OPTIMIZATION OF A GAS CHROMATOGRAPHIC/MASS SPECTROMETRIC METHOD FOR DETECTION AND QUANTITATION OF THE CHEMICAL INDICATORS OF ATYPICAL AGING IN WINE

by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In the

Chemistry

Program

YOUNGSTOWN STATE UNIVERSITY

August 2007

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ABSTRACT

The chemical composition of wine represents an extremely complex matrix, where a specific compound of interest may often be present in only trace amounts in the midst of many other components of much higher concentration, as well as many other trace components. In order to isolate, identify and quantify a particular compound, it is advantageous to simplify the matrix and optimize a method for extraction and separation of the compound, allowing for the achievement of ideal sensitivity, precision and level of detection and/or quantitation. In this work, a complete method for the detection, identification and quantitation of the chemical compound 2-aminoacetophenone (2-AAP), is presented, from initial solvent extraction to analysis via gas chromatography/mass spectrometry (GC/MS). 2-AAP is believed to be responsible for the wine defect known as atypical aging (ATA). With minor modifications this method could also be used to target other compounds typically found in the wine matrix, particularly those with a chemical structure similar to that of 2-AAP.

ACKNOWLEDGEMENTS

I would like to offer sincerest thanks to my research advisor, Dr. Jim Mike, not only for his endless patience, guidance and support toward the completion of this project, but more importantly to me, for his role as a trusted friend and confidant. I would also like to thank Dr. Tim Wagner and Dr. Larry Curtin for their time and valuable suggestions as members of my thesis committee. Dr. Roland Riesen, for introducing me to the field of enology and sharing his knowledge and experience. Ray Hoff, for his invaluable technical assistance and many hours spent with me at the mass spectrometer. And Dr. John Jackson for remaining a faithful friend and source of comedic relief in times of stress.

I would also like to express my deepest gratitude to my entire family for their continued love and consideration through this arduous process, without their support, both moral and financial, this never would have been possible. To Lorraine's family as well, for accepting me as one of their own and for supporting the both of us in every way possible. To Lorraine, for being patient and understanding with me when I was at my most difficult and for having faith in me when I had lost all faith in myself. I am also thankful to Lorraine for bestowing upon me the greatest gift of all, my beautiful daughter, Margaret Rose, who provided the impetus needed for the completion of this work. Lorraine and Maggie, I love you both very, very much.

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LIST OF ABBREVIATIONS AND SYMBOLS

2-AAP	2-aminoacetophenone
3-AAP	3-aminoacetophenone
4-AAP	4-aminoacetophenone
A_s	Peak asymmetry
ATA	Atypical Aging
cm	centimeter
C	Celsius
C_m	Molar concentration in mobile phase
C_s	Molar concentration in stationary phase
eV	Electron volt
F	Flow rate
FID	Flame Ionization Detector
GC	Gas Chromatography
HETP	Height Equivalent to a Theoretical Plate
i.d.	Internal diameter
k'	Capacity factor
K_D	Partition coefficient
l	Column length
L	Liter
LOD	Level of Detection
LOQ	Level of Quantitation
MA	Methyl Anthranilate

LIST OF ABBREVIATIONS (CONTINUED)

min	Minute
mL	Milliliter
mm	Millimeter
MS	Mass Spectrometry
m/z	Mass-to-charge ratio
N	Number of theoretical plates
π	pi (~3.14)
ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
r_c	Column radius
R_s	Resolution
S/N	Signal-to-noise ratio
sec	Second
t_0	Dead time
t_r	Retention time
ū	Linear velocity
µg	microgram
µL	microliter
µm	micrometer
UTA	Untypical Aging
W or W_b	Peak width at the base

CHAPTER I

Introduction

A. Gas Chromatography

Chromatography is a physical method where separation of compounds is achieved through the distribution (partitioning) of analytes (solutes) between two distinctly different phases; the mobile and the stationary. Those chromatographic techniques utilizing a gaseous mobile phase are called gas chromatography (GC). The mobile phase in GC is an inert gas. Commonly used gases include nitrogen, helium, argon and carbon dioxide. The stationary phases used in GC can either be a solid with wall-bonded modifiers or a viscous liquid that is chemically supported on a solid. Because of its relative low-cost, ease-of-use and powerful analytical properties, GC has currently become the most popular method of separation for volatile analytes (*1*).

B. Sample Introduction

Within any chromatographic system, the method of injection impacts the quality of the chromatographic separation. The injection system, therefore not only provides a means of introducing the sample onto the GC column, it simultaneously maintains the system pressure and chromatographic flow, as well as prevents the introduction of air onto the column – a potentially disastrous (and column destroying) event. There are various types of injection systems available, but two of the more popular methods used for capillary column GC are termed “split” and “splitless” injection.

In both “split” and “splitless” the injection device is fitted with an independently controlled injection heater for the injection throat. To help ensure reproducibility, the

device is also fitted with a replaceable throat liner made from glass or silica, as well as a self-sealing (and replaceable) silicone-based septum, for syringe injection. Also present are a connection for the carrier gas along with an automatically controlled split valve that is used for sample diversion during the injection process.

The injection process begins with introduction of the sample via a syringe into the injector throat, where it is vaporized as a result of the elevated temperature. The split valve controls the amount of sample that is allowed to enter the head of the column. During a "split" injection, the split valve is allowed to remain open and a majority of the sample volume is swept out of the injector into a waste line, which leaves only a relatively small, but representative portion of the injected sample that will ultimately partition between the stationary phase and the mobile phase within the chromatographic column. The principle reason for using split injection is to prevent overloading of the column, which could result in diminished detectability and poor chromatographic resolution and peak shape. Split injection thus allows the opportunity for maximizing detectability, while still maintaining chromatographic quality.

In contrast, during a "splitless" injection, the split valve remains closed for a short period of time (typically from 0.5-1.5 minutes), before opening to purge the sample. This allows a significantly greater proportion of the sample to be passed onto the column and partition between the stationary phase and the mobile phase. Splitless injection is used primarily to increase the sensitivity of an analysis, although there is often the sacrifice of chromatographic quality (i.e., resolution and peak shape.) (*2*).

C. Component Retention

As analyte solutes pass through a chromatographic system they are separated based upon their individual distribution coefficients (K_D) with respect to portioning between the gaseous mobile phase and the stationary phase. The distribution coefficient of a solute is described by the equation:

$$K_D = C_s/C_m$$

Where C_s is the concentration of the solute in the stationary phase and C_m is the concentration of the solute in the mobile phase. Retention of a solute corresponds to a longer time within the stationary phase. What that means is that the more a solute is 'retained' within the stationary phase of a chromatographic column, the longer will be its retention time (t_r). A species that is considered unretained by the stationary phase will pass through the column with virtually no partitioning. The time required for a non-retained solute to traverse the chromatographic column is called the chromatographic dead time (t_0). Importantly, the measured dead time (and, indeed, the retention of any solute) also includes the amount of time required to traverse the entire chromatographic system, not just the column. Due to the indistinct nature of the bonded or thin-film stationary phase, the actual volume of a stationary phase at any given time is difficult or impossible to determine. Because of this difficulty, the calculation or measurement of actual analyte partition coefficients is virtually impossible. Because of this, an experimental parameter called the capacity factor (k') is used as a descriptor of chromatographic retention. Capacity factor is defined by the equation:

$$k' = t_r\text{-}t_0 / t_0$$

Where t_r is the retention time and t_0 is the dead time of the column. Optimally, for quantitative analysis of solutes, the capacity factor value for an analyte should be approximately within the range of 1 to 5. Higher values of k' indicate elution times that are too long (increased partitioning between the analyte and stationary phase), while lower values indicate relatively little partitioning between the two, resulting in shorter retention times.

GC mobile phases consist of a continuously flowing inert gas (e.g. helium). The mobile phase gas carries the analyte sample through the column, but because of the nature of gases, plays virtually no direct role in the separation process. That is, gas molecules do not interact with one another or with solutes, so the mobile phase is truly only a solute carrier.

