solid-phase microextraction (SPME) was reported in 1990 by Arthur and Pawliszyn (*2*). This technique offers many advantages over others: it is solvent-free, environmentally friendly, relatively inexpensive, simple and portable. It also offers the advantage of concentration in the same step as extraction, thus reducing handling error while improving sensitivity. Furthermore, the lack of a large solvent component, which might otherwise overload the column, allows the use of splitless injection if desired. In this technique virtually the entire sample is placed onto the column, further improving the sensitivity of the method.

Solid-phase microextraction attaches a stationary phase coating to a silica fiber via covalent linkage or surface polymerization. Several coatings are available which are similar to the stationary phases used in capillary GC columns. Fiber coating selection is based primarily upon target analyte polarity and molecular weight, as some coatings demonstrate preferential extraction of lower molecular weight or more polar analytes and others are more suited to high molecular weight or nonpolar analytes. The fiber is inserted into a syringe assembly that is used to pierce the septa of sampling vials and the GC injection port while protecting the delicate fiber. The researcher has the option of lowering the fiber into the liquid sample itself (direct immersion) or suspending it in the headspace above the sample, as in Figure 1.2 below (*3*).

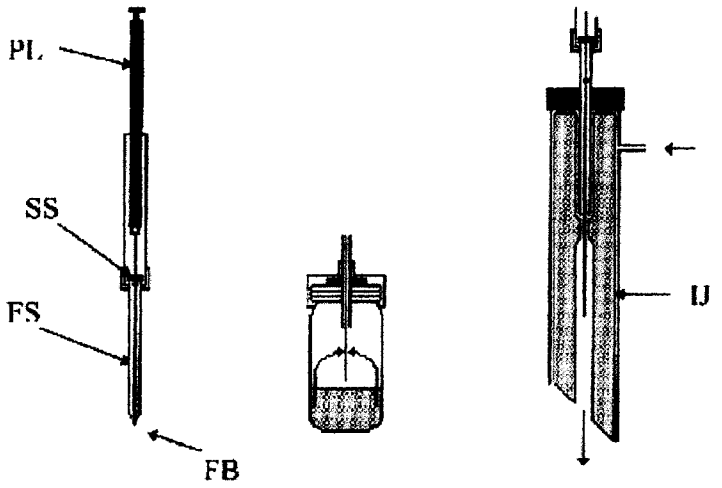


Figure 1.2

Schematic of the 'SPME' headspace sampling system. PL = Plunger, SS = sealing septum, FS = fiber sheath (pierces septum of vial and GC-injector), FB = SPME fiber, IJ = GC-injector.

For complex sample matrices, as in wine, headspace extraction avoids interference by nonvolatile components such as sugars, proteins and other high molecular weight

compounds. Headspace sampling also has the advantage of prolonging the lifetime of the fiber.

If the SPME fiber has a liquid coating such as the common polydimethylsiloxane (PDMS) or polyacrylate (PA) coatings, extraction occurs via absorption of the analytes into the coating. If one of the newer mixed coatings such PDMS–DVB (divinylbenzene), Carbowax–DVB or Carboxen is used, the primary extraction phase is a porous solid and extraction occurs via adsorption onto the surface as in Figure 1.3 below (*4*).

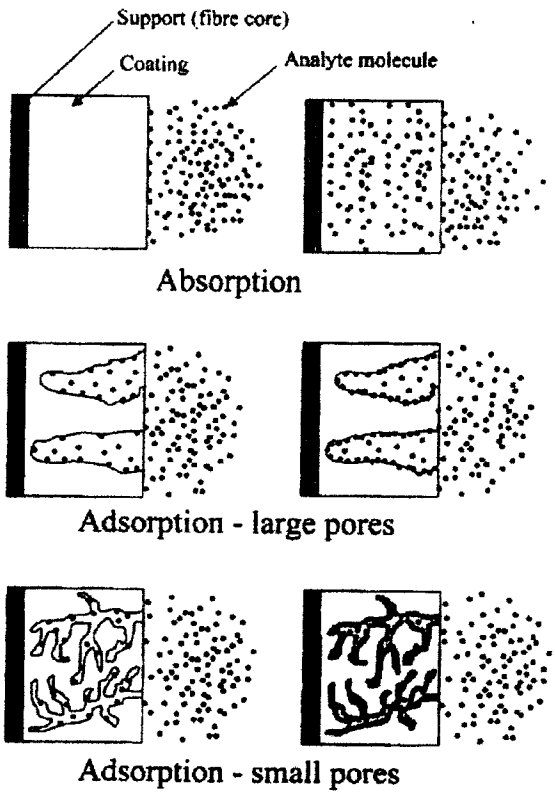


Figure 1.3 The frames on the left indicate conditions at the start of analyte exposure to the fiber; those on the right show the equilibrium conditions.

Analytes are retained in the pores or on the surface of the particles. DVB contains mesopores that extract larger analytes while Carboxen contains micropores that are ideal

for extracting smaller analytes. One drawback to the adsorption mode of extraction is the possibility of competition for limited sites on the surface, affecting linearity of extraction.

A layered DVB/Carboxen/PDMS fiber is available to expand the analyte range that can be extracted with one fiber. This fiber consists of a 110 μm fused silica core coated with a 25 μm inner layer of Carboxen particles suspended in PDMS and a 60 μm outer layer of DVB particles suspended in PDMS (see Figure 1.4).

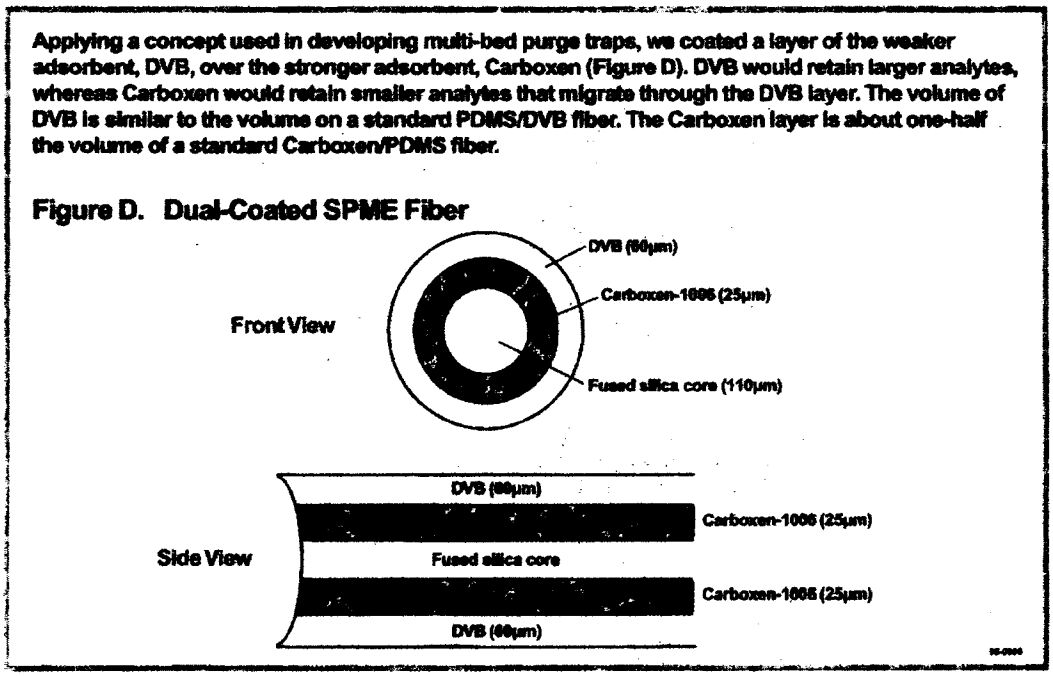


Figure 1.4 Obtained from Supelco, Inc. (Bellefonte, PA) by personal correspondence; used with permission.

Analytes can adsorb to the Carboxen and DVB particles as well as diffuse through the layers of PDMS coating (absorption), thus exhibiting both modes of interaction; however, Supelco lists adsorption as the dominant mode.

CHAPTER II

The Problem and Its Setting

A. Statement of the Problem

This research study involved development and validation of a solid-phase microextraction (SPME), gas chromatographic (GC) method for the analysis of essential trace components in Pinot Gris and Chardonnay wines produced by the Lake Erie Enology Research Center (LEERC). This study was designed to provide the LEERC with an efficient, validated method for the analysis of aroma components in these wines.

The first subproblem was the development of a protocol for extraction and separation of aroma volatiles typically found in wines. The second subproblem was the validation of this method to include determination of analyte specificity, linearity of standard curves, analyte recovery as a measure of method accuracy, definition of the analytical concentration range, analytical precision, limits of quantitation and detection, and long- and short-term method stability. The third subproblem was the identification and quantitation of the differences in the aroma composition of Pinot Gris and Chardonnay samples using the developed SPME/GC protocol.

B. The Hypotheses

At the onset of the research there were three hypotheses, one for each subproblem. First, that the protocol could be synthesized from various conditions presented in the literature and by experimentation with available equipment. The second hypothesis was that the developed method could be validated for all target components in both wine varieties. Third, that the spontaneous and inoculated musts would show

quantifiable differences in the date of appearance and final concentrations of aroma components.

C. The Delimitations

Early in the planning process it was decided that alternative conditions for separation, extraction, and quantitation would not be compared except as needed to find a valid method for analysis of the target aroma components present in Chardonnay or Pinot Gris wines. It was also determined that this study would not ascertain the applicability of the developed method to other varietal wines.

D. The Assumptions

As with all research, certain assumptions were made prior to beginning the study. The first assumption was that the sampling method employed by the LEERC gives repeatable samples that are representative of the entire batch. This is the most critical assumption, as the sampling process ultimately determines the fitness of the entire data set for any analysis. This is particularly true in trace analysis. Ideally, this assumption will be tested in future studies.

A second assumption focused again on the sampling process. The samples were drawn from the fermentation vessel, placed into plastic vials, capped and frozen to await analysis. Total storage time was more than one year. The assumption was that the concentrations of the aroma components present in the wines at the time of sampling would remain unchanged by the freezing and storage process. This assumption should also be tested in future research by the Lake Erie Enology Research Center.

A third assumption was that a single SPME/GC method would be suitable for analysis at any stage of fermentation, despite changes in wine composition. The fourth assumption was that the components on the LEERC list are critical components for this analysis. This assumption received some modification during the design portion of the research as a small number of components were deleted from the list of target analytes due to the unavailability of standards and were replaced by other components of interest. The overall change in the composition of the list was minor.

CHAPTER III

Literature Review

A. Purposes of Wine Analysis

There are several motives for the determination of trace components in wines. Volatile compounds, even in minute quantities, can greatly impact the flavor and aroma of wine. It is therefore important to identify these compounds, their origins in either the grape or the fermentation and aging processes, and the minimum levels at which they become detectable to the human senses of taste and olfaction.

Several traditional issues commonly propel the analysis of wine chemistry. First is the determination of wine origin. In Europe—and more recently in California (*5*)—objective determination of appellations (origin) has driven much research into chemical markers that can be used to identify wines (*6, 7*). Protection of the value provided by certifications and official designations prompts growers and governing bodies to detect and verify region of production and grape variety for wines.

Wine analysis can also be motivated by a desire to track and control chemical changes during fermentation. The fermentation process causes numerous changes in the chemical character of the must. These changes are influenced by a variety of factors including yeast species, temperature, grape variety, pH and levels of nutrients and sulfites. The concentrations of many macroscopic compounds change dramatically during fermentation; glucose, fructose, and other sugars decrease while ethanol and organic acid levels increase.

A third concern prompting analysis of wines is the detection of detrimental changes in wine flavor and aroma during aging and storage. These changes are

particularly characteristic of white wines, which lose their fruity aroma and flavor primarily due to hydrolysis of acetates (*8*). In a study of acetate and fatty acid ethyl esters, Vianna and Ebeler (*9*) discovered complex two-peak patterns that reveal rising and falling ester levels as fermentation progresses. This degradation in quality is accelerated by shipping and storage at elevated temperatures. Significant changes in the aroma of young Chardonnay wines occur in just five to seven days at 40°C (*10*).

Gonzalez-Viñas et al. (*11*) reported statistically significant increases in the levels of succinic acid, diethyl esters and acetaldehyde during aging. Each of these components negatively impacts the aroma. A corresponding decrease in acetate ester levels contributes to the loss of the fresh fruity qualities present in young wines (*11*). Chisholm and colleagues, in a study of Vidal blanc wines from the Lake Erie region (*12*), correlated gas chromatographic data with sensory evaluation by a trained panel. They reported a significant increase in vegetative and 'straw' odors upon aging, attributing these results to changes in terpene composition.

B. Key Trace Components of Wine

The relationship between chemical composition and sensory evaluation is complex. The major (macroscopic) components of wines are few in number and include ethanol, glucose, fructose, and glycerol as well as tartaric, malic, lactic and acetic acids. The minor or trace components are generally volatile and semi-volatile. For the purposes of this study these fall into five main groups: esters, organic acids, alcohols, monoterpene alcohols and ketones; a few target compounds do not fit easily into any of these categories.

Previous studies have identified levels of terpenoids and norisoprenoids (*5, 12, 13*), esters (*8, 12–14*), and alcohols (*12, 13*) in representative wines using various extraction and separation methods. Other researchers have focused on the odor impact of these components using gas chromatography/olfactometry based upon dilution analyses (*15*) or panel detection thresholds (*16–18*).

The sensory impression received from a wine involves not only the actual concentrations of various substances but also interactions within the wine matrix. The release of volatiles from the matrix is a complex series of equilibrium processes greatly affected by temperature. These equilibria can produce noticeable differences in the flavor impact of the various volatile components. Ferreira et al. (*18*) attempted to relate dilution values and odor unit values through a study of the gas-liquid partition coefficients of the key odorants. Although this research had only moderate success in classifying the odor spectrum of a Grenache red wine, the focus upon the role of equilibrium in the volatile fraction of wines represents a key step forward.

C. Traditional Extraction Methods

Many studies of wine aroma extract the entire volatile fraction using solvent extraction (*9, 11, 12, 17–23).* While this gives the total concentrations of all volatiles present, it exaggerates the odor impact of each by including the amount present in the liquid fraction. Only the amount that actually volatilizes under organoleptic conditions will contribute to the aroma. This limitation also applies to solid-phase extraction as demonstrated by Arrhenius et al. (*5*). In addition, each process has multiple handling steps; this increases the possibility that error will be introduced to the results.

Dynamic headspace sampling techniques such as purge-and-trap may appear to alleviate this difficulty. However, as described by Zhang et al. (*24*), the carrier gas is bubbled through the aqueous matrix, again extracting volatile components from the liquid phase rather than focusing exclusively on the amounts present at equilibrium in the headspace. Conversely, static headspace sampling (*25*) normally involves direct injection of the headspace sample into a gas chromatograph, giving a more accurate representation of headspace composition. A major drawback exists, however, in its use; many components require concentration before they can be detected by typical GC detectors.

D. Solid-Phase Microextraction

Despite advantages over other extraction methods as detailed in Chapter I, studies utilizing SPME have detected difficulties as well. Górecki and colleagues (*4*) determined that the newer porous solid coatings show a non-linear relationship between the amount of analyte extracted by the fiber and the concentration of that analyte in solution. Murray (*26*) determined that volatiles exhibit competition for the active sites on Carboxen/PDMS fibers, with higher molecular weight compounds displacing those with lower molecular weights. This may preclude the use of such fibers for complex samples such as wines.

Non-linear extraction appears to be an issue even when using the liquid-phase SPME coatings. Vaz Freire et al. (*27*) determined that PDMS fibers are most suitable for the analysis of esters, a conclusion verified by Vianna and Ebeler (*8*), while the polyacrylate coating is best for low molecular weight alcohols and terpenes. In 1996, Steffen and Pawliszyn (*28*) found that PA fibers were most suitable for polar compounds, whereas PDMS is recommended for the nonpolar constituents. However, while using

PDMS fibers, Bartelt (*29*) ascertained that polar compounds such as amines and alcohols were absorbed by the fibers in greater amounts than were hydrocarbons for a given retention index.

Matich and colleagues (*30*) found while using PDMS-coated fibers that higher molecular weight volatiles equilibrate much more slowly between the sample, headspace, and fiber coating than do lower molecular weight volatiles. In some cases, the headspace may be depleted of one or more high MW components without reaching equilibrium. They concluded that this limits the use of SPME for quantitation of complex systems.

Inconsistent results with standardization methods also appear in SPME studies. For instance, Vaz Freire et al. (*27*) concluded that internal standards gave more reliable data than the method of standard additions, while Vianna and Ebeler (*8*) found the internal standard method to be unreliable and recommended the use of external standards. Ortega and colleagues (*22*) used four internal standards, one for each of the four major classes of trace components studied in their research. This approach would certainly help to alleviate the possibility that the internal standard behaves differently than the target components.

E. Matrix Effects and Sample Preparation

Attempts to mimic organoleptic conditions for wine analysis still fall short. It is usual to add salts to a wine sample (*6, 7*); this promotes greater release of many analytes from the aqueous matrix into the headspace. It is even common practice to "salt out" the volatiles when doing solvent extraction (*17, 19–22*); this extracts polar compounds such as alcohols and acids from the aqueous matrix into the organic layer much more efficiently. However, it should be noted that this technique works against a goal of

mimicking volatile composition in the nose or mouth since it enhances extraction of less volatile components.