The carrier gas pressure at the column head (head pressure) determines both the linear velocity ($\bar{u}$) and the flow rate (F) of the gas through the column. Linear velocity is the flow rate expressed in units of distance/time and is described by the equation:

$$\bar{u} = l / t_0$$

and flow rate, which is a volumetric parameter (i.e., volume/time), is described by:

$$F = \pi(r_c^2)l / t_0$$

where l is the column length (cm), r_c is the radius of the column (cm) and t_0 is the dead time. Use of these parameters is vital when comparing experimental retention times with literature values for retention times of a given compound. Variability of gas pressures, as well as inconsistencies in chromatographic column parameters and GC systems would make such comparisons difficult or impossible otherwise.

A solute introduced onto the head of a chromatographic column, when properly injected, results in the formation of a (short) regular cylinder of distributed molecules known as a solute band. As a solute band passes down a chromatographic column, it becomes broadened by three kinetically controlled diffusion processes: eddy diffusion, longitudinal diffusion and mass transfer diffusion.

Eddy diffusion is diffusion resulting from the various paths taken by the solutes as they migrate down the column. Longitudinal diffusion is an entropic process resulting from the natural tendency of molecules to travel from areas of high concentration to areas of low concentration. Mass transfer diffusion (sometimes called resistance) reflects the speed with which solute molecules diffuse back and forth between the mobile and stationary phases as it passes down the column.

A parameter known as column efficiency can be defined. It is the ability of a chromatographic column to minimize diffusion resulting in low dispersion of solute bands which results in sharp, narrow peaks on the chromatographic record. The Van Deemter equation relates column efficiency to the above-defined band-broadening processes:

$$H = A + B/\bar{u} + C\,\bar{u}$$

where $\bar{u}$ is the linear velocity of the mobile phase, A is eddy diffusion, B is longitudinal diffusion and C is the rate of mass transfer. To calculate column efficiency the equation:

$$H = L / N$$

is used, where L is the length of the column and N is the total number of theoretical plates. The number of theoretical plates and the plate height (H) or height equivalent to a theoretical plate (HETP) are commonly used terms that describe column performance.

For symmetrically shaped peaks, the equation that can be used to calculate this parameter from the chromatographic record is:

$$N = 16(t_r / W_b)^2$$

Where N is the number of theoretical plates, t_r is the retention time of the peak and W_b is the peak width at the peaks base, measured in minutes.

In order to achieve an optimum separation of solutes, baseline separation (or resolution) must exist between adjacent peaks on the chromatogram. Ideally this must occur within an acceptably short period of time. Peak resolution is defined using a parameter known as the resolution factor, R_s, which can be calculated from the chromatogram using the following equation:

$$R_s = 2[(t_r)_B - (t_r)_A] / W_A + W_B$$

where t_r is the retention time of adjacent peaks for solutes *A* and *B*, and *W* is the width of each peak at its base. As long as peaks are symmetrically shaped, an $R_s < 1$ indicates that peaks are unresolved, an $R_s = 1$ indicates peaks that are quantifiable, but display a lack of baseline resolution and an $R_s > 1$ indicates peaks for which baseline resolution have been achieved.

The resolution of peaks on a chromatogram is strongly affected by the symmetry of those peaks. The movement of solute molecules through a GC column is random, and therefore their distribution will be statistical in nature (i.e. they are approximately Gaussian) and barring extraneous effects will be, by definition, symmetrically shaped. However, unusual interactions between analyte molecules and the stationary phase may result in molecular distributions within the solute bands that result in peak fronting or tailing in the chromatogram. Peak asymmetry can be calculated using the equation:

$$A_s = b/a$$

where *b* is the distance after the peak center and *a* is the distance before the peak center to be measured at 10% of the total peak height. Ideally, A_s values should be very close to 1.0. Values of $A_s > 1$ indicates a tailing peak while a value of $A_s < 1$ indicates peak fronting.

D. Solute Detection in GC

As solutes elute from a GC column after separation, they must be detected in some manner in order to create a chromatographic record. There are various detection devices available for this purpose, some that measure the changes in the bulk properties of the eluent, such as thermal conductivity and others that measure properties unique to the solute, as with mass spectroscopy. The chromatographer must choose the detector that is best suited for each individual application.

The flame ionization detector (FID) is among the most commonly used for gas chromatography. The FID consists of a hydrogen/air flame that is used to combust organic analytes as they elute from the column. Ions that form during the combustion process are collected on an electrode, which results in the production of an electric signal proportional to the concentration of the analyte. The FID is an extremely sensitive method of detection and has a wide linear dynamic range. However, it is a destructive method, and the sample is destroyed by the detection process. Furthermore, its detection is limited largely to those molecules containing carbon.

Another widely used method for GC detection is the mass spectrometer (MS). The MS ionizes solutes in a high vacuum, propels and focuses them and their

fragmentation products through a mass analyzer based upon their mass-to-charge ratio (m/z). After focusing, the MS collects and measures the amounts of each selected ion in a detector. In comparison to other detection methods, MS is expensive, but has the advantage of allowing chemical and structural information to be gained for each analyte based upon the mass to charge ratio (m/z) and fragmentation pattern (*3*).

CHAPTER II

Statement of the Problem

Due to the complex chemical matrix of wine, the development of a method of separation, identification, and quantitation that is reliable, sensitive, and specific to the compound or compounds of interest, is necessary for analytical analysis. The chemical compound 2-aminoacetophenone (2-AAP) was first reported by a German research group in the 1980's as the key component in connection with the flavor defect known as "atypical aging" (ATA) or "untypical aging" (UTA) that is sometimes found in white wine varietals. This defect causes an affected wine to lose its varietal flavors very quickly, and with this disappearance, atypical flavors described as furniture polish, wet wool, mothball, fusel alcohol and/or acacia blossom begin to appear. Often atypical aging is cited as a crucial reason why some vintages are rejected at the official quality examination. It has been estimated that the percentage of wines affected by ATA may be as high as 20%, which could potentially cause serious economic loss to the wine and grape industry. Due to the increase in atypical aging off-flavor in wines globally and particularly in American white wines, the necessity for analytical methods targeting the compound 2-aminoacetophenone, which is thought to be responsible, has gained importance. Because of 2-AAP's low threshold in wine, coupled with the number and variety of differing chemical compounds also present in the matrix, development of extraction, separation and quantitation techniques specific to this compound would be quite useful.

In this work a complete method of isolation, detection and quantification of the chemical compound 2-AAP in wine is presented. This method is the expansion and modification of a basic extraction/separation protocol originally developed in Germany (*13*). The method presented here includes solvent extraction, evaporation and re-solvation, compound separation via gas chromatography and chemical detection and quantification through mass spectrometry. The method is both fully optimized and partially validated with determinations of linearity, recovery as a measure of method accuracy, limits of detection and limits of quantitation.

CHAPTER III

Literature Review

Under normal conditions, as wine ages, its aroma compounds undergo slow changes due to oxidation and other processes. This effect generally occurs over the course of years. A wine classified as having "atypical (ATA) aging" possesses a chemical defect. Atypical aging in a wine is characterized by the development of an off-odor or an off-flavor either in the bottled wine or the cask. Atypical aging also occurs rather quickly, within a few months of fermentation. The off-flavor is described as naphthalene, floor polish, dirty wet towel, acacia blossom and/or fusel alcohol. The appearance of these off-flavors is accompanied by the disappearance of the wine's normal varietal flavor. It's estimated that atypical (or untypical (UTA)) aging affects up to 20% of all wines and is therefore associated with significant economic impact.

First identified and reported in the Frankonian wine-growing region of Germany in 1988 (*4*), the defect was named *untypischen alterungsnote* (UTA). Although primarily occurring in white wines, the defect has been found in most types and styles of wines of a particular year, but does not necessarily occur in all wines of that vintage. Since first reported, the number of cases of the defect has increased, as has the number of wine-growing regions reporting occurrences, not only in Europe, but worldwide.

Acree et al. in 1990 was the first to suggest that the chemical compound 2-aminoacetophenone (2-AAP), in combination with other compounds, may be what causes the 'foxy' flavor found in certain wines, specifically native American and French-American hybrids (*5*). An amount of 0.3 μg/L (ppb) was reported as the limit sufficient

for human perception. In 1993 Rapp et al. reported a flavor threshold of 0.7-1.0 μg/L (ppb) for 2-AAP as the compound responsible for off-flavor (*6*). Christoph later reported the threshold to be about 1.5-2.0 μg/L, but cautioned that this was dependant upon the aroma matrices of the individual wines (*4*). Using stable isotope dilution assay, Dollman et al. reported in 1996 that concentrations of 2-AAP detectable in off-flavored wines range between 0.7-12.8 μg/L (*7*). 2-aminoacetophenone has also been reported to cause off-flavors in other products such as corn flour (*9*) and beer (*10*).

Statistical studies have shown that wines produced from grapes grown under the stress caused by dry growing seasons and/or poor nitrogen uptake, exhibit an increase in the occurrence of untypical aging. The same studies show a significant relationship between the concentration of 2-AAP present in the wines and UTA off-flavor intensity (*8*). An amino acid metabolite, 2-AAP's route of formation has been investigated in several studies. In 2002, in separate studies, Hoenicke et al. suggested that the formation of 2-AAP is caused by either an oxidative degradation of the phytohormone indole-3-acetic acid (IAA) in the presence of superoxide radicals, triggered by sulfuration following fermentation (*8*) or by degradation of the tryptophan metabolite kynurenine (*11*).

Chapter IV

Materials and Methods

A. Materials

All reagents used in this work were of the highest purity available and were used as received. The wine used as a standard in all analytical determinations was Carlo Rossi Chablis (Modesto, CA). All chemicals used with their purity and source are listed in Table 4.1.

Table 4.1 Chemicals, Purity and Source

Chemicals	Percent Purity	Source
Ethyl-2-aminobenzoate	99+	Aldrich
Indole	99+	Aldrich
2-Aminoacetophenone	98	Aldrich
3-Methylindole	98	Aldrich
4-Aminoacetophenone	99	Sigma-Aldrich
Methyl-2-aminobenzoate	99+	Aldrich
3-Aminoacetophenone	97	Sigma-Aldrich
2,6-Dichloroaniline	98	Aldrich
Acetonitrile (HPLC Grade)	99.9	Fisher
Ethyl ether (Anhydrous)	99.9	Fisher
n-pentane (HPLC Grade)	99.3	Fisher
Sodium chloride	99.9	Fisher
Potassium hydroxide	99.5	Fisher
Acetone (HPLC-UR Grade)	99.9	Pharmco
Helium (Ultra-High Purity)	99.999	Praxair

Source	Full Name and Location
Aldrich	Milwaukee, WI
Sigma-Aldrich	St.Louis, MO
Fisher	Pittsburgh, PA
Pharmco	Richmond, VA

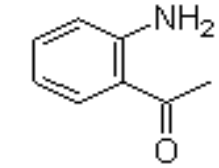
The original protocol suggested using a 60 meter Supelco SPB 5; 0.32 mm i.d. chromatography column for separation with hydrogen as a carrier gas (*13*). In the present work, all gas chromatography was performed using a Varian Factor Four wall-coated, open tubular, fused silica, capillary column with the following specifications:
length- 30 meter, 0.25 mm i.d. VF-5ms with 0.25 μm film thickness.
Column ends were cut with a ceramic scribe to ensure the most favorable results (i.e. the cuts were flush and even). Ultra-high purity helium obtained from Praxair (Cleveland, OH) was used as the carrier gas with Finnigan MAT GCQ Purifier water and oxygen traps installed on the gas lines. All injections were performed using a Finnigan MAT A200S autosampler (San Jose, CA) fitted with a Hamilton 10 μL, fixed-needle glass syringe purchased from Supelco (Bellefonte, PA).

Gas chromatography separations were performed using a Finnigan MAT GC system (San Jose, CA) coupled with a Finnigan MAT ion trap mass spectrometer (San Jose, CA). The GC was fitted with a 4 mm i.d. split/splitless injection liner packed with deactivated glass wool, Thermogreen LB-2 17 mm septa and vespal graphite ferrules, all purchased from Supelco (Bellefonte, PA). Gas chromatograph flow rates were measured using a Varian Intelligent Digital Flowmeter. A Denver Instrument Company TR-04 analytical balance was used to for all weighings. All samples were stored in 2 mL glass vials with screw caps and Teflon® lined septa.

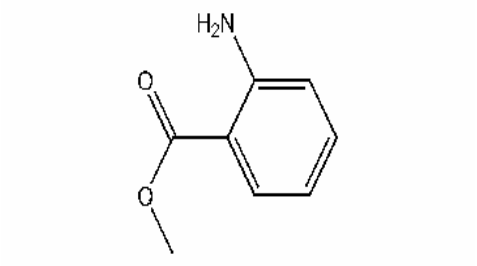
B. Methods

1. Selection of Standards

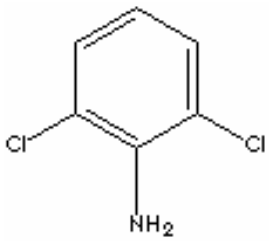
The selection of internal standards for use in this work was based primarily on the suggestion from the original extraction/separation protocol delivered via personal communication to Dr. Roland Riesen at Youngstown State University from Dr. R. Sponholz, Geisenheim, Germany (*13*). The internal standards were selected due to their structural similarity to the compound 2-aminoacetophenone (2-AAP) (believed to be the ATA-causing component) or based on the fact that they are possible degradation products of the potential precursors to 2-AAP, kynurenine and indole-3-acetic acid (*8*). Initially, the compounds 3-aminoacetophenone (3-AAP) and 4-aminoacetophenone (4-AAP) were chosen as internal standards. However, these compounds proved to be unacceptable for this study due to unresolvable co-elution in the wine matrix (3-AAP) and excessive peak broadening in the chromatogram (4-AAP). Several different internal standards were used in this work in order to determine which of the compounds behaved optimally as an internal standard in the given method. The names and chemical structures of each standard, along with that of 2-AAP are shown in Figure 4.1.



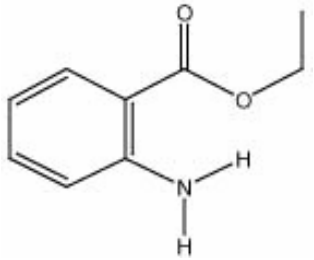
2-aminoacetophenone



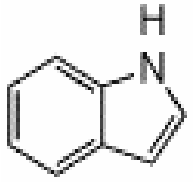
methyl anthranilate



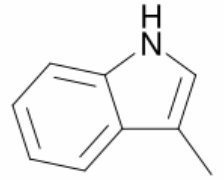
2,6-dichloroaniline



ethyl anthranilate



indole



skatole

Figure 4.1 Chemical Structures of 2-AAP and Standard Compounds

2. Extraction of Wine

The original extraction protocol, which was used as an outline for that presented below, suggests beginning with a 250 ml volume of wine and using methylene blue as a dryness indicator during addition of sodium sulfate (*13*). For this work, wines were generally tested in 100 mL aliquots delivered into a beaker via a volumetric pipette. After adjusting the pH of the wine samples to 5.0 with potassium hydroxide solution, approximately 1.0 grams of sodium chloride per 5.0 mL of wine was dissolved into the samples with the aid of a magnetic stir bar and plate. Samples were then transferred into individual separatory funnels. The beakers were rinsed with 3.0 mL portions of 1:1 ethyl ether/n-pentane, with each

wash then also being added to the sample in order to minimize loss of analytes during the transfer. A mixture of 1:1 ethyl ether/n-pentane in a volume equivalent to about 10% of the total aqueous volume of the samples was then added to the separatory funnels and the samples were vigorously shaken with frequent venting for about five minutes. At this point, approximately 10-20 grams of ice were added to the samples with swirling to minimize the formation of an emulsion. Initially, centrifugation was used as a means to break the emulsion formed in the extraction process with good results. However, ice addition as a means of emulsion minimization proved to be more convenient and equally effective, with less risk of analyte loss during liquid transfer. The layers were given time to separate (5-7 minutes) and then the aqueous phase was drained and discarded. The organic phase was drained into 50 mL conical, centrifuge tubes with Teflon® lined screw caps and granular, oven-dried sodium sulfate was added as a drying agent. The samples were swirled in a vortex mixer (vortexed) to aid in drying and then transferred, minus the drying agent, to clean, 50 mL, oven-dried, conical centrifuge tubes. The remaining drying agent (Na_2SO_4) was rinsed and vortexed twice with 3.0 mL portions of organic solvent which was then added to the sample to again minimize loss of analytes. The samples were then concentrated to dryness with nitrogen using an Organomation Associates Inc. N-EVAP III Nitrogen Evaporator, re-solvated with 0.2 mL of acetonitrile and transferred via pipette to a 2.0 mL screw-cap GC/MS sample vial with a Teflon® lined septa for instrumental analysis.