Another area where most research has departed from organoleptic conditions is in the adjustment of temperature to enhance absorption. Most studies (*8, 15, 26, 30*) test samples at ambient temperature, near 25°C. This practice could increase error due to daily variations in ambient laboratory temperature. One study (*7*) used an extraction temperature of 40°C, close to organoleptic conditions. It is generally accepted that increasing the sample temperature will release more volatiles from the matrix into the headspace (*24*); however, a higher temperature also results in greater release of the analytes from the fiber back to the headspace, and can degrade the fiber coating to the point where it loses its ability to absorb analytes (*24*).

De la Calle Garcia et al. tested extraction efficiency versus temperature by measuring flame ionization detector response (*6*). They found a sharp increase in FID response with an extraction temperature between 25° and 40°C, with a slower but continued rise through a maximum temperature of 70°C. A point of interest is the sharp increase in response at temperatures corresponding to mouth conditions. In contrast, Favretto and colleagues (*23*) found a general decrease in the levels extracted with a temperature increase from 25°C to 50° and 75°C, and recommend the lower temperature for extraction. It is interesting to note that this study did not add salts to the wine matrix.

A research group at the Cornell University Department of Food Science and Technology has done extensive work with a mouth simulator called the retronasal aroma simulator (RAS). The RAS is specifically designed to mimic mouth conditions, including temperature, airflow due to breathing, and saliva (*31*). SPME can be used to

sample the airflow that passes through the headspace in the RAS. This shows great promise for accurate analysis that gives results quite similar to actual organoleptic conditions. However, this method has not yet been tested on wine samples and is presently impractical for large scale, rapid testing of multiple samples.

F. Need for Present Research

Numerous studies have been published which claim to determine the levels of trace aroma components in wines. Unfortunately, many of these methods have never been fully validated and thus cannot be used for quantitative comparisons. An additional limitation is the focus of many previous studies on a small number of analytes, often comprising only one class of organic compounds. Such a method cannot succeed in truly characterizing aroma, which derives from complex interactions among a wide range of organic components.

Solid-phase microextraction shows much promise as a rapid, environmentally friendly, inexpensive, solventless method for analysis of wine. It has been used to analyze wine in many previous studies but is hampered by competitive selection of certain components in the matrix. An attempt to avoid this difficulty may be the reason the SPME studies already described have generally been limited to a narrow class of compounds such as esters or terpenes. Other research studies have cast their analytical net too wide, seeking to determine as many concentrations as possible regardless of the impact of each component on wine aroma.

Despite the vast quantity of wine analysis in the literature, a strong need existed to develop an efficient, validated method able to identify and quantitate key trace volatile components of wine, thus providing for the traditional analytical needs: origin

determination, quality control of fermentation and analysis of the aging process. Ideally, the method must extract those components most essential to the aroma and character of wine in ratios that are consistent with their concentrations in the headspace and should be easily adaptable to needs that may arise in the future. This study presents such a method.

CHAPTER IV

Materials and Equipment

A. Chemical Standards

All reagents used in this research were of the highest purity available and were used as received. The chemical standards are listed in Table 4.1 with their purity and source.

Table 4.1 Chemical Standards, Purity, and Source

STANDARD	PERCENT PURITY	SOURCE
ESTERS		
Ethyl acetate	99.9	Fisher
Isoamyl acetate	98	Aldrich
Ethyl hexanoate	99+	Aldrich
Ethyl lactate	98	Sigma
Ethyl octanoate	99+	Aldrich
Ethyl decanoate	99+	Aldrich
Diethyl succinate	99	Aldrich
2-Phenethyl acetate	99+	Fluka
Ethyl isovalerate	98	Aldrich
Hexyl acetate	99	Aldrich
Ethyl butyrate	99	Aldrich
Ethyl isobutyrate	99	Aldrich
Ethyl benzoate	99+	Aldrich
ORGANIC ACIDS		
Isobutyric acid	99	Aldrich
Butyric acid	99+	Aldrich
Hexanoic acid	99.5	Aldrich
Octanoic acid	99.5	Aldrich
Decanoic acid	99+	Aldrich
Isovaleric acid	99	Aldrich
ALCOHOLS		
2-Methyl-1-propanol	99.5	Aldrich
2-Methyl-1-butanol	99+	Aldrich
3-Methyl-1-butanol	98	Aldrich
Hexanol	99+	Aldrich
Phenethyl alcohol	99	Aldrich
cis-3-Hexen-1-ol		Aldrich

Table 4.1 (continued)

STANDARD	PERCENT PURITY	SOURCE
MONOTERPENE ALCOHOLS		
Linalool	97	Aldrich
Linalool oxides	97+	Fluka
α-Terpineol	90	Aldrich
Nerol	97	Aldrich
Geraniol	98	Aldrich
β-Citronellol	95	Aldrich
CYCLICS, KETONES, AND OTHERS		
4-Vinylguaiacol	97	Research
Guaiacol	98	Aldrich
Furaneol (HDMF)	99+	Fluka
Acetaldehyde	99	Aldrich
2,4,6-Trichloroanisole	99	Aldrich
γ-Butyrolactone	99+	Aldrich
Diacetyl	97	Sigma
INTERNAL STANDARDS		
Ethyl 2-hydroxyvalerate		Sigma
Ethyl nonanoate	97	Research
Valeric acid	99	Eastman
4-Methyl-2-pentanol	99	Aldrich
9-Decenol	97	Aldrich
1,6-Heptadien-4-ol	97	Aldrich
4-Hydroxy-4-methyl-2-pentanone	99	Aldrich
REAGENTS		
Ethanol	95	Pharmco
Tartaric acid	99.8	Fisher
Sodium sulfate decahydrate	98	Acrōs
Sodium hydroxide	98	Fisher
Modulab water		In-House

Source	**Full Name and Location**
Acrōs	Acrōs Organics (Geel, Belgium)
Aldrich	Aldrich Chemical Company Inc. (Milwaukee, WI)
Eastman	Eastman Organic Chemicals (Kingsport, TN)
Fisher	Fisher Scientific (Fair Lawn, NJ)
Fluka	Fluka (Switzerland)
Pharmco	Pharmco Products Inc. (Brookfield, CT)
Research	Research Chemicals Ltd. (Heysham, Lancaster, UK)
Sigma	Sigma Chemical Co. (St. Louis, MO)

B. Chromatographic Supplies

The chromatography gases were as follows: ultra-high purity helium as the carrier gas, ultra-high purity hydrogen to fuel the FID flame, and hydrocarbon-free air as an oxidizer for the FID flame. All gases were obtained from Praxair (Cleveland, OH). Water and oxygen traps were installed on all carrier gas lines.

The primary chromatographic phases (Supelcowax-10 column and DVB/Carboxen/PDMS SPME fibers) were purchased from Supelco (Bellefonte, PA). The DB-1 and DB-5 columns were from Alltech Associates (Deerfield, IL). Columns included the following:

- A 10-meter, 100 μm i.d. DB-1 with 0.25 μm film thickness
- A 30-meter, 250 μm i.d. DB-5 with 0.25 μm film thickness
- A 30-meter, 250 μm i.d. Supelcowax-10 with 0.25 μm film thickness
- Polar and non-polar retention gaps ranging from one to three meters in length.

SPME fibers tested included the manual injection polydimethylsiloxane (PDMS) fiber with 7 μm phase coating thickness (known as the 7 μm PDMS), a 100 μm manual injection PDMS fiber, and both the manual injection and autosampler versions of the 50/30 μm DVB/Carboxen/PDMS fiber described in detail in Chapter I. The autosampler DVB/Carboxen/PDMS fibers were custom-fitted by Supelco with a 23-gauge fiber guide to interface with a Merlin Microseal septum.

C. Wines

One goal of this research was an analysis and comparison of fermentation lots of Chardonnay and Pinot Gris wines fermented at Youngstown State University under

different yeast conditions. The grape must was obtained from regional wineries. Markko Vineyards provided 20 gallons of Chardonnay must from their October 4, 2001 harvest; the Kingsville Grape Research Branch (OARDC) provided 20 gallons of Pinot Gris must from their October 1 harvest. Each must was divided evenly into 10-gallon lots for inoculated (control, C) and spontaneous (S) fermentations. The musts were allowed to settle by gravitation for 24 hours at 4°C.

After settling, the musts were racked and portioned into 3-gallon glass fermentation vessels. This resulted in three control fermentations and three spontaneous fermentations for each grape variety. The control lots were treated with 50 ppm SO_2 to inhibit the indigenous yeast and then inoculated with commercial freeze-dried yeast (Prise de Mousse, EC 1118). The spontaneous lots were left untreated and were allowed to ferment via native yeasts present in the must. Twelve fermentations were thus established, identified as: Chardonnay C-1, C-2, C-3, S-1, S-2, and S-3 plus Pinot Gris C-1, C-2, C-3, S-1, S-2, and S-3.

The vessels were kept at a room temperature of approximately 25°C during fermentation. A 50-mL sample of juice was drawn each day by pipette from the center of the fermentation vessel and divided into portions for analysis. Of this total, 25 mL were reserved for the analysis that was conducted through this study. This aliquot was placed into a plastic vial with screw cap and stored frozen at –80°C until analysis. Once the fermentation process was completed, the wine was racked to separate it from the settled yeast and other sediment (the *lees*). Cold stabilization allowed tartrates to precipitate for removal. The final 50-mL sample from each fermentation batch was taken after cold stabilization; the wine was then bottled.

D. Equipment

Initial method development was conducted on a Varian STAR 3400 CX gas chromatograph using a flame ionization detector and STAR Version 4.0 chromatography software. All injections were done manually. The injector was initially fitted with an open glass inlet liner having an internal diameter of 3.4-mm for direct injection of samples. A 10-μL glass syringe with fixed needle was used for injections. A 0.8-mm I.D. glass inlet liner was used in the injector during the development of the solid-phase microextraction method. Sample vials with volumes of 2- and 4-mL with screw caps and Teflon-lined septa were used for all samples and standards. These vials were stored at room temperature when not in use.

GC method fine-tuning, mass spectrometric method development and all method validation procedures were conducted on a Varian 3800 GC using a Varian Saturn 2000 ion trap MS/MS as the detector and STAR Version 5.52 chromatography software. This instrument was fitted with a Combi-Pal Autosampler (CTC Analytics, Zwingen, Switzerland) used in SPME mode throughout validation. The injector was fitted with a Merlin Microseal septum and a 0.75-mm I.D. glass inlet liner. During validation, 10-mL sample vials with magnetic crimp caps and Teflon-lined septa were used. These vials were refrigerated when not in use. Each was used for only one sample, although perhaps for multiple injections, and then discarded.

CHAPTER V

Methods

A. Column Selection and the GC Separation Program

The first column tested for suitability was coated with a DB-5 stationary phase. This stationary phase was poly(5% diphenyl/95% dimethylsiloxane) coated on the inner walls of a fused silica column. It was a fully bonded phase with wide temperature limits. The reasons for starting with this column included not only its reputation as a rugged, durable method development column but also because the intent of the work was for adoption by other laboratories, the wide prevalence of DB-5MS columns made its selection attractive. Because the goal was to move this method to a GC/MS instrument, use of a DB-5 column for initial development seemed prudent.

The DB-5 is a fairly nonpolar phase that can be expected to release polar and low molecular weight analytes easily while retaining the high molecular weight and highly nonpolar compounds. This proved to be the case. Unfortunately there were column interactions with some compounds that affected the separation. Several clusters of compounds proved essentially impossible to separate. In particular, the organic acids demonstrated a strong fronting effect. This caused difficulty in achieving a well-resolved separation from neighboring peaks. Even when different temperature ramps and head pressures were tested, no significant improvement in the fronting of these peaks was attained.

To assist in improving the separation, various retention gaps were placed in front of the DB-5 column to act as a pre-separation phase. Highly polar segments of 1 to 3 meters in length proved unsuccessful; the same was true of highly nonpolar segments.

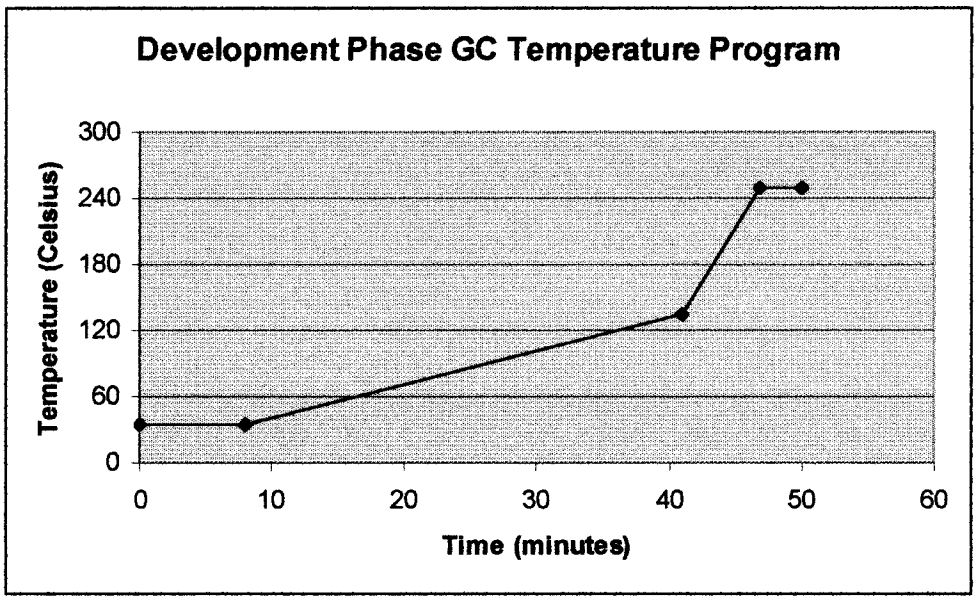
Finally, a 10-meter length of a microbore (0.100 mm I.D.) DB-1 column was coupled in front of the 30-meter DB-5 column. The DB-1 stationary phase was 100% poly(dimethylsiloxane), or PDMS, fully bonded and with a wide operating temperature range. Although this pre-column greatly improved resolution of several analytes, separation was not optimal and this column combination was abandoned. In part this was prompted by concern over extra-column band broadening that was expected to occur within the glass connector used to couple the columns.

A wide range of temperature programs was utilized throughout this phase of the column selection process. Isothermal and various gradient programs were checked with each column configuration. Continual gradient runs with no hold periods were tested, as well as programs incorporating several hold periods or temperature plateaus.

The final column tested was a Supelcowax-10 column consisting of a 100% polyethylene glycol (PEG) phase. Although it is listed by the manufacturer as a bonded phase, deeper investigation into the actual nature of the phase led to the conclusion that a more accurate description was 'highly crosslinked' rather than bonded. This stationary phase had a relatively high polarity and a more limited temperature range than the DB- phases that were tested. Supelco (Bellefonte, PA) does not warrant this column to be low-bleed for mass spectrometric analysis; however, it was the lowest-bleed column available with a strongly polar stationary phase. It was hoped that this phase would retain the acids much more effectively than the less polar columns already tested, pulling the acids away from other analytes and thus removing their interfering effect. As hoped, this was the case and peak resolution improved greatly. The first of six acids eluted from the column nearly 35 minutes into the temperature program.

For the optimal GC separation program, carrier gas column flow at the initial temperature of 35°C was approximately 1.0 mL per minute and was measured at the detector with an electronic flowmeter. The corresponding injector head pressure at this flow rate was approximately 13 psi. The detector gases were hydrogen (30 mL/min) and air (300 mL/min), again measured at the detector. The injector temperature was set at 250°C with the FID heated to 270°C. Note that the maximum column temperature for the Supelcowax-10 column was 280°C. The column oven temperature program held at 35°C for 8 minutes, then increased at a rate of 3°C per minute to 150°C with no hold period; the final segment of the program was a ramp of 15°C per minute to 250°C with a 2-minute hold. The overall GC run time was 55 minutes. The temperature program is graphically represented in Figure 5.1 below.

Figure 5.1



Good resolution was achieved with this program for all target analytes, with full baseline resolution for all but four closely eluting components.

Injections were performed manually during method development. In addition, significantly higher concentrations of analytes were used for ease of peak identification and determination of resolution. SPME injections were made by extracting the analytes from the headspace above the sample for periods ranging from 30 seconds to 10 minutes, depending on analyte concentrations. The SPME fiber was desorbed in the injector in splitless mode for 1.5 minutes and then remained in the injector for an additional 3-minute period in split mode with a 100:1 split ratio. This extra desorption time in split mode was used to remove any remaining analytes from the fiber in order to reduce carryover from one sample to the next. Initially a 10-minute split mode desorption was utilized. However, the Thermogreen septa that were used would sometimes leak when the fiber was removed after this length of time, releasing head pressure.

B. Selection of the SPME Fiber

Throughout the GC program development and column selection processes described above, the SPME fiber used was a 7 μm PDMS fiber. The coating on this fiber is virtually identical to the stationary phase that coats a DB-1 column and was expected to have nearly identical selectivity for high molecular weight and strongly nonpolar compounds. Once an optimal GC separation was obtained, the fiber selection phase began.

A more diluted mixture of standards was made to examine the extraction of the analytes using various fibers. This mixture was tested using the 7 μm PDMS fiber, the 100 μm PDMS fiber, and the 50/30 μm DVB/Carboxen/PDMS fiber (the 'mixed' fiber). As expected, the PDMS fibers exhibited greater extraction of the high MW, nonpolar

compounds than did the mixed fiber. However, the mixed fiber exhibited significantly greater extraction of both the lower molecular weight and the more polar compounds. Overall, it was determined that the gain in extraction for the polar compounds exceeded the loss of extractability for the nonpolar components, and the DVB/Carboxen/PDMS fiber was chosen as the preferred fiber for this research.