3. Chromatographic Method

In all instances, the autosampler was programmed to first wash the syringe three times with solvent (HPLC-Grade acetonitrile) and four times with the sample itself. A fifth fill was the sample to be analyzed. After a hesitation of 1.0 second, the autosampler then injected a sample volume of 2.0 μL, minus an air plug, into the injector, set at 200 ^{0}C and allowed the needle to reside there for 4.0 seconds prior to withdrawal. The temperature of the chromatographic column was initially set at 50.00 ^{0}C and increased upon injection at a rate of 8.00 ^{0}C/min. to 100.00 ^{0}C. The column was held at this temperature for 10.00 minutes and then increased again at a rate of 2.00 ^{0}C/min to a temperature of 130.00 ^{0}C. The column was then immediately increased at 10.00 ^{0}C/min. to a temperature of 180.00 ^{0}C. Finally, the column temperature was ramped to 250.00 ^{0}C at a rate of 40.00 ^{0}C/min. and held at that temperature for the last 5.00 minutes of the chromatographic run.

Splitless injection was used with the split vent closing 0.20 minutes prior to injection and opening again 0.75 minutes into the run. The split vent flow was set at 45.0mL/min. with the septum purge set at 0.5mL/min. The carrier gas pressure (ultra-high purity helium) was set at 7.50 psi for the duration of the separation. Surge pressure was disabled. The ion trap source (electron impact, 70eV) was activated 3.00 minutes after injection with the temperature set at 200 ^{0}C, scanning a mass range between 100-200 m/z at 0.50 seconds per scan. The transfer line was set to a temperature of 280 ^{0}C.

All compound retention, identification and quantification were performed using Finnigan MAT GCQ data processing systems.

4. Method Optimization

By systematically varying specific instrumental conditions (listed below) of a method and measuring the resultant effect in the signal/noise ratio, the resolution, detection limits and levels of quantitation of a method were optimized with respect to a particular chemical compound or group of compounds. Noise level is defined as that signal which is produced by the instrument when a sample containing no analyte, or a blank sample, is analyzed with the instrument or when the area on a chromatogram containing no sample signal is analyzed. The ratio between the signal produced by an analyte and that which is produced by a blank is called the signal/noise ratio (S/N). For this work, in all instances, the signal-to-noise ratio was calculated by comparing the chromatographic peak height of the standard to the average peak height of the surrounding noise level. A number of instrumental variables were tested in this manner with the method being adjusted based on the results to provide an optimal separation, level of detection and level of quantitation for the analytes. A list of these variables along with a short definition of each is provided below:

Depth of Column – The distance or "depth" that the chromatographic column extends from the chromatograph oven into the GC injector.

Air Volume – Often in GC analysis, a small amount of air, known as an "air gap" is drawn into the injection syringe after uptake of the sample and prior to injection, in order to prevent loss of volatiles and minimize contamination of the sample.

Injector Temperature – The temperature of the GC injector where all analytes will be vaporized immediately prior to introduction to the GC column for separation. If the temperature is too low, separation will be poor with little or no peak resolution. If the temperature is too high, decomposition or alteration of chemical structures of analytes may occur.

Pre-injection Hold Time – The amount of time the autosampler pauses between insertion of the syringe needle into the injector port and injection of the sample.

Split Vent Flow – Rate at which the carrier gas flows through the split outlet after it's been opened.

Injection Volume – The volume of sample introduced to the injector for analysis.

Needle Residence – Amount of time the syringe needle pauses inside the injector following an injection.

Septum Purge Flow – Rate at which the carrier gas flows through the septum purge vent.

Split Vent Open – The amount of time that elapses between the injection of a sample and the opening of the split vent.

CHAPTER V

Results and Discussion

A. Peak Identification

Three methods were used to achieve chromatographic peak identification. First, a comparison of retention times (t_r) between standards run in isolation and those extracted from a wine matrix was employed. Secondly, separate wine samples were spiked with an increased concentration of each of the analytes of interest. The resulting chromatograms displayed a marked increase in peak height and area for the appropriate analyte. Lastly, the mass spectral data of each peak was compared with the NIST library spectral data available on the Finnigan MAT ion trap mass spectrometer to assist in providing a more positive identification. A typical chromatogram of standards run in isolation showing target peaks identified and labeled is shown in Figure 5.1 for comparison to a typical chromatogram with spiked concentrations extracted from a wine matrix as shown in Figure 5.2.

B. Method Optimization

The compound of interest, 2-aminoacetophenone and the chemical standards 2,6-dichloroaniline, ethyl anthranilate, indole, methyl anthranilate and skatole each of identical concentration, were analyzed via gas chromatography using a fixed method, with a single variable (listed below) being systematically modified in each analysis. The peak height of the standard was then compared to the peak height of the noise level and the resulting signal/noise ratio (S/N) of each chromatogram was analyzed to determine