C. Selection of Internal Standards

The decision was made early in the research to include several internal standards covering a range of molecular weights and polarities in order to more closely match the varying interactions of the target analytes during the various phases of extraction and analysis. A search was conducted to determine a group of appropriate internal standards. Ortega et al. (*22*), used a mixture of 2-butanol, acetone, ethyl 2-hydroxypentanoate (ethyl 2-hydroxyvalerate), pentanoic acid (valeric acid), 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, and 2-octanol. This group of compounds could be used to mimic the chromatographic behavior of the esters (ethyl 2-hydroxyvalerate), organic acids (valeric acid), alcohols (4-methyl-2-pentanol, 2-butanol, and 2-octanol), and ketones (acetone and 4-hydroxy-4-methyl-2-pentanone). In selecting internal standards for the monoterpene alcohols, both the functionality (diene alcohols) and molecular weights were considered. Two potential internal standards were selected: 1,6-heptadien-4-ol to match the functionality, and 9-decenol to more closely match the molecular weight while still retaining some common functionality.

These potential internal standards were tested using the identical extraction and separation conditions already established, and six were selected based upon retention

times that showed no interference with any target analytes. The literature was searched to verify that none of these six components had ever been identified as a natural component of wine. Thus an internal standard mixture containing at least one component for each major class of target analytes was in place, as listed below in Table 5.1.

Table 5.1 Internal Standards and Related Organic Classes

TARGET ORGANIC CLASS	INTERNAL STANDARD SELECTED
Esters	Ethyl 2-hydroxyvalerate
Acids	Valeric acid
Alcohols	4-Methyl-2-pentanol
Ketones	4-Methyl-4-hydroxy-2-pentanone
Monoterpene Alcohols	9-Decenol and 1,6-Heptadien-4-ol

Two of these internal standards were subsequently removed from the study. Ethyl 2-hydroxyvalerate was determined to be an inappropriate internal standard for the esters and was replaced by ethyl nonanoate. Further details of this change will be given in Chapter VI. Also, one of the monoterpene alcohol internal standards, 9-decenol, was removed when a closely matching mass spectrum was detected in unspiked wine at approximately the same retention time. It was not replaced because a second monoterpene alcohol internal standard (1,6-heptadien-4-ol) was already in place.

D. Development of the Mass Spectrometric Detection Method

At this point in the investigation, the method was moved to the Varian 3800 GC–Saturn 2000 MS/MS for refinement and validation. The only significant changes in

instrumentation were the use of an autosampler, the addition of the mass selective detector (MSD) and the use of a Merlin Microseal septum. The Merlin Microseal necessitated the use of custom-made 23-gauge SPME fibers.

An initial volume of 500 mL of the highest concentration mixed standard (MS 150) was prepared, with target analytes added at approximately 150% of the expected concentrations in wine (see Table 5.2).

Table 5.2 Preliminary Composition of the MS 150 Mixed Standard

Name	Conc. (mg/L) or (ppm)
Ethyl acetate	225.0
Isoamyl acetate	7.77
Ethyl hexanoate	3.04
Ethyl lactate	75.27
Ethyl octanoate	4.65
Ethyl decanoate	3.15
Diethyl succinate	18.2
2-Phenethyl acetate	1.55
Ethyl isovalerate	0.23
Hexyl acetate	0.99
Ethyl butyrate	1.39
Ethyl isobutyrate	0.31
Ethyl benzoate	0.30
Isobutyric acid	5.72
Butyric acid	4.21
Hexanoic acid	15.2
Octanoic acid	17.2
Decanoic acid	4.52
Isovaleric acid	2.81
Isobutanol	61.7
2- and 3-Methyl-1-butanol	300.3 each
Hexanol	7.50
2-Phenethyl alcohol	5.83
cis-3-Hexen-1-ol	2.28
Linalool oxides	0.22
Linalool	0.53
α-Terpineol	0.24
Nerol	0.33

Geraniol	0.73
β-Citronellol	0.61
4-Vinylguaiacol	0.42
Guaiacol	0.08
γ-Butyrolactone	15.31
Furaneol	1.14
Acetaldehyde	138.6
Diacetyl	3.14
2,4,6-TCA	0.07
Valeric acid	**25**
4-Methyl-2-pentanol	**25**
Ethyl 2-hydroxyvalerate	**25**
1,6-Heptadien-4-ol	**25**
4-Hydroxy-4-methyl-2-pentanone	**25**
9-Decenol	**25**

Note: Components listed in bold print are internal standards.

This batch of MS 150 was used for final development and initial validation work including self-depletion and early linearity experiments.

The MS 150 solution was serially diluted as needed to produce mixed standards that had 50%, 75%, 100%, and 125% of the expected concentration of each analyte in wine. These were labeled MS 50, MS 75, MS 100 and MS 125. The diluting solution was a synthetic blank model wine prepared using in-house Modulab deionized water plus 12% aqueous ethanol and 7.0 g/L tartaric acid, with the pH adjusted to 3.3 using 6M sodium hydroxide. These conditions were selected to mimic the wine matrix as closely as possible for ethanol content, total acidity, and pH.

A new batch of master mixed standard solution (MS 150) was prepared prior to the final validation runs. Some components were eliminated from the analysis during the study and a new internal standard was added as previously mentioned. Concentrations were also adjusted to match the levels found in wine during preliminary validation. The master standard was then diluted 1:1 with synthetic blank wine to bring the

concentrations to levels appropriate for mass spectral analysis. The master MS 150 standard was then diluted with synthetic blank wine to form four additional solutions with concentration ranges of approximately 50 – 125% of the levels expected in wine. Table 5.3 indicates the concentrations of the mixed standard solutions; all analytes were present in the parts per million (ppm) or parts per billion (ppb) range. The abbreviation 'IS' indicates an internal standard.

Samples of wine (2001 Chardonnay, batch C-1 and 2001 Pinot Gris, batch S-1) were spiked with internal standards for use during validation. The spiked wine was then diluted 1:1 to reduce the analyte concentrations to appropriate levels for mass spectral analysis. The concentrations of internal standards present in the final diluted wine are shown in Table 5.4.

The GC portion of the separation method was maintained in essentially the same form as that used during development with a few notable exceptions. The program was modified to run at a constant column flow rate of 1.0 mL/min rather than at constant head pressure. The constant head pressure method was used during development due to instrument limitations; constant flow was not possible. However, when this method was monitored on the Varian 3800 GC, the constant head pressure combined with changing column temperature caused a significant drop in column flow during the program. The rate fell from 1.0 mL/min to a final rate of just 0.4 mL/min. Although this decrease in column flow is not a difficulty when using the FID, the mass selective detector needed to be calibrated with a consistent amount of material entering the ion trap. A steady drop in the rate at which material exited the column during the run altered the calibration and could have produced inaccurate results.

Table 5.3 Final Composition of the Mixed Standard Solutions

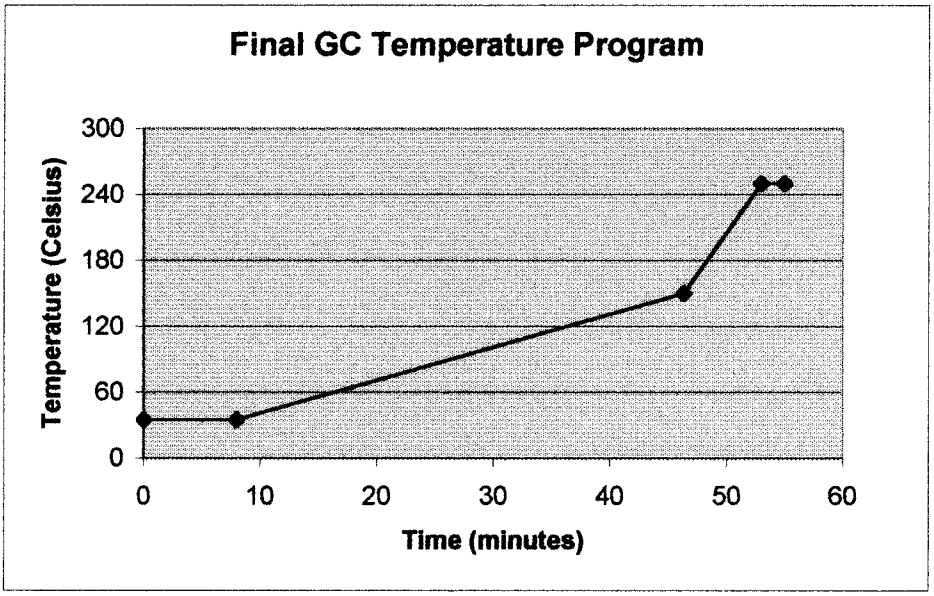
Analyte	Concentrations (parts per million)				
	MS 50	MS 75	MS 100	MS 125	MS 150
Acetaldehyde	17.7	26.55	35.4	44.25	53.1
Ethyl acetate	16.73	25.10	33.47	41.83	50.20
Ethyl isobutyrate	0.04360	0.06540	0.08720	0.1090	0.1308
Ethyl butyrate	0.08540	0.1281	0.1708	0.2135	0.2562
Ethyl isovalerate	0.00617	0.00925	0.0123	0.0154	0.0185
Isobutanol	3.42480	5.13720	6.84960	8.56200	10.2744
Isoamyl acetate	0.33537	0.50305	0.67073	0.83842	1.0061
4-Methyl-2-pentanol (IS)	3.1858	4.7787	6.3715	7.9644	9.5573
3-Methyl-1-butanol	33.15	49.73	66.30	82.88	99.45
Ethyl hexanoate	0.61010	0.91515	1.2202	1.5253	1.8303
Hexyl acetate	0.01938	0.02908	0.03877	0.04846	0.05815
1,6-Heptadien-4-ol (IS)	3.23048	4.84573	6.46097	8.07621	9.69145
Ethyl lactate	19.08	28.63	38.17	47.71	57.25
Hexanol	0.36867	0.55300	0.73733	0.92167	1.1060
4-Hydroxy-4-methyl-2-pentanone (IS)	16.88	25.33	33.77	42.21	50.65
Ethyl octanoate	2.56380	3.84570	5.12760	6.40950	7.69140
Linalool oxide	1.0835	1.6253	2.1671	2.7088	3.2506
Ethyl nonanoate (IS)	2.8016	4.2024	5.6031	7.0039	8.4047
Ethyl decanoate	3.72843	5.59265	7.45687	9.32108	11.1853
Diethyl succinate	0.74620	1.1193	1.4924	1.8655	2.2386
2-Phenethyl acetate	0.03973	0.05960	0.07947	0.09933	0.1192
2-Phenethyl alcohol	53.47	80.20	106.9	133.7	160.4

Table 5.4 Internal Standard Concentrations in Wine Samples

	Concentration in Spiked Wine (parts per million)	
Internal Standard	in Chardonnay	in Pinot Gris
4-Methyl-2-pentanol	25.642	21.942
1,6-Heptadien-4-ol	33.184	26.656
Ethyl nonanoate	26.220	28.430
4-Hydroxy-4-methyl-2-pentanone	98.874	107.138

Difficulties also occurred with matching known analyte peaks to the expected mass spectra from the stored libraries. It was determined that chemical ionization was occurring in the trap due to high analyte concentrations reaching the trap. A split method was developed to alleviate this problem (refer to section E.2 below). After extensive comparison of the results from the split and splitless methods, the split method was selected for the remainder of the research. Conditions were as follows: a starting temperature of 35°C was held for 8 minutes then raised by 3°C per minute to 134°C. A final ramp of 20°C per minute increased the temperature to 250°C where it was held for 3.2 minutes. The overall GC runtime was 50 minutes; the final temperature program is represented graphically in Figure 5.2 below.

Figure 5.2



The mass spectrometer was set in electron ionization (EI) mode and programmed to begin scanning 1.0 minutes into the GC run, using a scan time of 0.37 seconds per scan and covering a mass-to-charge (m/z) range from 25 to 215.

E. Determination of Sampling Parameters

1. Salting Out

Virtually all published methods for the analysis of aroma volatiles in wine utilize salting out before extraction. Salting out involves the addition of a non-competing inorganic salt to the aqueous matrix, altering the equilibrium distribution of analytes between the matrix and the headspace and increasing the levels of many analytes in the headspace.

A variety of inorganic salts can be used in both headspace and direct immersion extraction; saturated NaCl (*6, 7, 27, 28*) and $(NH_4)_2SO_4$ (*18-22*) have both been used with success. The use of mixed salts was tested by Sáenz-Barrio et al. (*32*). They found a mixture of $(NH_4)_2SO_4$ and NaH_2PO_4 [2.5:1 (w/w)] more effective for increasing recoveries during solvent extraction than sodium chloride alone or in mixtures.

Although salting out opposes a goal of matching organoleptic conditions, it was decided for this research to focus instead on getting a complete analysis of the target aroma components present in the matrix. Thus salting out was utilized. This study used $Na_2SO_4 \cdot 10\ H_2O$ that had been heated at 210°C for 24 hours to remove the waters of hydration and any low-boiling organic contaminants. The resulting anhydrous salt was stored in a desiccator at room temperature. Sodium sulfate was selected for its high solubility in aqueous solutions and the production of triple-molar amounts of ions upon dissociation. Both of these factors lead to high ion loads in the wine, enhancing the effects of salting out. For anhydrous

sodium sulfate the saturation level was found to be 2.0 grams of Na_2SO_4 per 6.0 mL of aqueous sample. This level was used throughout the validation.

2. Extraction Time

The length of time that the SPME fiber was exposed to the headspace had a significant effect on the extraction. Highly volatile analytes were present in the headspace at high levels; as they were extracted they continued to exit the matrix to the headspace at rapid rates due to their high vapor pressures. Headspace concentrations of low volatility analytes, even when allowed to reach equilibrium with the matrix prior to extraction, were soon depleted in the headspace by absorption/adsorption to the fiber. Because they volatilized much more slowly, they did not replenish in the headspace during extraction. The result of this difference in volatility could have been an overrepresentation of relatively more volatile analytes on the fiber as compared to the other analytes.

Extraction studies were conducted to determine the optimum time to hold the fiber in the headspace of the sample. If the fiber remained in the headspace too long, it was feared that the relative amounts of various analytes might not be accurately represented. However, an abbreviated sampling time could have led to inconsistencies in concentration because the analyte levels in the headspace and the fiber coating did not have sufficient time to come to equilibrium. Autosamplers such as the Combi-Pal that give consistent sampling and injection times for multiple runs can help to alleviate analytical variability due to shortened sampling times.

In this study, six vials were prepared using MS 100 and salt. Each vial was heated to 40°C and agitated to equilibrate the sample with the headspace prior to the extraction. The samples were extracted for a pre-determined exposure time of 5, 10, 15, 20, 30 or 45 minutes. Each vial was extracted three times to observe possible differences due to depletion of analytes. The average peak areas of selected analytes for the triplicate runs were plotted against extraction time (see Figure 6.4) to determine the time required for equilibration of each analyte between the headspace and the fiber. This data was used to determine an optimum extraction time to maximize analyte extraction while minimizing sampling time.

Based upon these results, the final Combi-Pal program was set as follows: vials were heated at 40°C for a 5 minute pre-extraction period with agitation of 500 rpm. The SPME fiber was then inserted into the headspace, where extraction occurred for 30 minutes with continuation of the temperature and agitation. The fiber was subsequently desorbed in the injector for 8 minutes with a 50:1 split.

3. Self-Depletion

Five vials were prepared using MS 100 and sodium sulfate. The first vial was extracted five times using the extraction time and program described above. The second vial was extracted in triplicate and the remaining vials were extracted once each. Peak areas were determined and the relative standard deviations (RSD) of the runs were calculated. Run-to-run depletion of several esters was deemed to be significant (refer to Chapter VI for details); for the remainder of the

research, only the first extraction from each vial was used to quantitate these components.

F. Validation of the Method

1. Stability

Stability is a measure of the length and conditions of sample storage that produce consistent results for a given method. Because run-to-run depletion had already been established within a single sample vial, it was necessary to test stability using sets of vials rather than repeatedly injecting from the same vial. Chardonnay (2001 C-1) was spiked with internal standards and used to prepare three sets of fifteen vials each. Each vial contained 6.0 mL of spiked wine and 2.0 g of salt.

One vial from each set was analyzed to provide baseline data; the remaining vials were stored at different temperatures – one set of vials at room temperature (25°C), one set in the refrigerator (4°C), and one set in the freezer (-16°C). Each day for one week a fresh vial was prepared using spiked wine that had been frozen in plastic, screw-cap tubes. The fresh vial and one vial from each storage set were analyzed. Analysis was conducted twice weekly for three additional weeks. Four representative compounds, one from each organic class, were selected for analysis to track changes occurring as a result of storage.

Analyte peak areas for the stored vials were divided by the analyte peak areas from the fresh vial to produce a daily peak area ratio for each analyte. The

peak area ratios were plotted against time of storage for each of the three storage temperatures and analyzed for a pattern of stability.

2. Linearity

Five vials were prepared containing 6.0 mL of sample (MS 50, MS 75, MS 100, MS 125 or MS 150) and 2.0 g of salt. Each vial was extracted in triplicate and the average peak area for the three runs was determined for each analyte. The only exception was in the analysis of the esters as discussed above. The percent relative standard deviation (%RSD) of the triplicate runs were determined for each analyte.

The average peak area, peak area ratio (peak area analyte/peak area relevant IS) and response factor [(peak area ratio – y-intercept)/concentration] were plotted versus concentration for each analyte. The resulting plots were analyzed for linearity.

Five new vials were then prepared by standard addition using 3.0 mL of wine with internal standards combined with 3.0 mL of one of the mixed standards (MS 50 in the first vial, MS 75 in the second vial, etc.). Each vial was extracted in triplicate. Average peak areas were plotted against the appropriate mixed standard (MS) concentration for each analyte. The concentration of each analyte present in the original wine was determined from the x-intercept. A comparison of this plot with the one generated from the full-strength mixed standard vials led to a determination of the boosting (or damping) effect of the actual wine matrix on the signal of each analyte.

3. Recovery

Three vials were extracted in triplicate, Vial 1 with 6.0 mL MS 100 solution, Vial 2 with 3.0 mL MS 100 and 3.0 mL wine with internal standards, and Vial 3 with 6.0 mL of wine with internal standards. The average peak areas for the triplicate runs were determined and the data analyzed to determine recovery. The expected peak area (PA) of Vial 2 for each analyte and internal standard (IS) was calculated according to the following equation:

$$Expected\ PA\ Vial\ 2 = (\tfrac{1}{2}\ PA\ Vial\ 1) + (\tfrac{1}{2}\ PA\ Vial\ 3)$$

The percent recovery (%R) for each analyte and IS utilized the following:

$$\%R = [PA\ Vial\ 2 / Expected\ PA\ Vial\ 2] \times 100$$

Corrected recoveries were also calculated by using peak area ratios (PA of the analyte/PA of the relevant IS) in place of peak areas.

4. Specificity

One run of Vial 1 (MS 100) and one run of Vial 3 (spiked wine) from section 3 were selected. For each analyte and internal standard component, the following parameters were calculated: resolution (R_s), number of theoretical plates (N), tailing factor or peak symmetry (A_s) and capacity factor (k'). The equations are given below, with the appropriate measurements indicated on Figure 5.3.

Resolution is measured by comparing each peak (A) with an adjacent peak (B) in the chromatogram.

$$R_s = \frac{2\,[(t_r)_B - (t_r)_A]}{W_A + W_B}$$

Width is determined by drawing an extended tangent line down each side of the peak. This helps to correct for unresolved peaks or those which tail or front. The adjacent peak could be either to the right or to the left of the target peak; it is generally only necessary to calculate resolution from an adjacent peak that is not clearly baseline-resolved from the peak of interest.

The number of theoretical plates was calculated independently for each peak. The equation for N is used for symmetric peaks, while N* is calculated for asymmetric peaks.

$$N = 16\left(\frac{t_r}{w_b}\right)^2 \qquad N^* = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2$$

Peak symmetry:

$$A_s = \frac{b}{a}$$

Capacity factor:

$$k' = \frac{t_r - t_o}{t_o}$$

A more detailed discussion of these parameters is found in Chapter I, section B.

5. Sensitivity

The sensitivity of the method was the amount of change in the signal per unit change in the concentration of each analyte. This was determined as the slope of the linearity (standard calibration) curve plotted from average peak area versus concentration for each analyte.

Figure 5.3

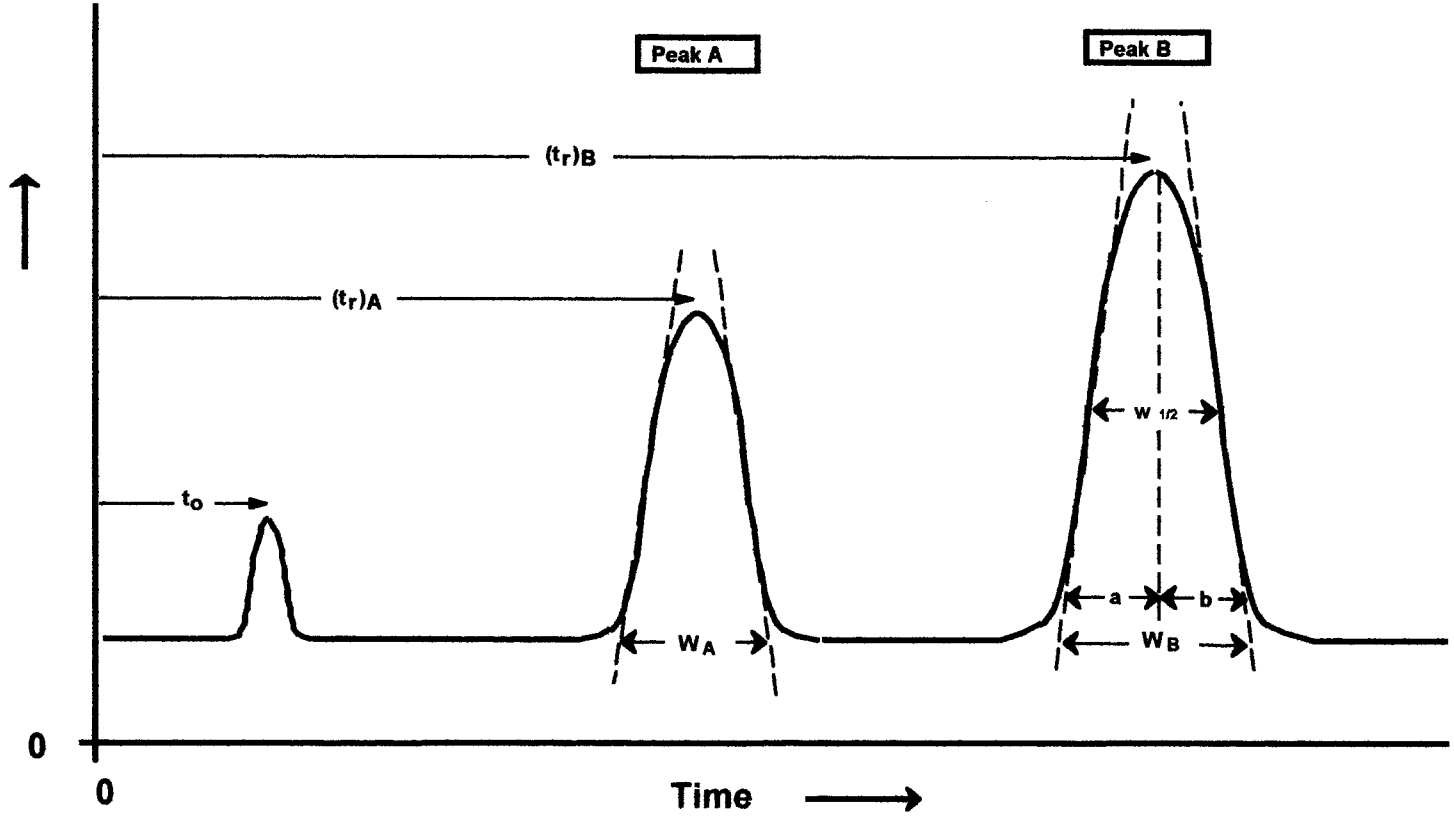


Figure 5.3 Measurements taken to calculate resolution (R_s), peak asymmetry (A_s), capacity factor (k') and number of theoretical plates (N).

6. Limit of Quantitation

The limit of quantitation (LOQ) was the lowest concentration that generated a signal of at least 10 times the signal of the adjacent noise. The purpose of the minimum signal-to-noise (S/N) ratio of 10 is to ensure that the error due to noise does not exceed 10% of the signal.

Data from the standard mixtures (MS 50, MS 75, etc.) were checked to determine a starting concentration for each analyte such that the signal for that analyte was at least 10 times the noise level. Serial dilutions were conducted until the lowest concentration was reached where the S/N ratio was greater than or equal to 10. This was identified as the limit of quantitation, or the lowest concentration of each analyte that is able to be reliably quantified.

7. Limit of Detection

The limit of detection (LOD) was defined as the lowest concentration of an analyte that could be qualitatively detected by the method. This parameter was determined in a manner similar to the LOQ, except that the S/N ratio for the limit of detection was only 3 rather than 10. A signal at least three times higher than the adjacent noise indicated the presence of an analyte rather than random noise.

8. Precision

The precision of the method consisted of two primary parts – injection repeatability and intra-assay repeatability. Injection repeatability was a measure of the random variability that occurred when the same sample was injected in the same manner several times. For this purpose, the data from the self-depletion portion of the research was used (refer to section E.3 of this chapter). The RSD of

the peak areas and the retention times of five extractions from the same vial were determined for selected analytes.

Intra-assay repeatability was the variation between samples prepared and analyzed separately using the same method. This was determined from the RSD of the peak areas and the retention times for the five 'first extractions' in the self-depletion study.

9. Range

The range was defined as the set of concentrations across which the method was deemed to be valid. This was determined by synthesizing the data from the linearity, precision and limit of quantitation and detection sections, and was expected to be somewhat different for each analyte of interest.

CHAPTER VI

Results and Discussion

A. Determination of the Sampling Parameters

1. Peak Identification

Peak identification was accomplished using two primary methods. Standards of increasing concentration were tested with peak identification based upon the corresponding increase in peak area relative to other peaks in the chromatogram. As an additional confirmation, mass spectral data for each peak was compared with stored library spectra from the Saturn software and from the NIST 98 library. A typical chromatogram with target peaks labeled and identified is given as Figure 6.1.

2. Standard Solution Concentrations

During the analysis of development runs it was noted that some analytes present in the target list were not present in either Chardonnay or Pinot Gris (see Figures 6.2 and 6.3). Several other components were present in wines at much different levels than originally anticipated. Thus, several changes were made to the composition of the mixed standard solutions as noted in Chapter V. Future work may include an increase in organic acid concentrations for the standard solutions based upon the significant peak areas observed for these components in Pinot Gris. Chardonnay showed similar peak areas for octanoic and decanoic acids but exhibited no detectable level of nonanoic acid.

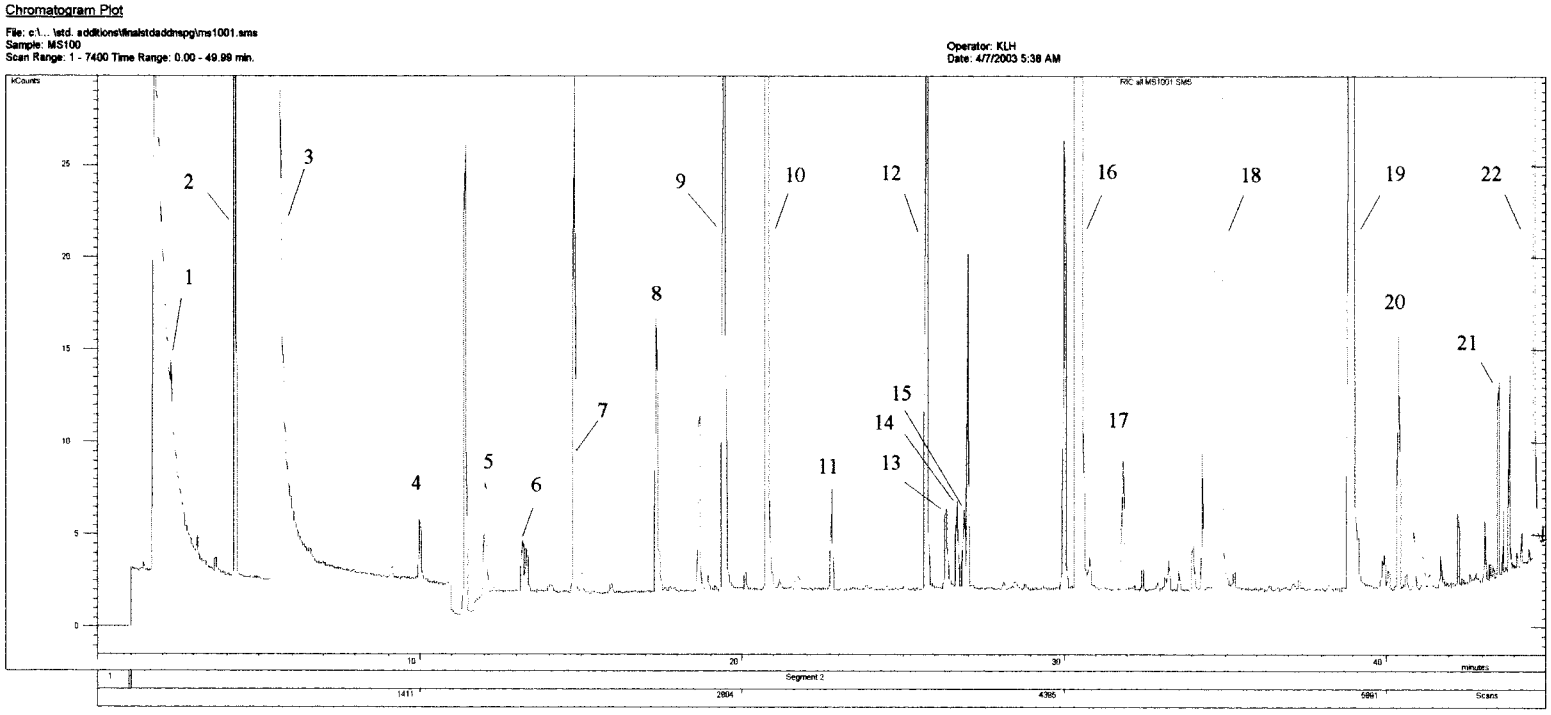


Figure 6.1 Peak identification chromatogram.

1	Acetaldehyde	9	3-Methyl-1-butanol	16	Ethyl octanoate
2	Ethyl acetate	10	Ethyl hexanoate	17	Linalool oxide
3	Ethanol	11	Hexyl acetate	18	Ethyl nonanoate (IS)
4	Ethyl butyrate	12	1,6-Heptadien-4-ol (IS)	19	Ethyl decanoate
5	Ethyl isovalerate	13	Ethyl lactate	20	Diethyl succinate
6	Isobutanol	14	Hexanol	21	2-Phenethyl acetate
7	Isoamyl acetate	15	4-Hydroxy-4-methyl-2-pentanone (IS)	22	2-Phenethyl alcohol
8	4-Methyl-2-pentanol (IS)				

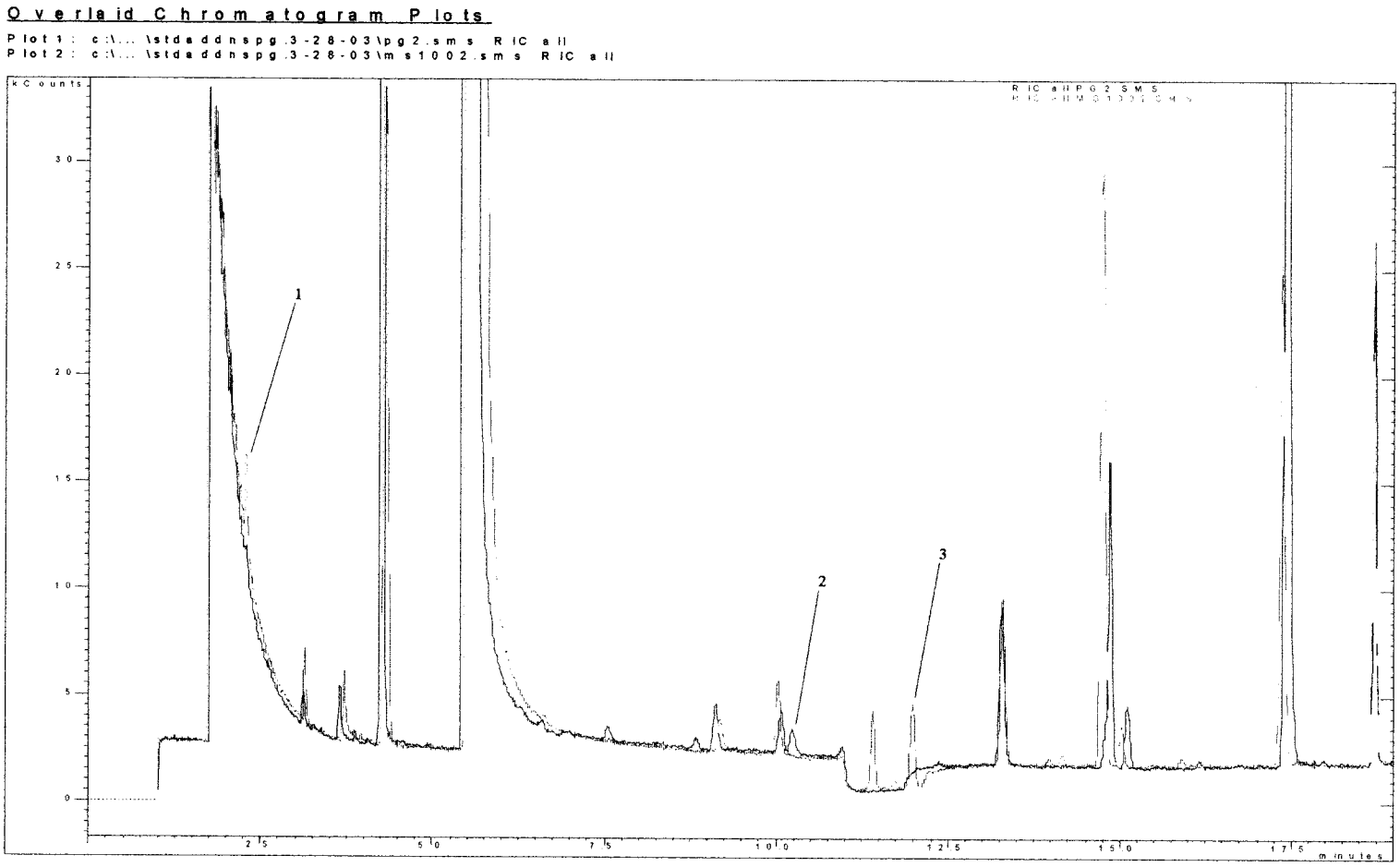


Figure 6.2 Standard solution (blue) and Pinot Gris (red) differed at several points.
(1) acetaldehyde (2) 1-propanol (3) ethyl isovalerate