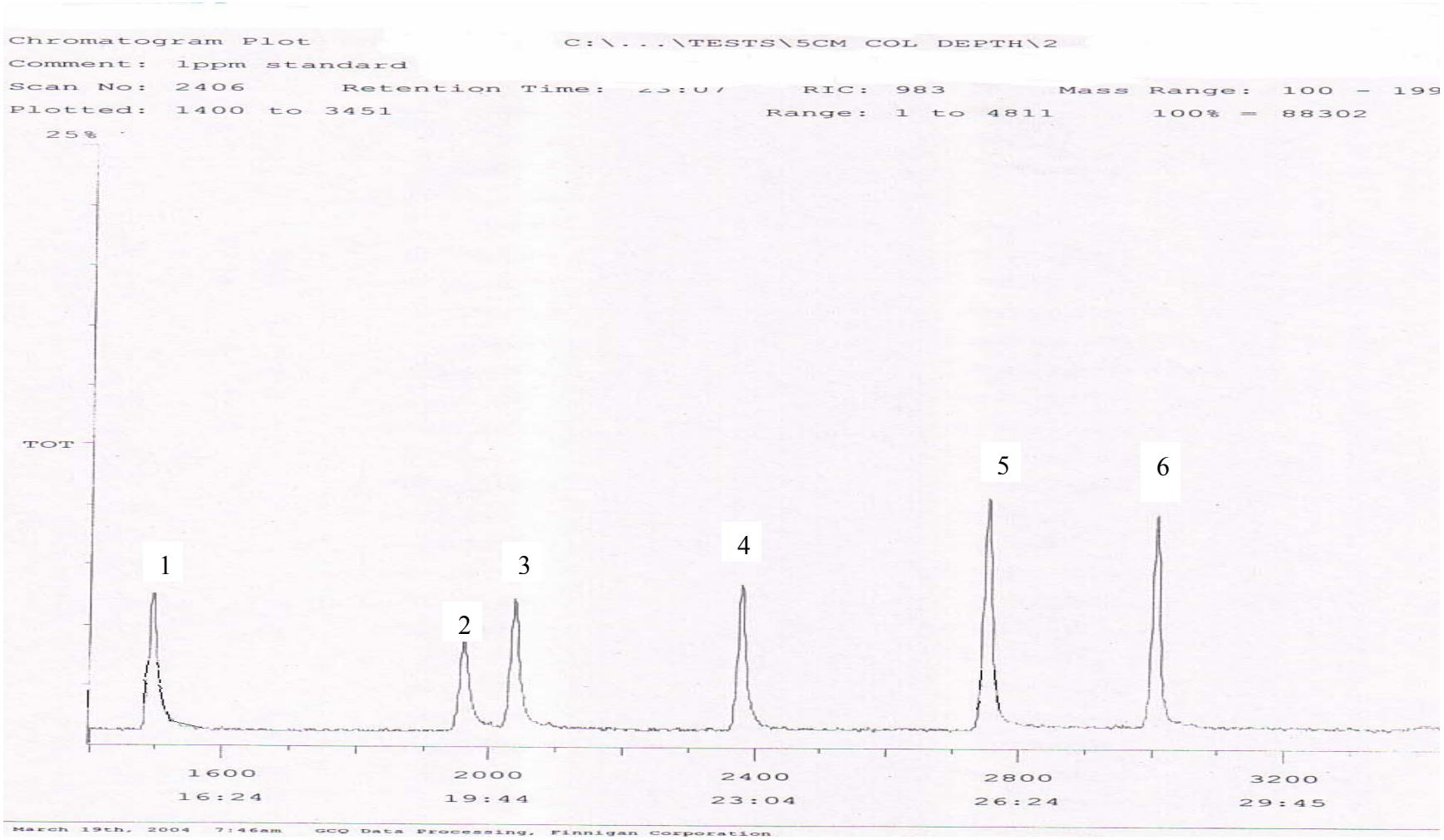


Figure 5.1 Peak Identification in Solvent

1	2,6-Dichloroaniline	4	Methyl anthranilate
2	Indole	5	Skatole
3	2-Aminoacetophenone	6	Ethyl anthranilate

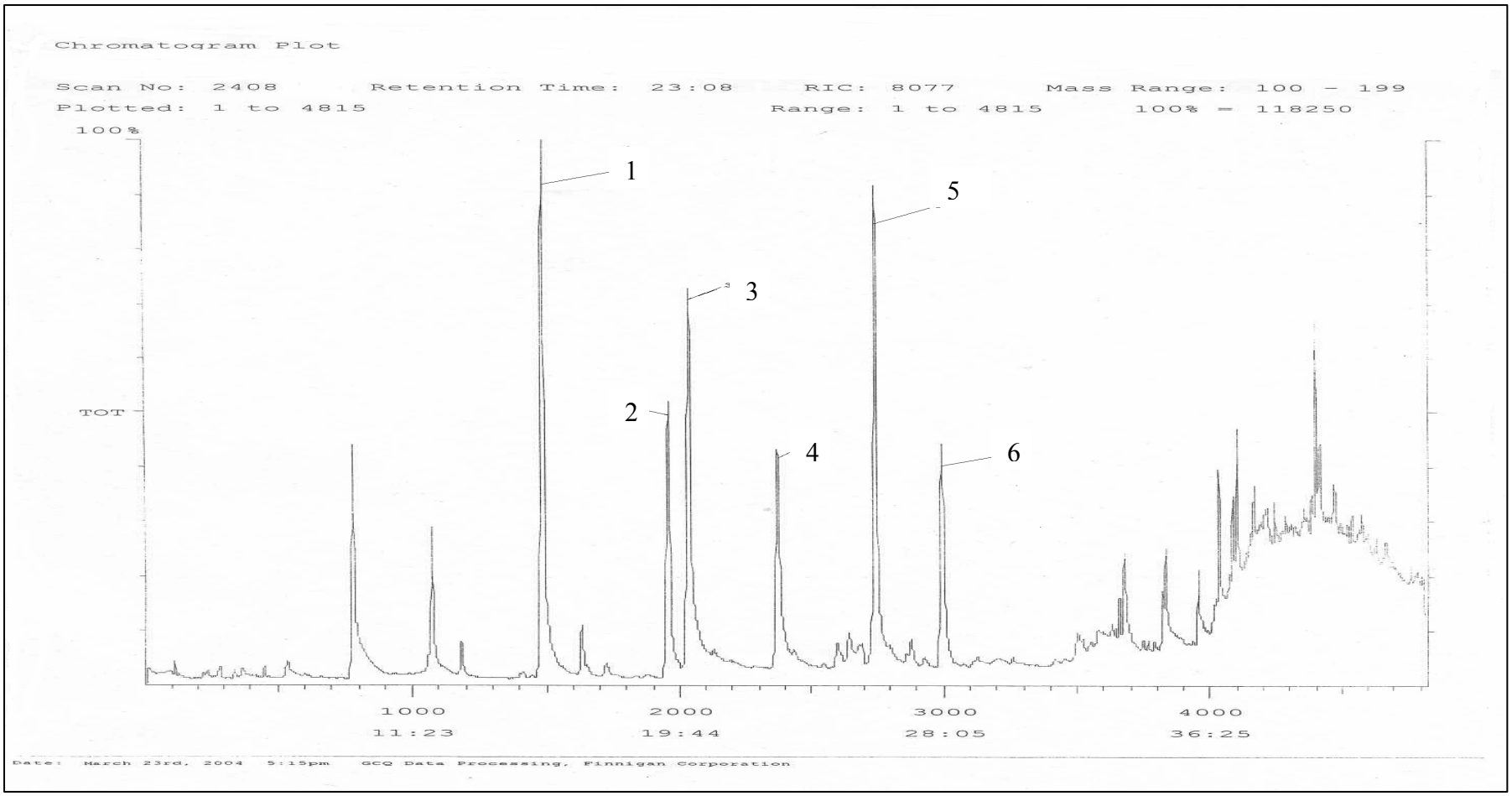


Figure 5.2 Peak Identification in a Wine Matrix

1	2,6-Dichloroaniline	4	Methyl anthranilate
2	Indole	5	Skatole
3	2-Aminoacetophenone	6	Ethyl anthranilate

the optimal value of each of the following instrumental conditions**:** Depth of column, air volume, injector temperature, pre-injection hold time, split vent flow, injection volume, needle residence, septum purge flow and split vent open. Maximizing the signal-to-noise ratio of each parameter increases specificity of the method and lowers the levels of detection and quantitation. The results of each these tests were tabulated and are represented graphically in Figures 5.3 – 5.11.

1. Depth of Column

In this work, the instrumental variable first tested was the depth of the chromatographic column into the injector. It was decided that this parameter should be initially determined as a point by which all other parameters would be based. The reasoning for this was practical, as altering the depth of the column requires some disassembly of the chromatograph, which includes the risk of column contamination and/or breakage, whereas altering most other parameters simply involves computer software manipulation or basic mechanical adjustments. The chromatographic column was inserted into the injector from the chromatograph oven and secured with a ferrule and a column nut (see Figure 5.12). By shifting the position of the ferrule and column nut on the column, the depth of the column into the injector could be altered.

It should be noted that the results of the signal-to-noise ratios for the column depth experiment were somewhat higher than the results of the experiments of remaining parameters because a higher standard concentration was used in the column depth trials. Results of the column depth experiment show that a signal-to-noise ratio of 51.22 was achieved when the chromatographic

column was inserted 5.0 cm into the injector, the next highest value achieved was 44.65 when the column was at 5.5 cm depth. The ratios steadily declined as the depth increased or decreased from a depth of 5.0cm.