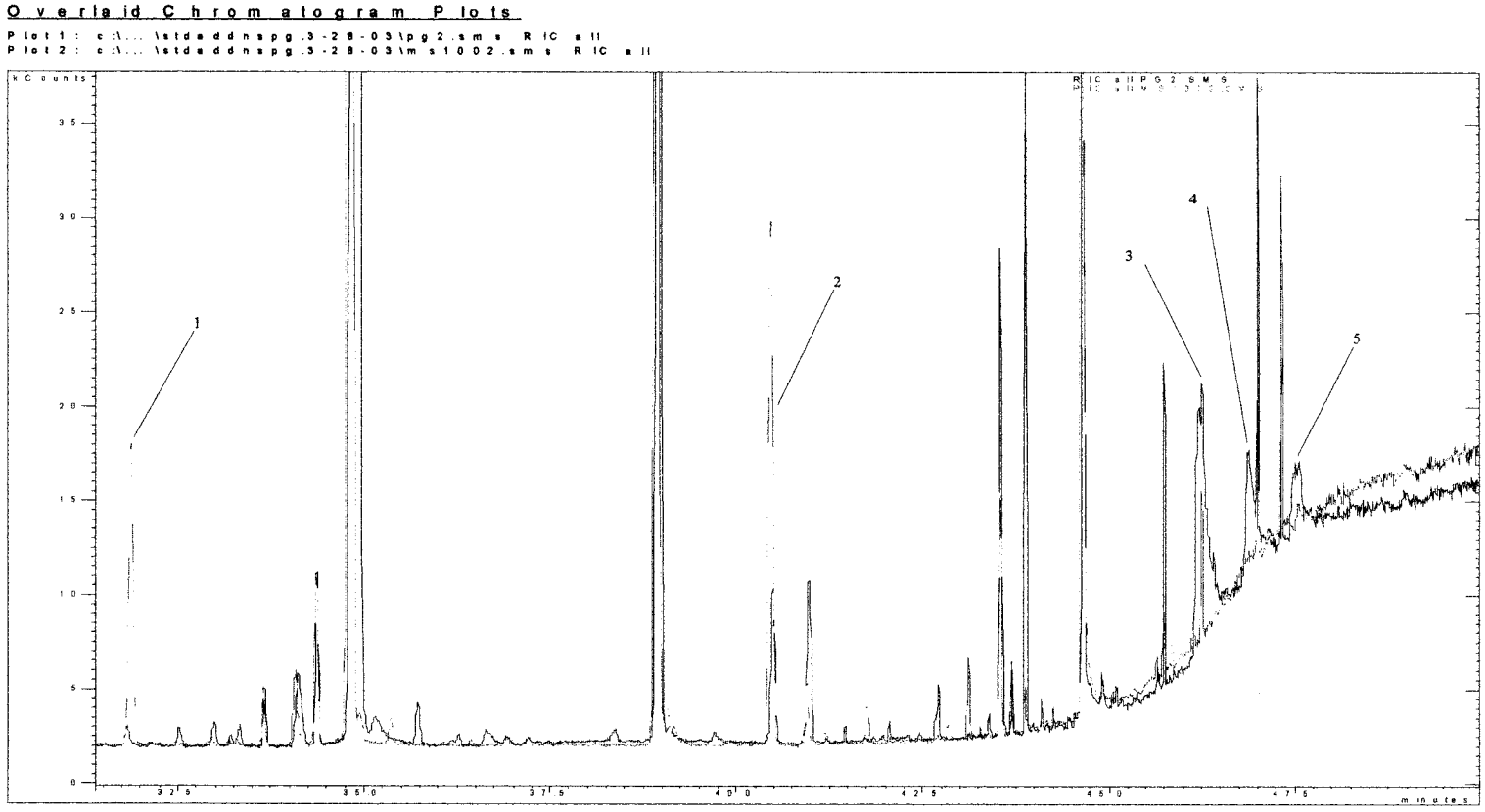
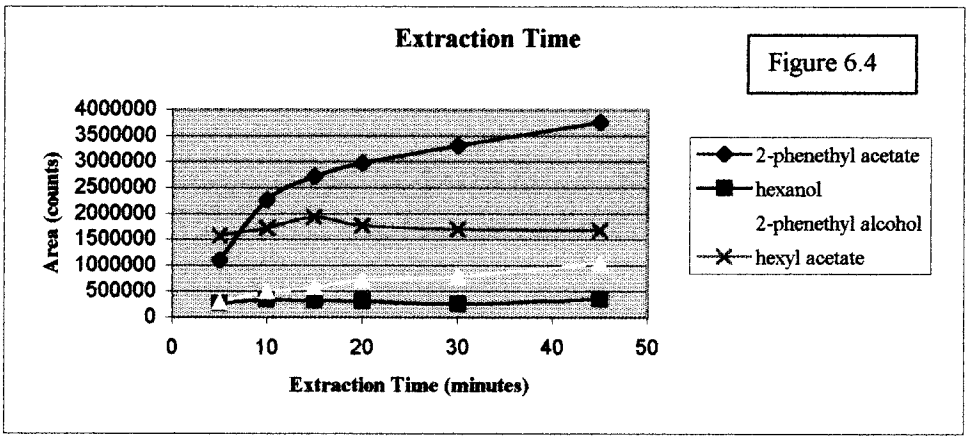


Figure 6.3 Standard solution (blue) and Pinot Gris (red) exhibited several differences near the end of the separation as well. (1) linalool oxide (2) diethyl succinate (3) octanoic acid (4) nonanoic acid (5) decanoic acid

3. Extraction Time

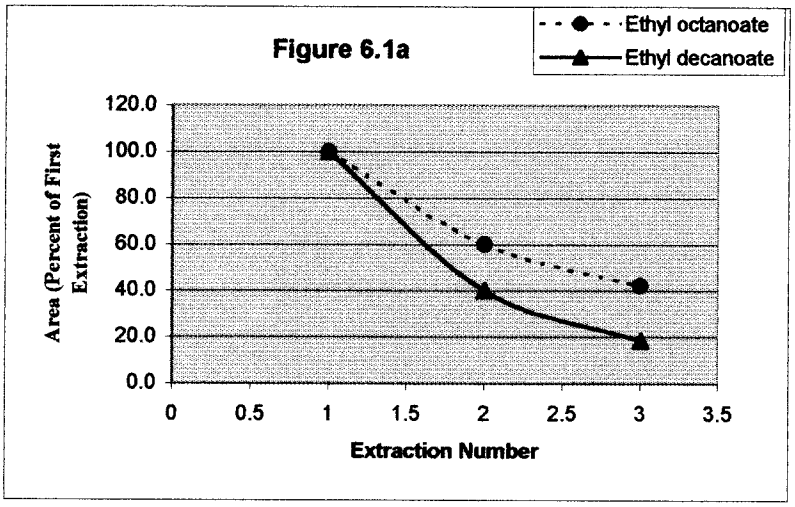
The average peak areas of selected analytes for the triplicate runs were plotted against extraction time (see Figure 6.4) to determine the time required for equilibration of each analyte between the headspace and the fiber. This data was used to determine an optimum extraction time of 30 minutes,which was selected to maximize analyte extraction while minimizing sampling time.

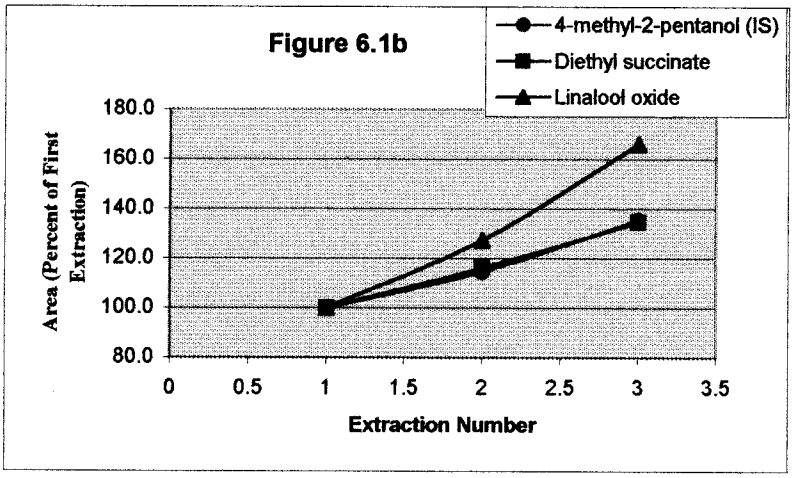


4. Headspace Depletion

Five vials were prepared using the middle standard (MS 100). The first vial was extracted five times using the extraction time and program described above. The second vial was extracted in triplicate, and the remaining vialswere extracted once each. Table 6.1 contains the raw peak area data for the first three extractions from Vial 1. Peak areas shown in red exhibited a downward trend indicative of headspace depletion. Peak areas shown in blue exhibited the opposite trend and provide evidence for potential competition on the coating of the fiber.

Figures 6.5a and 6.5b illustrate the trends for representative analytes.





Peak areas for the multiple runs were averaged and relative standard deviations (RSD) were calculated. Relative standard deviations were also calculated for the first extractions from each of the five vials. Results are shown in Table 6.2.

Table 6.1 Headspace Depletion with Multiple Extractions
(peak areas given in counts)

Analytes	Extraction Number (Vial 1)		
	1	2	3
Acetaldehyde	**18437**	**20242**	**20704**
Ethyl acetate	**867916**	**1009346**	**1032319**
Ethyl isobutyrate	ND	ND	ND
Ethyl butyrate	**34399**	**37369**	**49453**
Ethyl isovalerate	ND	ND	ND
Isobutanol	37982	51817	48712
Isoamyl acetate	324405	396919	385620
4-methyl-2-pentanol (IS)	**200632**	**229834**	**271786**
3-methyl-1-butanol	1054538	1078629	1076407
Ethyl hexanoate	**1872673**	**1546175**	**1370870**
Hexyl acetate	**279755**	**246255**	**225793**
1,6-heptadien-4-ol (IS)	**293039**	**340065**	**389697**
Ethyl lactate	ND	ND	19729
Hexanol	coeluted	29711	39172
4-hydroxy-4-methyl-2-pentanone (IS)	coeluted	84641	66450
Ethyl octanoate	**12767639**	**7676238**	**5395299**
Linalool oxide	**33472**	**42630**	**55590**
Ethyl decanoate	**6658381**	**2675350**	**1221179**
Diethyl succinate	**130529**	**151689**	**175856**
2-Phenethyl acetate	**137308**	**147101**	**150161**
2-Phenethyl alcohol	**768611**	**963490**	**1053290**

Table 6.2 Relative Standard Deviations of Multiple Extractions

Analytes	%RSD Vial 1 (5 extractions)	%RSD Vial 2 (3 extractions)	%RSD Vials 1-5 (first extractions)
Acetaldehyde	14.8	27.9	12.5
Ethyl acetate	9.0	3.8	4.9
Ethyl butyrate	16.8	2.2	10.2
Isobutanol	12.4	26.9	12.3
Isoamyl acetate	8.5	5.8	8.0
4-Methyl-2-pentanol	14.2	15.4	14.7
3-Methyl-1-butanol	6.5	28.8	15.8
Ethyl hexanoate	24.7	17.5	2.9
Hexyl acetate	20.2	19.8	2.4
1,6-Heptadien-4-ol (IS)	13.1	8.7	8.3
Hexanol	27.1	4.7	28.9
4-Hydroxy-4-methyl-2-pentanone (IS)	18.7	3.7	7.4
Ethyl octanoate	46.9	23.8	2.6
Linalool oxide	24.2	10.0	9.2
Ethyl decanoate	114.8	80.1	4.0
Diethyl succinate	14.4	12.7	2.9
2-Phenethyl acetate	4.2	4.1	5.0
2-Phenethyl alcohol	14.3	12.0	5.9

Run-to-run headspace depletion of several components, particularly the higher molecular weight esters, was noticeable. The difference between the RSD for three extractions from Vial 2 and the RSD for the first extractions only from all five vials is striking for components such as ethyl hexanoate, hexyl acetate, ethyl octanoate, ethyl decanoate, and diethyl succinate. These components had triplicate extraction RSD values ranging from 12.9% to 80.1%. However, the RSD for the first extractions from each vial ranged from 2.4% to 4.0%. The latter values indicate that overall repeatability of the peak areas for these components was good; therefore, it was concluded that headspace depletion was a significant factor. For the remainder of the research, only the first extraction from each vial was used to quantify these particular components rather than an average of triplicate runs.

B. Validation of the Method

1. Stability

Chardonnay (2001 C-1) was spiked with internal standards and used to prepare three sets of fifteen vials each. One vial from each set was analyzed to provide baseline data; the remaining vials were stored at different temperatures – one set of vials at room temperature (25°C), one set in the refrigerator (4°C) and one in the freezer (-16°C). On selected days during storage, a fresh vial was prepared and analyzed along with one vial from each storage set. Analyte peak areas for the stored vials were divided by the analyte peak areas from the fresh vial to produce daily peak area ratios for each analyte. A ratio of 1.0 indicates no

difference between the peak areas of the fresh and stored vials. The peak area ratios were plotted against time of storage for each of the three storage temperatures and analyzed for a pattern of stability (see Figure 6.6). It is important to note that data from the first five days was questionable due to the use of a fiber later determined to be flawed.

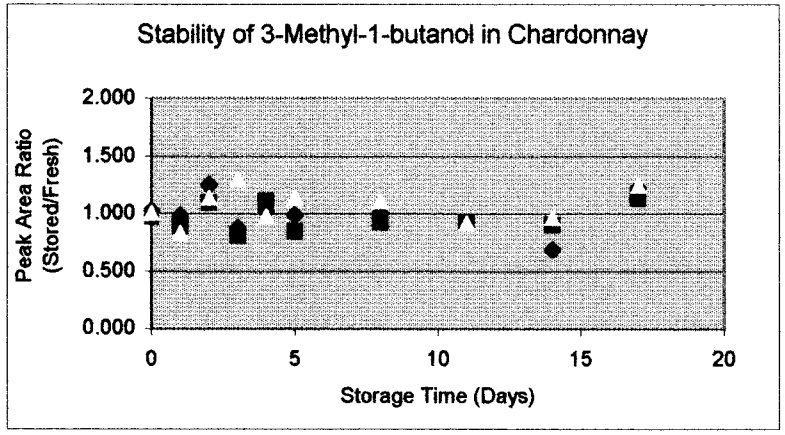
All four components were stable for at least the first 11 days, with ethyl octanoate consistently above a ratio of 1.0 and 1,6-heptadien-4-ol consistently below that ratio.

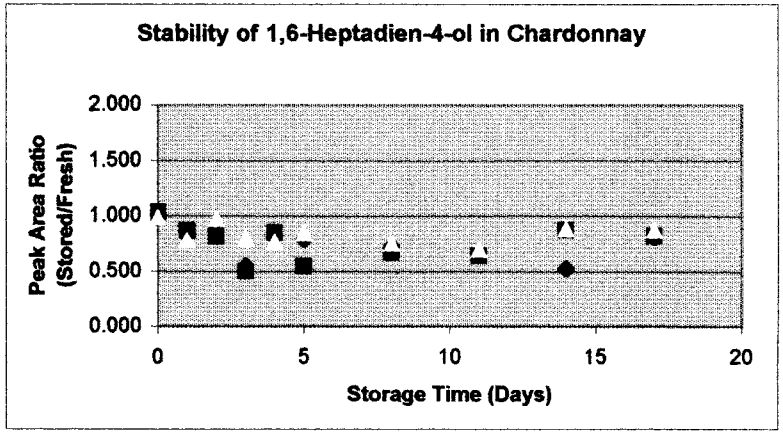
2. Linearity

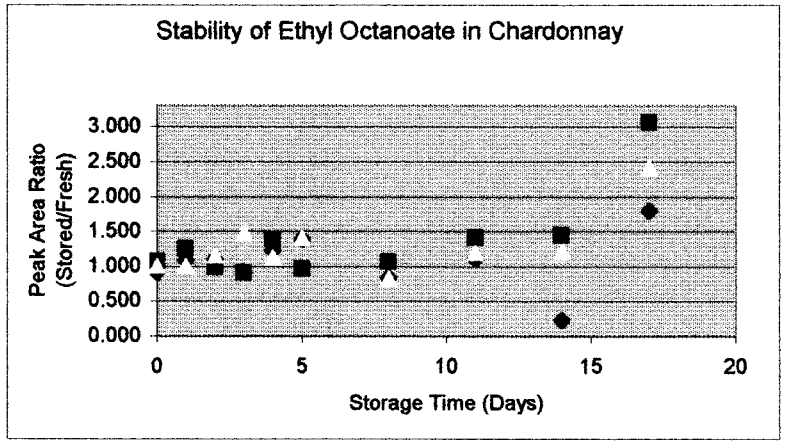
Vials were prepared at each standard concentration (MS 50 to MS 150) plus additional concentrations above and below these levels. Each vial was extracted in triplicate and the average peak area for the three runs was determined for each analyte. The only exception was in the analysis of the esters, as discussed previously. The average peak area was plotted versus concentration for each analyte (see Figure 6.7). The resulting plots were analyzed for linearity (see Table 6.3). Isobutanol was the only component with a linear range not including the model wine (0 ppm analyte concentration). The lowest concentrations for this analyte showed a slope near zero, indicating that these concentrations were below the detection limit.

Figure 6.6 Peak area ratios during storage

Legend: ◆ Refrigerator (4°C)
■ Freezer (-16°C)
Room Temperature (25°C)







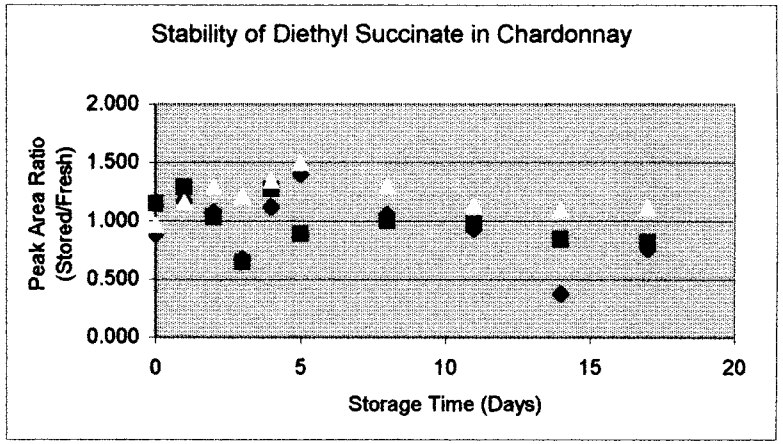


Figure 6.7 Representative Linearity Plots (one per organic class)

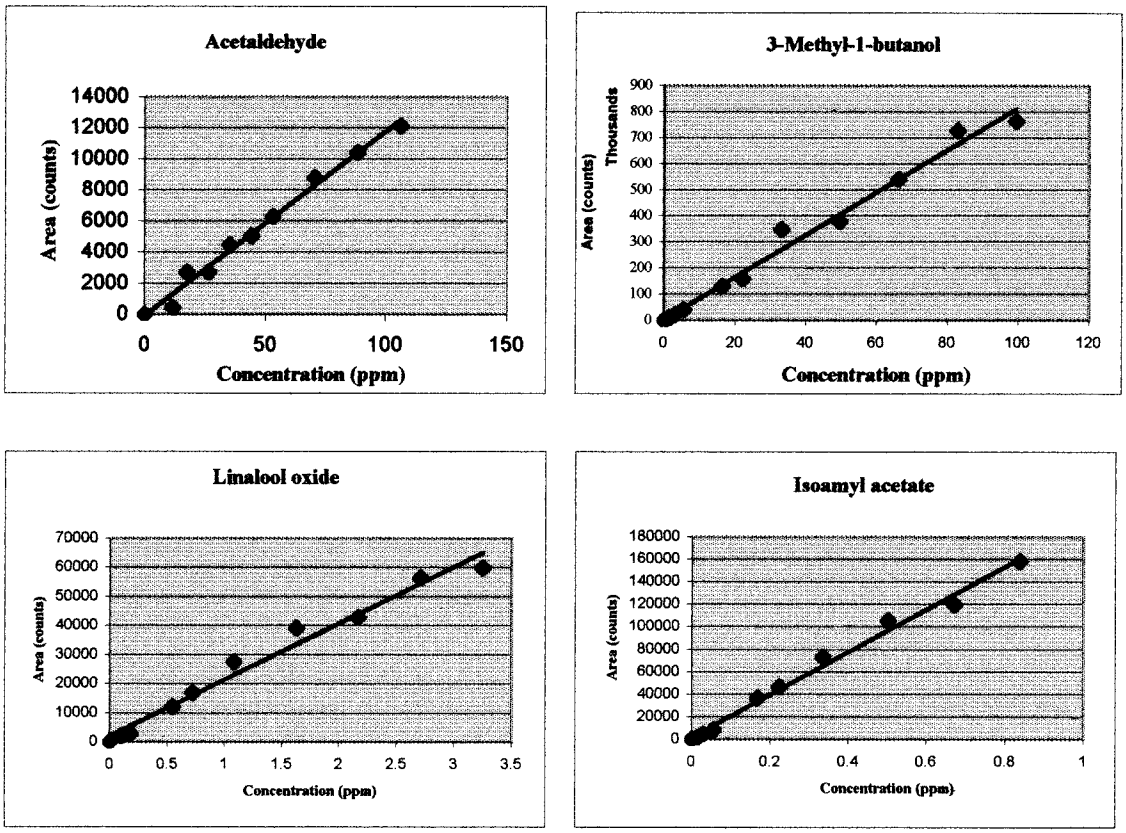


Table 6.3 Linearity Data

Analyte	Equation of the Line	R^2	Linear Range (ppm)	
Acetaldehyde	y = 118.46x - 85.133	0.9875	0.0	106.1
Ethyl acetate	y = 3249.6x + 2044.9	0.9941	0.00	83.67
Ethyl butyrate	y = 58373x + 995.25	0.9863	0.0000	0.5123
Ethyl isovalerate	y = 1317943.9x + 336.6	0.9921	0.0000	0.0370
Isobutanol	y = 3394.7x - 85.6	0.9645	3.42480	10.2744
Isoamyl acetate	y = 189359x + 1729.5	0.9904	0.00000	0.83842
4-Methyl-2-pentanol (IS)	y = 13693x + 664.38	0.9883	0.00000	9.55730
3-Methyl-1-butanol	y = 8138x + 1406.9	0.9818	0.00	132.6
Ethyl hexanoate	y = 594415x + 5950.7	0.9692	0.00000	1.2202
Hexyl acetate	y = 457373 x + 48.018	0.9830	0.00000	0.02908
1,6-Heptadien-4-ol (IS)	y = 26789x + 4459.1	0.9822	0.00000	9.69145
Ethyl lactate	y = 825.44x - 1797.6	0.9686	0.00	57.25
Hexanol	y = 33452x + 876.29	0.9480	0.0000	1.8433
4-Hydroxy-4-methyl-2-pentanone (IS)	y = 829.19x - 1376.3	0.9754	0.00	50.65
Ethyl octanoate	y = 1504771x + 55174	0.9960	0.00000	2.56380
Linalool oxide	y = 19408x + 1937.4	0.9786	0.0000	3.2506
Ethyl nonanoate (IS)	y = 1128644.6x + 43303.1	0.9910	0.00000	2.80157
Ethyl decanoate	y = 752759.8x - 6041.9	0.9840	0.00000	3.72843
Diethyl succinate	y = 55111x + 893.5	0.9841	0.0000	1.8655
2-Phenethyl acetate	y = 252157x - 459.77	0.9914	0.0000	0.1589
2-Phenethyl alcohol	y = 10878x - 1526.6	0.9984	0.00	53.47

3. Recovery

Recovery is one measure of the accuracy of a method, and is often used when a standard of exactly known concentrations and identical matrix to the sample is not available for comparison. Three vials were extracted in triplicate, Vial 1 (MS 100), Vial 2 (50/50 mixture of MS 100 and wine with internal standards added), and Vial 3 (wine with internal standards). The expected peak area (PA) of the mixed vial (Vial 2) for each analyte and internal standard (IS) was calculated according to the following equation:

$$Expected\ PA\ Vial\ 2 = (\tfrac{1}{2}\ PA\ Vial\ 1) + (\tfrac{1}{2}\ PA\ Vial\ 3)$$

The percent recovery (%R) for each analyte and IS utilized the following:

$$\%R = [PA\ Vial\ 2 / Expected\ PA\ Vial\ 2] \times 100$$

Corrected recoveries were also calculated using peak area ratios (PA of the analyte/PA of the relevant IS) in place of peak areas. Table 6.4 indicates the internal standard used for correction. Recovery results are given in Table 6.5.

Table 6.4 Internal Standards Used for Corrected Recoveries

Ethyl nonanoate used as IS for:	**4-Methyl-2-pentanol used as IS for:**
Ethyl acetate	Isobutanol
Ethyl butyrate	3-Methyl-1-butanol
Ethyl isovalerate	Hexanol
Isoamyl acetate	2-Phenethyl alcohol
Ethyl hexanoate	
Hexyl acetate	**4-Hydroxy-4-methyl-2-pentanone**
Ethyl lactate	**used as IS for:**
Ethyl octanoate	Acetaldehyde
Ethyl decanoate	
Diethyl succinate	**1,6-Heptadien-4-ol used as IS for:**
2-Phenethyl acetate	Linalool oxide

Table 6.5 Recovery Results

Analyte	%R in Pinot Gris	Corrected %R in Pinot Gris	%R in Chardonnay	Corrected %R in Chardonnay
Acetaldehyde	176.6	46.9	131.5	142.5
Ethyl acetate	103.6	101.7	101.4	88.0
Ethyl butyrate	110.4	126.6	92.3	90.9
Ethyl isovalerate	77.4	100.7	45.8	76.9
Isobutanol	135.6	96.3	127.1	66.7
Isoamyl acetate	106.4	121.8	88.8	100.1
4-Methyl-2-pentanol (IS)	107.7	100.0	123.2	100.0
3-Methyl-1-butanol	123.7	63.1	113.1	55.2
Ethyl hexanoate	107.5	123.7	error	error
Hexyl acetate	109.4	112.9	76.1	91.4
1,6-Heptadien-4-ol (IS)	113.8	100.0	113.1	100.0
Ethyl lactate	112.0	113.6	113.5	153.4
Hexanol	96.9	63.3	100.0	30.9
4-Hydroxy-4-methyl-2-pentanone (IS)	124.1	100.0	145.8	100.0
Ethyl octanoate	77.1	93.2	93.6	100.0
Linalool oxide	129.3	33.9	81.9	43.9
Ethyl nonanoate (IS)	89.2	100.0	93.6	100.0
Ethyl decanoate	80.8	170.1	88.7	93.0
Diethyl succinate	125.8	147.1	115.8	110.5
2-Phenethyl acetate	112.7	114.3	100.2	106.7
2-Phenethyl alcohol	89.6	47.6	114.6	35.4

Figure 6.8 shows a chromatogram obtained from Pinot Gris (2001, C-1) spiked with internal standards. Figure 6.9 shows the same for Chardonnay (2001, S-1).

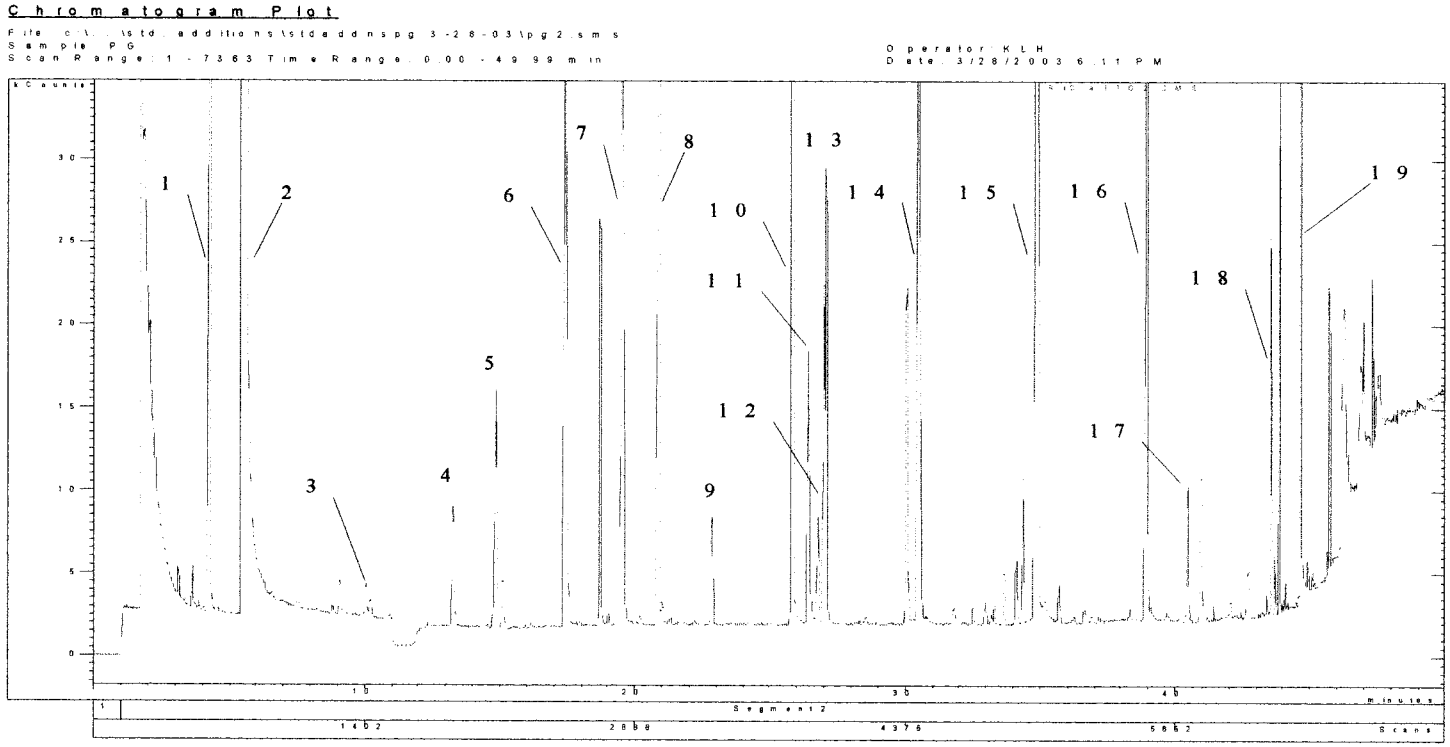


Figure 6.8 –Chromatogram of Pinot Gris (2001, C-1)

1	Ethyl acetate	7	3-Methyl-1-butanol	13	4-Hydroxy-4-methyl-2-pentanone (IS)
2	Ethanol	8	Ethyl hexanoate	14	Ethyl octanoate
3	Ethyl butyrate	9	Hexyl acetate	15	Ethyl nonanoate (IS)
4	Isobutanol	10	1,6-Heptadien-4-ol (IS)	16	Ethyl decanoate
5	Isoamyl acetate	11	Ethyl lactate	17	Diethyl succinate
6	4-Methyl-2-pentanol (IS)	12	Hexanol	18	2-Phenethyl acetate
				19	2-Phenethyl alcohol

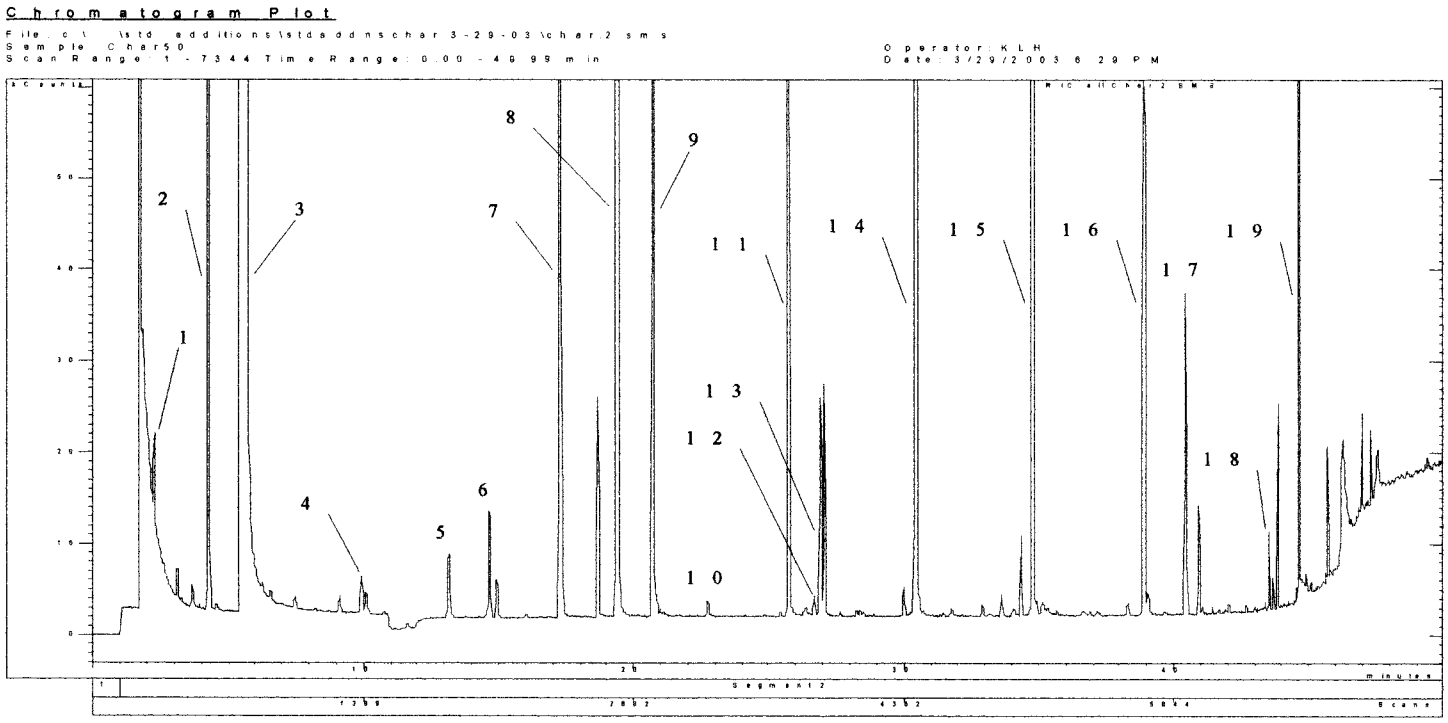


Figure 6.9 –Chromatogram of Chardonnay (2001, S-1)

1	Acetaldehyde	7	4-Methyl-2-pentanol (IS)	13	4-Hydroxy-4-methyl-2-pentanone (IS)
2	Ethyl acetate	8	3-Methyl-1-butanol	14	Ethyl octanoate
3	Ethanol	9	Ethyl hexanoate	15	Ethyl nonanoate (IS)
4	Ethyl butyrate	10	Hexyl acetate	16	Ethyl decanoate
5	Isobutanol	11	1,6-Heptadien-4-ol (IS)	17	Diethyl succinate
6	Isoamyl acetate	12	Hexanol	18	2-Phenethyl acetate
				19	2-Phenethyl alcohol

Components that had resolution difficulties showed poor recoveries. Acetaldehyde, for example, was a small tangent peak on the side of the air peak that resulted from insertion of the needle into the injector. Additionally, the ketone selected as the internal standard for use with acetaldehyde had a significantly higher molecular weight and a hydroxyl group substituent. These differences were likely contributors to the poor corrected recovery for this analyte. 3-Methyl-1-butanol and linalool oxide sometimes co-eluted with fiber bleed peaks. Run-to-run repeatability of these peaks was problematic and might have contributed to the poor recovery results.