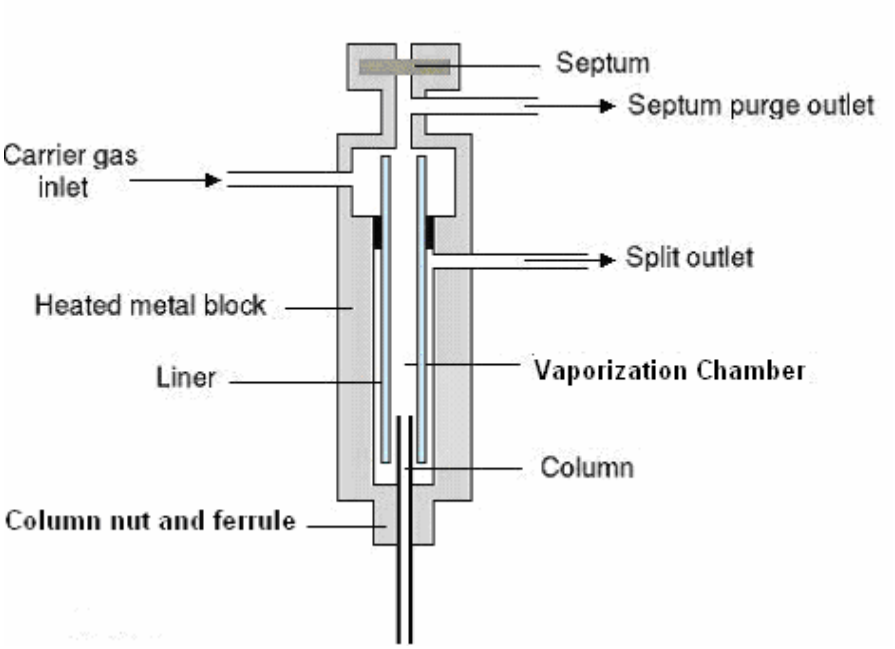
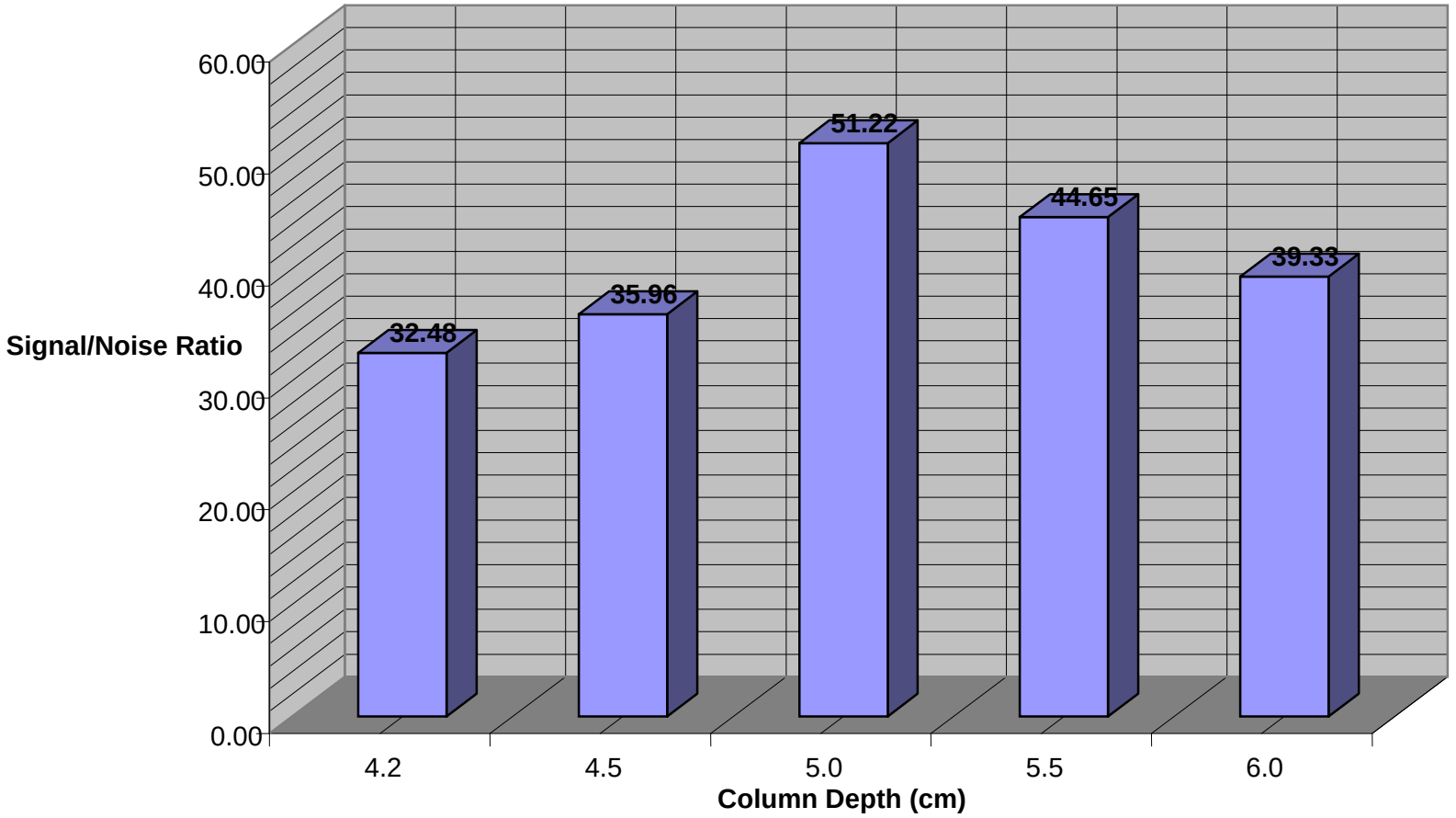


Figure 5.12 Chromatograph Injector Port (*12*)

Based on the empirical results of this experiment, the column depth into the injector was fixed at 5.0 cm for the remainder of the experiments. It was decided that the high (relative to the other depths) signal-to-noise ratio obtained from this column depth would provide optimal sensitivity on which the other tested parameters could then be based.

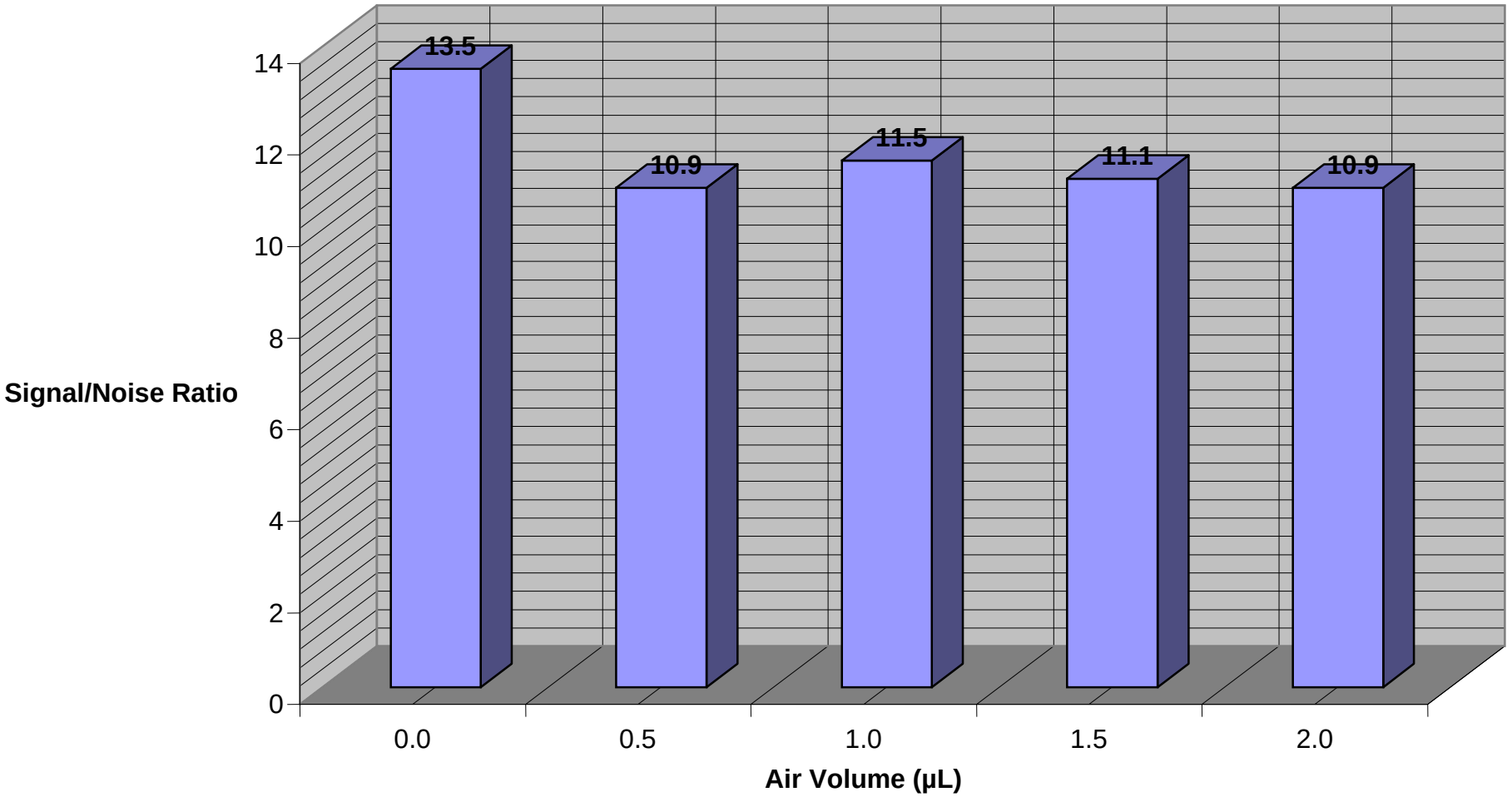
Figure 5.3: Column Depth into Injector



2. Air Volume

The next parameter to be tested was the volume of air that would be drawn into the injection syringe after uptake of the sample to be analyzed and prior to injection of the sample into the vaporization chamber. This volume of air, called an "air gap" was meant to minimize contamination of the sample and prevent loss of volatiles. A range from 0.0 μL to 2.0 μL (increasing 0.5 μL with each trial) of air was tested. Results indicate that a maximum signal-to-noise ratio of 13.5 was achieved when no air was in the syringe during injection. Based on these findings, no air gap was used in any injections made in this work. The S/N ratios for the other trials were relatively close in value to the maximum as well (11.5 – 10.9). Therefore, in any future work, this parameter may need to be altered due to individual considerations (e.g. analyte stability, room temperature, solvent volatility).

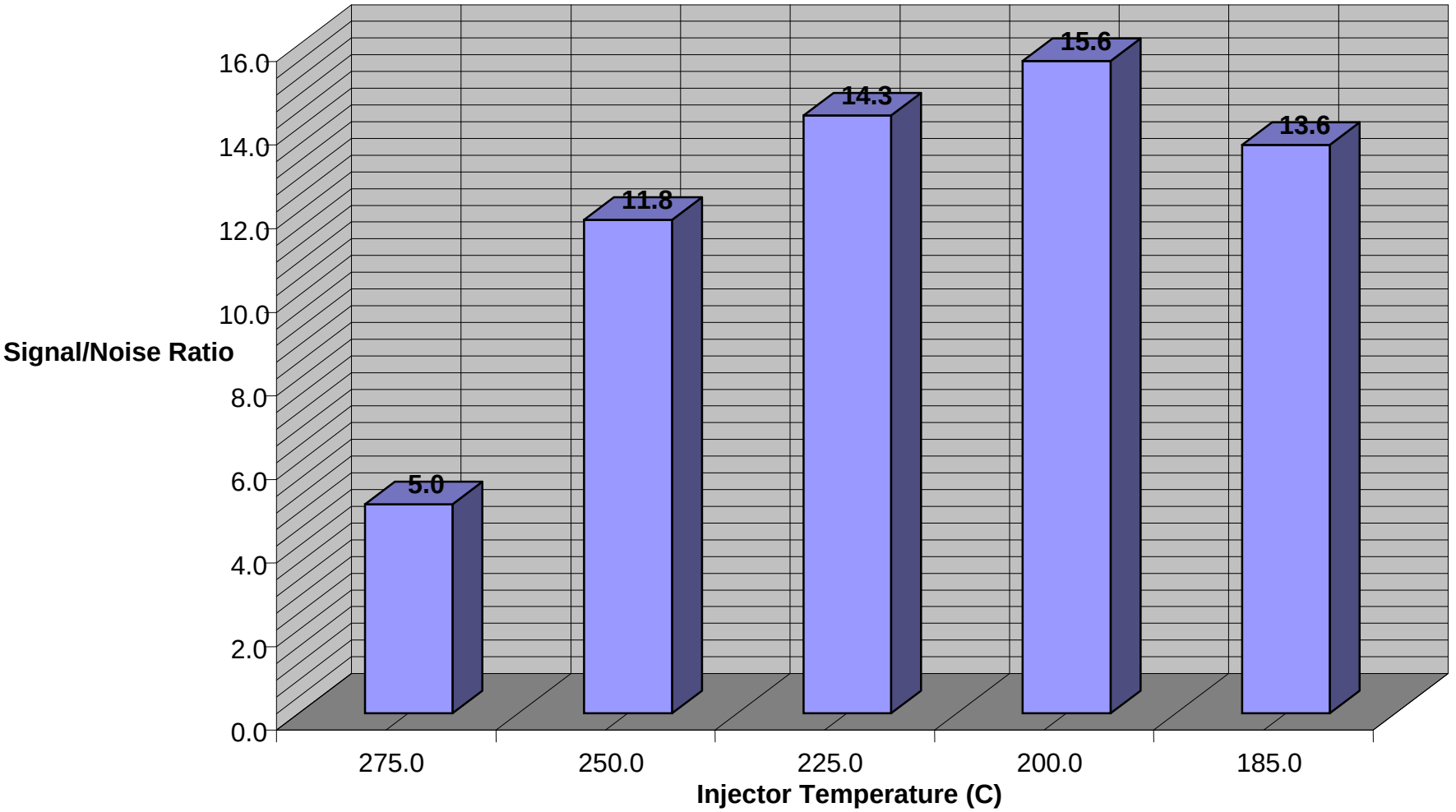
Figure 5.4: Air Volume



3. Injector Temperature

The injector port contains the vaporization chamber, in which vaporization of the sample occurs prior to introduction to the GC column. The temperature of the injector port was thermostatically controlled in order to optimize the volume of sample to be analyzed via partitioning between the mobile and stationary phases. If the temperature was too low, component separation would be poor. If the temperature was too high, decomposition and/or structural alteration of the analytes could occur prior to adequate separation. In this experiment, five different injector port temperatures were tested ranging from 185.0 ^{0}C to 275.0 ^{0}C. The results indicated that a temperature of 200.0 ^{0}C provided a maximum S/N ratio of 15.6. The S/N ratios at 185.0 ^{0}C (13.6) and 225.0 ^{0}C (14.3) were only slightly lower, but the values rapidly decreased as temperature increased beyond 225.0 ^{0}C. This decrease was most likely due to decomposition of the analytes at higher temperatures as discussed earlier. Based on these results, an injector temperature of 200.0 ^{0}C was used for all injections in this work.