4. Specificity

This validation parameter was actually a set of measurements that indicate how effectively each analyte was separated and identified from other components in the matrix. A sample of Pinot Gris wine was analyzed using the developed method and key measurements were made of each analyte and internal standard peak. A thorough discussion of each of these measurements and calculations is given in Chapter V section F.4. The results are summarized in Table 6.6.

A resolution of $R_s < 1$ indicates unresolved peaks while $R_s = 1$ generally indicates peaks which are adequately resolved for quantitation but do not exhibit baseline resolution. Baseline resolution occurs when the signal returns completely down to the background signal before rising for the start of the next peak. When $R_s \geq 1.5$, baseline resolution has been achieved. The ability to identify peak start and end by ion patterns with mass spectrometric detection

allows quantitation even with limited resolution. The two peaks with resolution less than 1.0 (ethyl acetate and 4-methyl-2-pentanol) were still quantifiable.

Table 6.6 Specificity Parameters

Analyte	Resolution (R_s)	Number of Theoretical Plates (N or N^*)	Peak Symmetry (A_s)	Capacity Factor (k')
Ethyl acetate	0.761	N^* = 59280	0.636	1.425
Ethyl butyrate	1.157	N = 91970	1.000	4.635
Isobutanol	11.298	N = 154600	1.030	6.475
Isoamyl acetate	1.773	N = 194900	0.930	7.330
4-Methyl-2-pentanol (IS)	0.533	N^* = 375700	0.863	8.827
3-Methyl-1-butanol	5.077	N^* = 376770	0.856	10.034
Ethyl hexanoate	1.024	N^* = 633400	0.816	10.793
Hexyl acetate	5.117	N^* = 789280	1.185	11.949
1,6-Heptadien-4-ol (IS)	4.918	N^* = 1048700	0.871	13.677
Ethyl lactate	2.874	N = 563700	0.919	14.025
Hexanol	1.865	N = 991270	1.038	14.228
4-Hydroxy-4-methyl-2-pentanone (IS)	1.895	N^* = 944670	0.851	14.346
Ethyl octanoate	3.912	N^* = 1288900	0.631	16.374
Ethyl nonanoate (IS)	1.012	N^* = 1223600	0.626	18.884
Ethyl decanoate	1.033	N^* = 2404100	0.740	21.222
Diethyl succinate	4.710	N^* = 2882500	0.889	22.095
2-Phenethyl acetate	1.373	N^* = 12470100	0.649	23.877
2-Phenethyl alcohol	3.088	N^* = 19147300	0.700	24.511

Column efficiency is the ability of a column to interact with a particular analyte in a way that allows elution of a sharp, clearly defined peak. There are two methods for calculating column efficiency based upon the historically used unit of N, or 'number of theoretical plates,' for peaks which exhibit a symmetrical Gaussian shape and N^* for asymmetric peaks.

Asymmetry generally has a significant effect on resolution and quantitation. A value of $A_s = 1$ indicates a mathematically symmetrical peak, a fronting peak is indicated by $A_s < 1$ and $A_s > 1$ indicates a tailing peak. In the analyzed sample for this validation, most peaks exhibited some degree of fronting.

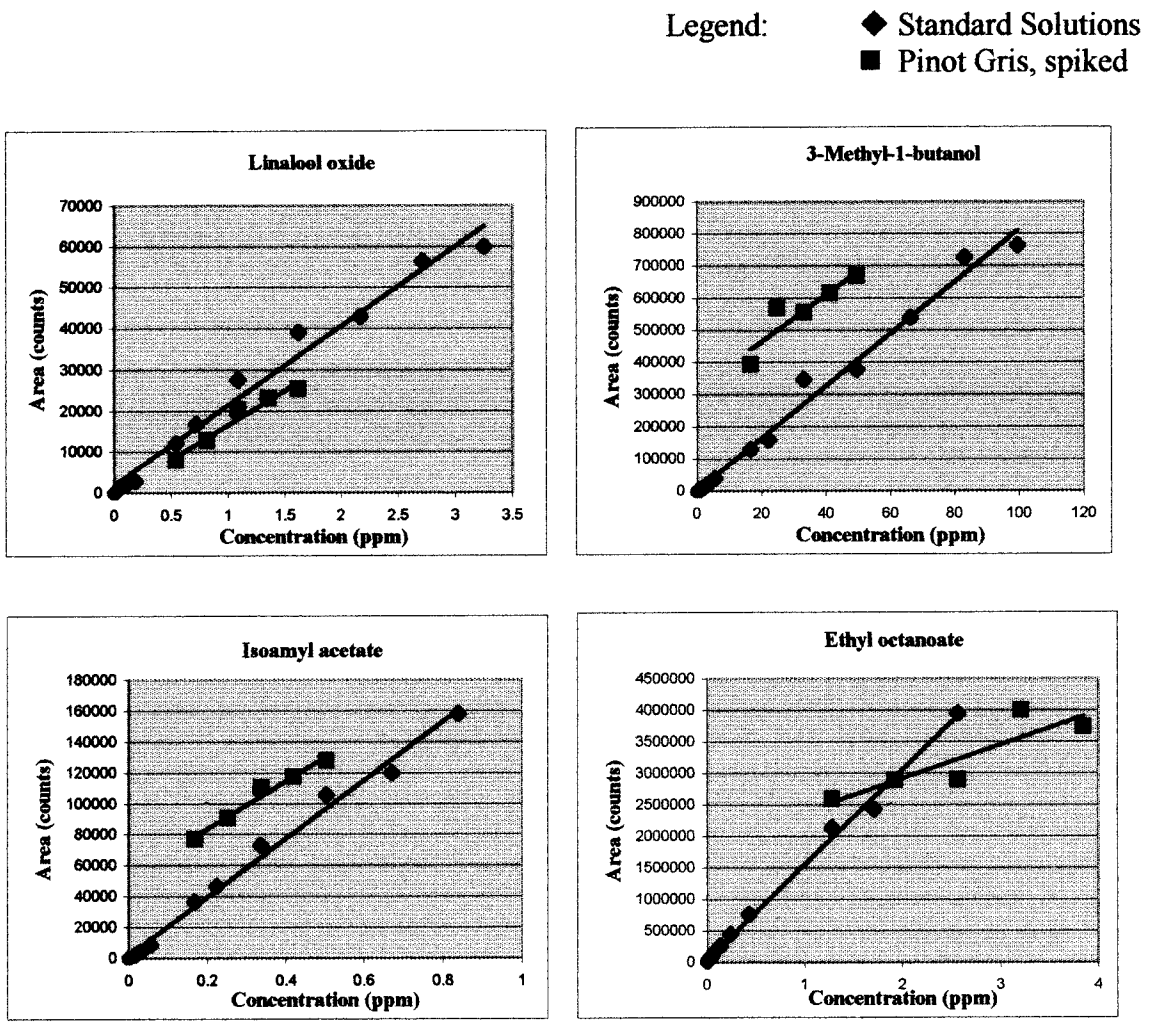
The capacity factor, or *k'*, for an analyte is an indicator of the type and degree of interaction between the analyte and the stationary phase in chromatography. Small values for *k'* indicate little interaction between the analyte and the stationary phase, resulting in shorter retention times, similar to those for the unretained reference peak. Higher values for *k'* correspond to longer retention times and indicate greater interaction between the analyte and the stationary phase.

5. Sensitivity

If a method is suitably sensitive for a particular analyte, increases in the concentration of that analyte should produce a measurable increase in response. The slope of the line of a calibration curve and the slope of the standard additions curve give an indication of the specificity of the method for each analyte in the standard solutions and in the wine matrix. Figure 6.10 shows sensitivity curves for representative analytes in the mixed standard solutions and in spiked Pinot Gris wine. It can be noted that in each case except linalool oxide, the lines representing the spiked Pinot Gris did not have y-intercepts of zero. However, these analytes were present in unspiked wine, with the x-axis representing the *additional* concentration spiked into wine. Thus, a y-intercept of zero was not

expected. Linalool oxide was the exception, as it was not found to naturally occur at detectable levels in Pinot Gris.

Figure 6.10 Sensitivity Curves for Representative Analytes



A significant difference in slope between ethyl octanoate in the standard solutions and in the spiked Pinot Gris was noted. This can be attributed to the additive effects of the spiked amount (1 to 4 ppm) to the amount naturally present in wine (approximately 2 ppm based upon the calibration curve). This brought the total amount of ethyl octanoate present in the spiked wine into a range of 3 to

6 ppm. The calibration curve for this analyte (see Figure 6.11) showed a leveling effect in this region that corresponded to the reduced slope seen in the spiked Pinot Gris curve.

Figure 6.11 Calibration Curve for Ethyl Octanoate

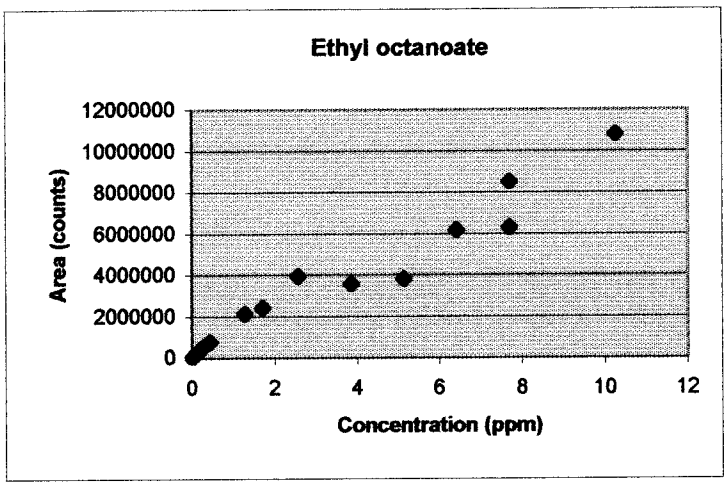


Table 6.7 displays the sensitivity of the method for all analytes in the standard solutions and in Pinot Gris. The wine matrix exhibited a damping effect on the sensitivity for nearly every analyte, particularly the higher molecular weight esters.

Table 6.7 Sensitivity of the Method in Standards and Pinot Gris

Analyte	Sensitivity (counts per ppm)	
	Standards	Pinot Gris
Acetaldehyde	118	78
Ethyl acetate	3249	2655
Ethyl butyrate	58373	82040
Ethyl isovalerate	1318000	1110300
Isobutanol	3395	2940
Isoamyl acetate	189400	154020
4-Methyl-2-pentanol (IS)	13690	9493
3-Methyl-1-butanol	8138	7251
Ethyl hexanoate	594400	420800
Hexyl acetate	457400	543500
1,6-Heptadien-4-ol (IS)	26790	8578
Ethyl lactate	825	427
Hexanol	33450	26970
4-Hydroxy-4-methyl-2-pentanone (IS)	829	731
Ethyl octanoate	1505000	530500
Linalool oxide	19410	16680
Ethyl nonanoate (IS)	1129000	-345010
Ethyl decanoate	752800	54350
Diethyl succinate	55110	41040
2-Phenethyl acetate	252200	186400
2-Phenethyl alcohol	10880	8827

6. Limit of Quantitation

The limit of quantitation (LOQ) is the lowest concentration that can be reliably quantified. This is defined as the concentration that generates a peak signal at least 10 times higher than the signal of the adjacent noise. The purpose of the minimum signal-to-noise (S/N) ratio of 10 is to ensure that the error due to noise does not exceed 10% of the signal. This was determined by analyzing a series of dilutions from the standards. Ethyl isobutyrate was not analyzed at concentrations sufficient for quantitation.

7. Limit of Detection

The limit of detection (LOD) is the lowest concentration that is able to be reliably detected as a peak rather than noise. This is defined as the concentration that generates a peak signal at least 3 times higher than the signal of the adjacent noise. Table 6.8 provides a summary of the limits of quantitation and detection for all analytes and internal standards. Ethyl isobutyrate was not present in wines at detectable levels. Acetaldehyde had high limits of detection and quantitation, due largely to its presence as a small tangent on the side of a significant air peak resulting from fiber insertion into the injector.

Table 6.8 Limits of Quantitation and Detection

Analytes	LOD (ppm)	LOQ (ppm)
Acetaldehyde	17.70	53.10
Ethyl acetate	0.3347	1.521
Ethyl isobutyrate	0.1308	>0.2616
Ethyl butyrate	0.01423	0.05693
Ethyl isovalerate	0.00056	0.00411
Isobutanol	0.311345	2.28320
Isoamyl acetate	0.0067073	0.030488
4-Methyl-2-pentanol (IS)	0.159288	0.289615
3-Methyl-1-butanol	0.1658	0.663
Ethyl hexanoate	0.0030505	0.0061010
Hexyl acetate	0.001762	0.009690
1,6-Heptadien-4-ol (IS)	0.0323048	0.161524
Ethyl lactate	1.735	9.542
Hexanol	0.033515	0.18433
4-Hydroxy-4-methyl-2-pentanone (IS)	2.814	16.88
Ethyl octanoate	<0.0008546	0.0008546
Linalool oxide	0.054177	0.098546
Ethyl nonanoate (IS)	0.000933856	0.00466928
Ethyl decanoate	0.00621406	0.186422
Diethyl succinate	0.014924	0.067836
2-Phenethyl acetate	0.003612	0.01987
2-Phenethyl alcohol	0.008911	0.2228

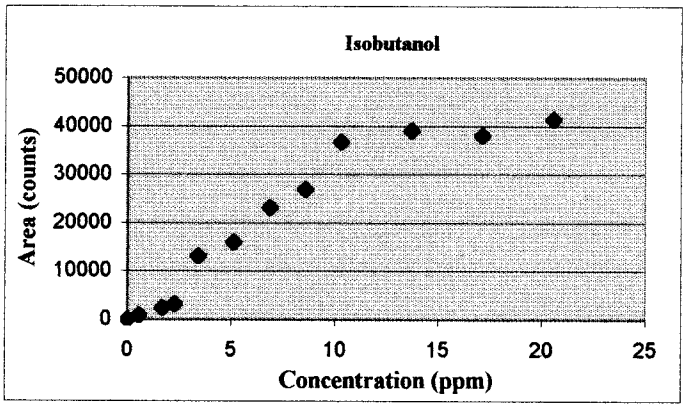
Concentrations of each analyte in wine were calculated by two methods and then compared to each other and to the limits of quantitation. Both calculations relied upon the data from spiking wine with 1:1 amounts of the mixed standard solutions. The first method calculated the x-intercept from the equation of the line and then doubled it to account for the dilution factor introduced in the spiking process. This indicated the probable concentration of the analyte in pure (unspiked) wine. The second method inserted the peak area of the analyte from an unspiked wine sample into the equation for the line of the calibration curve. See Table 6.9 for a comparison of the results for each analyte.

Table 6.9 Calculated Analyte Concentrations in Wine

Analytes	X-intercept of Spiked Pinot Gris Curve (ppm)	Concentration from Calibration Curve (ppm)
Acetaldehyde	19.16	0
Ethyl acetate	189.4	off scale
Ethyl isobutyrate	0	0
Ethyl butyrate	0.04054	0.2610
Ethyl isovalerate	0.000846	0
Isobutanol	16.1469	14.76728
Isoamyl acetate	0.68888	0.82336
3-Methyl-1-butanol	88.168	125.8216
Ethyl hexanoate	0.92342	0.97742
Hexyl acetate	0.05360	0.131
Ethyl lactate	379.0	204.6
Hexanol	1.1701	1.5622
Ethyl octanoate	7.02454	2.16606
Linalool oxide	0	1.02468
Ethyl decanoate	56.556	1.86144
Diethyl succinate	1.2030	1.22896
2-Phenethyl acetate	0.2766	0.3366
2-Phenethyl alcohol	30.58	50.9

Several early eluting analytes were found by both methods to be present in wines at levels below their limit of quantitation; these included acetaldehyde, ethyl isobutyrate, ethyl butyrate and ethyl isovalerate. This method is therefore unsuitable for analysis of these components at normal wine levels. Three additional components – ethyl acetate, ethyl lactate and 2-phenethyl acetate – were found at levels that exceeded the analyzed range of their calibration curves. The calibration curves must be demonstrated to be linear in an extended range in order for this method to be suitable for analysis of these components; time did not permit the expansion of the linear range during this study. Finally, isobutanol was found to be present in wine at a concentration (14.8 to 16.1 ppm) that was not in a linear region of the calibration curve (see Figure 6.12 below). This method is therefore unsuitable for analysis of isobutanol at these levels.

Figure 6.12 Calibration Curve for Isobutanol



8. Precision

The precision of a method consists of two primary parts – injection repeatability and intra-assay repeatability. Injection repeatability is a measure of the random variability that occurs when the same sample is injected in the same

manner several times. In this study, the RSD of the peak areas of five extractions from the same vial were within acceptable ranges for representative analytes that did not show significant run-to-run headspace depletion. The RSD of the retention times for both injection and intra-assay repeatability were quite good for all components in the research. This could be quite useful in future work if narrow time windows of selected-ion monitoring (SIM) mass spectrometry are desired for improvement of detectability of some components.

Intra-assay repeatability is the variation between samples prepared and analyzed separately using the same method. This was determined from the RSD of the peak areas and retention times of single extractions from five separate vials of identical concentration. Run-to-run headspace depletion did not impact these results, summarized in Table 6.10. Intra-assay repeatability was within acceptable limits for all components.

Table 6.10 Method Precision for Selected Analytes

Analytes	Injection Repeatability (5 x Same Vial)		Intra-Assay Repeatability (5 x Different Vials)	
	Peak Area RSD	Retention Time RSD	Peak Area RSD	Retention Time RSD
Ethyl acetate	9.0%	0.67%	4.9%	0.78%
Isoamyl acetate	8.5%	0.64%	8.0%	0.74%
3-Methyl-1-butanol	6.5%	0.40%	15.8%	0.57%
2-Phenethyl acetate	4.2%	0.03%	5.0%	0.07%
2-Phenethyl alcohol	14.3%	0.02%	5.9%	0.05%

9. Range

The range is defined as the set of concentrations across which the method is deemed valid. This was determined by synthesizing the data from the linearity, precision and limit of quantitation and detection sections. This method is valid across the ranges presented in Table 6.11, although observed wine concentrations did not always fall into these ranges as noted earlier.

Table 6.11 Validation Ranges

Analyte	Validation Range (ppm)
Acetaldehyde	53.10 - 106.1
Ethyl acetate	1.521 - 83.67
Ethyl butyrate	0.05693 - 0.5123
Ethyl isovalerate	0.00411 - 0.0370
Isobutanol	2.28320 - 10.2744
Isoamyl acetate	0.030488 - 0.83842
4-Methyl-2-pentanol (IS)	0.289615 - 9.55730
3-Methyl-1-butanol	0.663 - 132.6
Ethyl hexanoate	0.0061010 - 1.2202
Hexyl acetate	0.009690 - 0.02908
1,6-Heptadien-4-ol (IS)	0.161524 - 9.69145
Ethyl lactate	9.542 - 57.25
Hexanol	0.18433 - 1.8433
4-Hydroxy-4-methyl-2-pentanone (IS)	16.88 - 50.65
Ethyl octanoate	0.0008546 - 2.56380
Linalool oxide	0.098546 - 3.2506
Ethyl nonanoate (IS)	0.00466928 - 2.80157
Ethyl decanoate	0.186422 - 3.72843
Diethyl succinate	0.067836 - 1.8655
2-Phenethyl acetate	0.01987 - 0.1589
2-Phenethyl alcohol	0.2228 - 53.47

CHAPTER VII

Conclusions

The objective of this research study was the development and validation of a solid-phase microextraction (SPME), gas chromatographic (GC) method for the analysis of essential trace components in Pinot Gris and Chardonnay wines produced by the Lake Erie Enology Research Center (LEERC). This study was designed to provide the LEERC with an efficient, validated method for the analysis of aroma components in these wines.

At the onset of the research there were three hypotheses. The first hypothesis stated that the protocol could be synthesized from various conditions presented in the literature and by experimentation with available equipment. A method was developed and validated with extensive modifications to literature conditions. The most notable modification for available equipment was the selection of split injection and a 1:1 dilution of samples to avoid chemical ionization in the trap. The Saturn 2000 mass spectrometer ionizes the column effluent directly in the trap rather than prior to the trap as in most instruments. This results in a very high concentration of ions in the trap itself and leads to chemical ionization even at parts-per-million concentration levels. The transfer of this method to an instrument with a different trap design should allow expansion of the validation ranges for most analytes to higher concentrations.

The second hypothesis was that the developed method could be validated for all target components in both wine varieties. This hypothesis was disproved. Four components (acetaldehyde, ethyl isobutyrate, ethyl butyrate and ethyl isovalerate) were found to be present in wine at concentrations below their limits of quantitation with this method. Isobutanol exhibited nonlinear behavior at the wine concentrations determined

by this method. Several other components, notably the monoterpene alcohols, were undetectable in wines or standard solutions of corresponding concentrations with this method. The need for split injection and sample dilution as discussed above is a likely contributor to this problem.

The third hypothesis, that the spontaneous and inoculated musts would show quantifiable differences in the date of appearance and final concentrations of aroma components, was not tested. Time constraints prevented the analysis of the stored Chardonnay and Pinot Gris samples taken during their fermentation.

CHAPTER VIII

Future Work

There are a number of promising avenues of research based upon the results of this study. One group of studies could assess the accuracy of the assumptions upon which this study was based. For instance, it was assumed that the sampling method employed by the Lake Erie Enology Research Center gave repeatable samples that were representative of the entire batch. This was the most critical assumption, as the sampling process ultimately determined the fitness of the entire data set for any analysis. This was particularly true in trace analysis. One future study could analyze samples drawn from different regions of the batch to determine whether the single sample that was frozen to await analysis was representative.

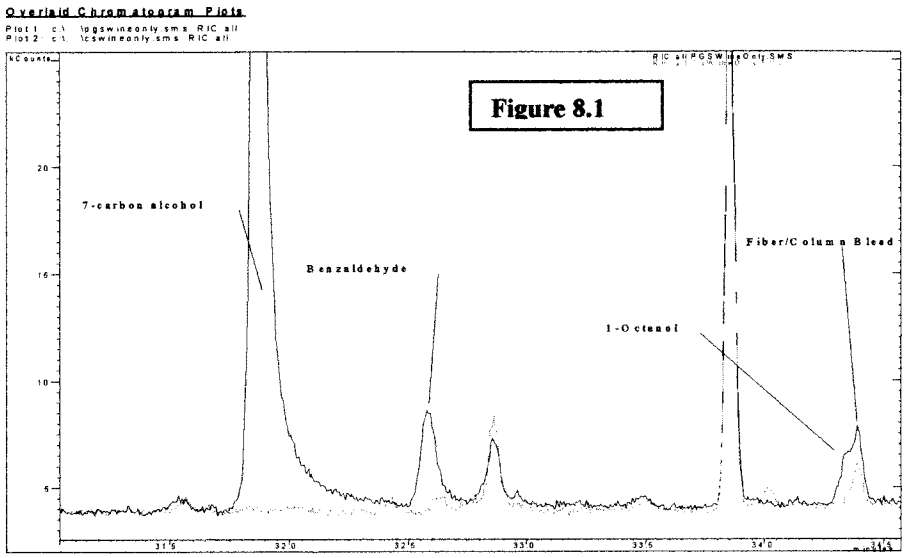
A second assumption focused again on the sampling process. The samples were drawn from the fermentation vessel, placed into plastic vials, capped and frozen to await analysis. Total storage time was more than one year. The assumption was that the concentrations of the aroma components present in the wines at the time of sampling would remain unchanged by the freezing and storage process. This assumption should be tested through a study that runs concurrently with the fermentation and sampling phase. Two samples should be drawn daily, with one analyzed immediately and the other sample frozen to await analysis. When the frozen sample is analyzed in the future, the results should be compared to the analysis done on the day of sampling. Multiple samples could be drawn and frozen, then analyzed individually over a period of several months to determine the freezer stability of the fermentation samples. Samples could also be frozen

in glass containers to determine whether the plastic storage vials adsorb any of the analytes during storage, thus interfering with accurate analysis.

A third assumption was that a single SPME/GC method would be suitable for analysis at any stage of fermentation, despite changes in wine composition. This assumption should be tested by analyzing samples with varying sugar and ethanol concentrations, as well as changing analyte concentrations that mimic those present at various stages of fermentation. The effects of changing pH over the levels normally seen from the beginning to the end of fermentation would also be a valuable addition to the knowledge gained in this study.

An additional area for future research may include working with the existing phase equilibria in the wine matrix rather than salting out to drive components into the headspace at artificially high levels. The data thus gathered will give a more accurate representation of wine aroma and flavor as experienced by the consumer.

A third area for future research is expansion of the list of target analytes. During analysis of the chromatograms, several esters and other components that were not on the original target list were noted in the wines in significant quantities (see Figure 8.1). These components, as well as others, could be added to the research to enhance the usefulness of the aroma analysis.



Finally, the problem of competition or saturation of the fiber coating during extraction is an area that strongly indicates the need for future research. Run-to-run headspace depletion of certain analytes, particularly high molecular weight esters, was evident in the results, and a corresponding run-to-run increase in the peak areas of other analytes was seen. This gives a strong indication that the available adsorption sites on the fiber become saturated by certain components, blocking others from being extracted in levels corresponding to their presence in the matrix. Future work could test whether this is true of other fiber coatings as well as the DVB/Carboxen/PDMS coating used in this research. Method modifications may alleviate this problem and allow lower limits of detection for some components such as the important monoterpene alcohols.

REFERENCES

(1) Jin, D.; Pardue, H. L. Algorithms for Time-Dependent Chromatographic Peak Areas 1. Algorithms Evaluated for Fully Resolved Peaks. *Anal. Chim. Acta.* **2000**, *422*, 1–10.

(2) Arthur, C.L.; Pawliszyn, J. Solid-phase Microextraction with Thermal Desorption using Fused Silica Optical Fibers. *Anal. Chem.* **1990**, *62*, 2145–2148.

(3) Kolb, B. Headspace Sampling with Capillary Columns. *J. Chromatogr. A* **1999**, *842*, 163–205.

(4) Górecki, T.; Yu, X.; Pawliszyn, J. Theory of Analyte Extraction by Selected Porous Polymer SPME Fibres. *Analyst* **1999**, *124*, 643–649.

(*5*) Arrhenius, S.P.; McCloskey, L.P.; Sylvan, M. Chemical Markers for Aroma of *Vitis vinifera* Var. Chardonnay Regional Wines. *J. Agric. Food Chem.* **1996**, *44(4)*, 1085–1090.

(*6*) De la Calle Garcia, D.; Magnaghi, S.; Reichenbächer, M; Danzer, K. Systematic Optimization of the Analysis of Wine Bouquet Components by Solid-Phase Microextraction. *J. High Resol. Chromatogr.* **1996**, *19*, 257–262.

(*7*) Pozo-Bayón, M.A.; Pueyo, E.; Martin-Álvarez, P.J.; Polo, M.C. Polydimethylsiloxane Solid-Phase Microextraction-Gas Chromatography Method for the Analysis of Volatile Compounds in Wines; Its Application to the Characterization of Varietal Wines. *J. Chromatogr. A* **2001**, *922*, 267–275.

(*8*) Pérez-Coello, M.S.; Martin-Álvarez, P.J.; Cabezudo, M.D. Prediction of the Storage Time in Bottles of Spanish White Wines Using Multivariate Statistical Analysis. *Z. Lebensm.-Unters.-Forsch.* **1999**, *208*, 408–412.

(*9*) Vianna, E.; Ebeler, S.E. Monitoring Ester Formation in Grape Juice Fermentations Using Solid Phase Microextraction Coupled with Gas Chromatography– Mass Spectrometry. *J. Agric. Food Chem.* **2001**, *49(2)*, 589–595.

(*10*) De la Presa-Owens, C.; Noble, A.C. Effect of Storage at Elevated Temperatures on Aroma of Chardonnay Wines. *Am. J. Enol. Vitic.* **1997**, *48(3)*, 310–316.

(*11*) Gonzalez-Viñas, M.A.; Perez-Coello, M.S.; Salvador, M.D.; Cabezudo, M.D.; Martin-Álvarez, P.J. Changes in Gas-Chromatographic Volatiles of Young Airen Wines During Bottle Storage. *Food Chem.* **1996**, *56(4)*, 399–403.

(*12*) Chisholm, M.G.; Guiher, L.A.; Zaczkiewicz, S.M. Aroma Characteristics of Aged Vidal blanc Wine. *Am. J. Enol. Vitic.* **1995**, *46(1)*, 56–62.

(*13*) Falqué, E.; Fernández, E.; Dubourdieu, D. Differentiation of White Wines by Their Aromatic Index. *Talanta* **2001**, *54*, 271–281.

(*14*) Dykstra, S.A.; Mike, J.H.; Levison, B. Youngstown State University, Youngstown, Ohio. National Science Foundation Research Experience for Undergraduates, unpublished work, 2001.

(*15*) Deibler, K.D.; Acree, T.E.; Lavin, E.H. Solid Phase Microextraction Application in Gas Chromatography/Olfactometry Dilution Analysis. *J. Agric. Food Chem.* **1999**, *47(4)*, 1616–1618.

(*16*) Martineau, B.; Acree, T.E.; Henick-Kling, T. Effect of Wine Type on the Detection Threshold for Diacetyl. *Food Res. Intl.* **1995**, *28(2)*, 139–143.

(*17*) Guth, H. Quantitation and Sensory Studies of Character Impact Odorants of Different White Wine Varieties. *J. Agric. Food Chem.* **1997**, *45(8)*, 3027–3032.

(*18*) Ferreira, V.; Ardanuy, M.; López, R.; Cacho, J.F. Relationship between Flavor Dilution Values and Odor Unit Values in Hydroalcoholic Solutions: Role of Volatility and a Practical Rule for Its Estimation. *J. Agric. Food Chem.* **1998**, *46(10)*, 4341–4346.

(*19*) Ferreira, V.; Sharman, M.; Cacho, J.F.; Dennis, J. New and Efficient Microextraction/Solid-Phase Extraction Method for the Gas Chromatographic Analysis of Wine Volatiles. *J. Chromatogr. A* **1996**, *731*, 247–259.

(*20*) Ferreira, V.; López, R.; Escudero, A.; Cacho, J.F. Quantitative Determination of Trace and Ultratrace Flavour Active Compounds in Red Wines Through Gas Chromatographic-Ion Trap Mass Spectrometric Analysis of Microextracts. *J. Chromatogr. A* **1998**, *806*, 349–354.

(*21*) Ferreira, V.; López, R.; Cacho, J.F. Quantitative Determination of the Odorants of Young Red Wines from Different Grape Varieties. *J. Sci. Food Agric.* **2000**, *80*, 1659–1667.

(*22*) Ortega, C.; López, R.; Cacho, J.; Ferreira, V. Fast analysis of important wine volatile compounds; Development and Validation of a New Method Based on Gas Chromatographic-Flame Ionisation Detection Analysis of Dichloromethane microextracts. *J. Chromatogr. A* **2001,** *923*, 205–214.

(*23*) Favretto, D.; Grandis, G.; Allegri, G.; Traldi, P. An Investigation of the Aroma Fraction of Some Italian Wines by Solid Phase Micro Extraction Gas Chromatography/Mass Spectrometry and Membrane Inlet Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1595–1600.

(*24*) Zhang, Z.; Yang, M.J.; Pawliszyn, J. Solid-Phase Microextraction. *Anal. Chem.* **1994**, *66(17)*, 844A–853A.

(*25*) Jeleń, H.H.; Wlazly, K.; Wasowicz, E.; Kamiński, E. Solid-Phase Microextraction for the Analysis of Some Alcohols and Esters in Beer: Comparison with Static Headspace Method. *J. Agric. Food Chem.* **1998**, *46(4)*, 1469–1473.

(*26*) Murray, R.A. Limitations to the Use of Solid-Phase Microextraction for Quantitation of Mixtures of Volatile Organic Sulfur Compounds. *Anal. Chem.* **2001**, *73(7)*, 1646–1649.

(*27*) Vaz Freire, L.M.T.; Costa Freitas, A.M.; Relva, A.M. Optimization of Solid Phase Microextraction Analysis of Aroma Compounds in a Portuguese Muscatel Wine Must. *J. Microcolumn Sep.* **2001**, *13(6)*, 236–242.

(*28*) Steffen, A.; Pawliszyn, J. Analysis of Flavor Volatiles Using Headspace Solid-Phase Microextraction. *J. Agric. Food Chem.* **1996**, *44(8)*, 2187–2193.

(*29*) Bartelt, R.J. Calibration of a Commercial Solid-Phase Microextraction Device for Measuring Headspace Concentrations of Organic Volatiles. *Anal. Chem.* **1997**, *69(3)*, 364–372.

(*30*) Matich, A.J.; Rowan, D.D.; Banks, N.H. Solid Phase Microextraction for Quantitative Headspace Sampling of Apple Volatiles. *Anal. Chem.* **1996**, *68(23)*, 4114–4118.

(31) Deibler, K.D.; Lavin, E.H.; Acree, T.E. Solid Phase Microextraction Application in GC/Olfactometry Dilution Analysis. Pre-print book chapter, **2001**.

(*32*) Sáenz-Barrio, C.; Cedrón-Fernández, T. Microextraction of Volatile Components from Wine Samples and their Determination by GC-FID. The Effect of Salts and Extraction Solvents Used. *Chromatographia* **2000**, *51(3/4)*, 221-225.