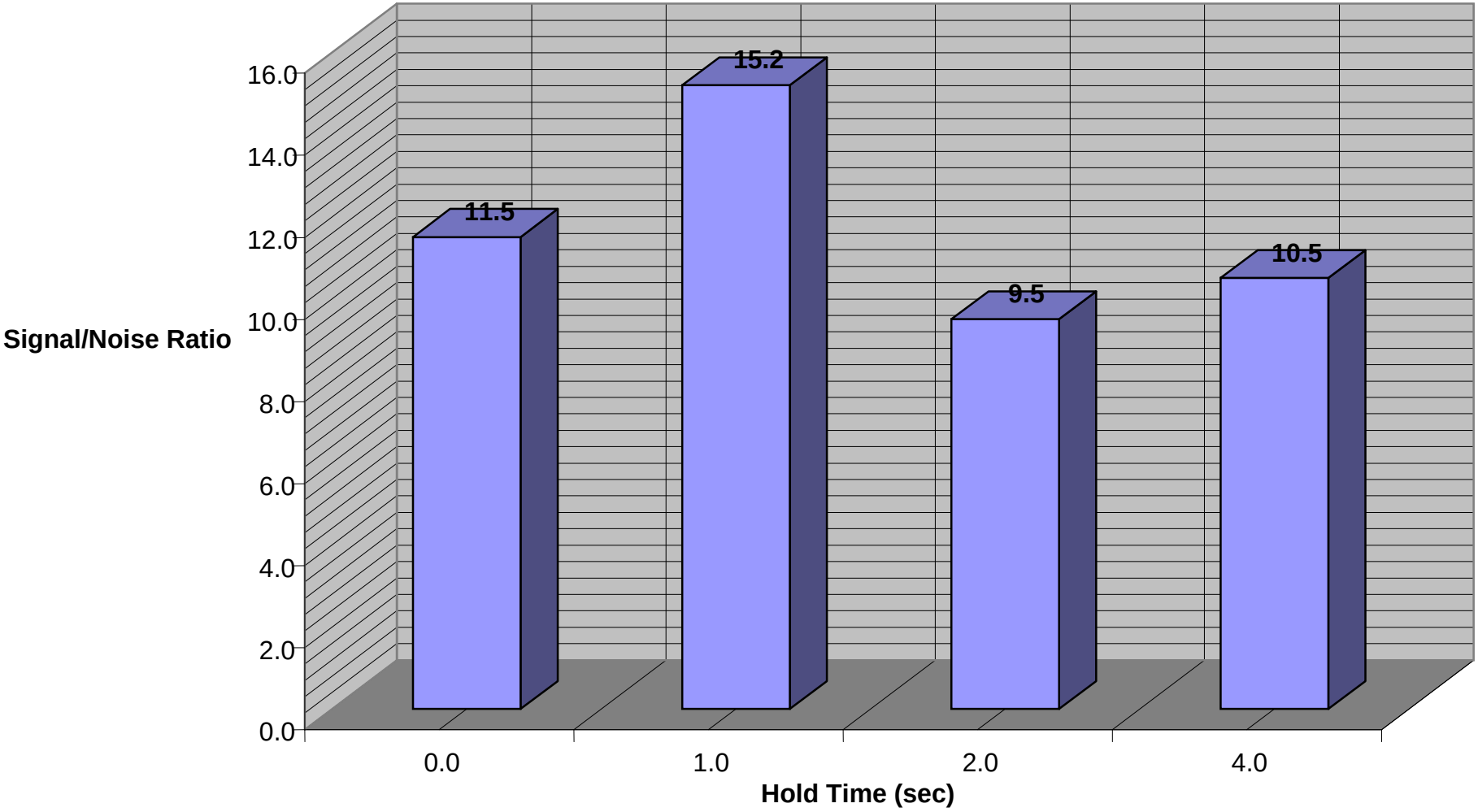
Figure 5.5: Injector Temperature



4. Pre-Injection Hold Time

Pre-injection hold time was the amount of time, in seconds, that the autosampler paused after insertion of the syringe needle into the injection port and before injection of the sample. Pausing allowed the needle to warm up in the injector port before the sample was transferred. This reduced the risk of fractionation inside the syringe or in the injection port prior to introduction of the sample onto the column head. Four time periods were tested ranging from 0.0 seconds to 4.0 seconds. Fractions of a second could not be tested using the Finnigan MAT A200S autosampler. The best signal-to-noise ratio (15.2) occurred when a 1.0 second pause was utilized. No pause (0.0 sec) recorded a 11.5 S/N ratio. Ratios fell significantly when the time was increased beyond 1.0 second. Based on these empirical results, the autosampler was programmed to reside in the injector port for a period of 1.0 second before injection of the sample for all injections in this work.

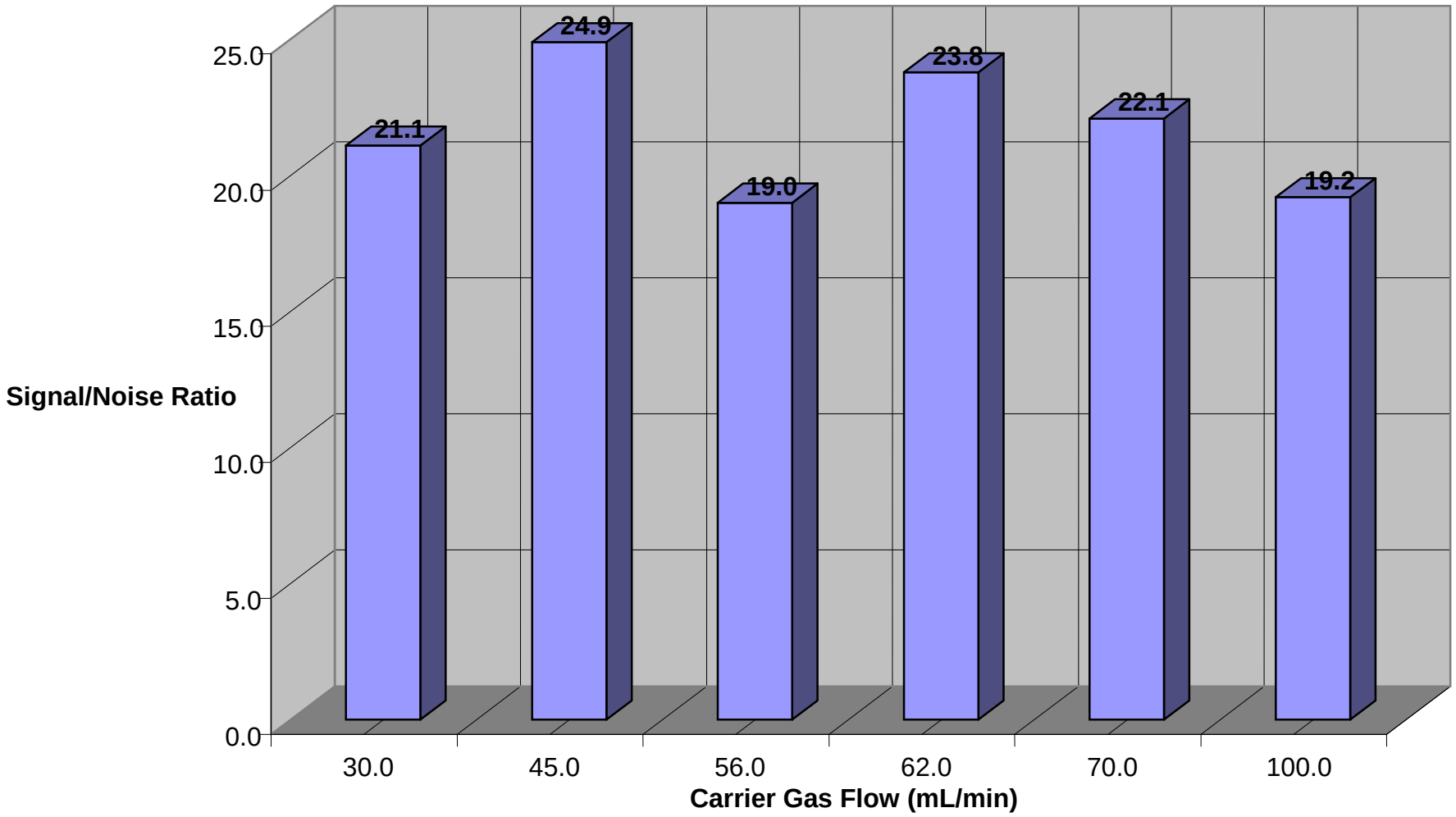
Figure 5.6: Pre-Injection Hold Time



5. Split Vent Flow

The split vent flow was the rate at which the carrier gas (helium) flowed through the injection port from the carrier gas inlet to the split outlet valve. In split injection mode, the flow of the carrier gas through the injection port was constant. In splitless injection mode, as used in the present work, the split vent remained closed for a short period of time (typically 0.5-1.5 minutes) before opening to purge the sample. The flow rate of the carrier gas was thus negligible until after the vent was opened. For the present experiment, the flow rate was measured using a Varian Intelligent Digital Flowmeter at the split outlet valve. Six different flow rates were tested ranging from 30.0 mL/min to 100.0 mL/min. The results of this parameter test showed no apparent trend, however a flow rate of 45.0 mL/min indicated the highest S/N ratio of 24.9. Based on this empirical evidence, a split vent flow rate of 45.0 mL/min was used in all analyses.

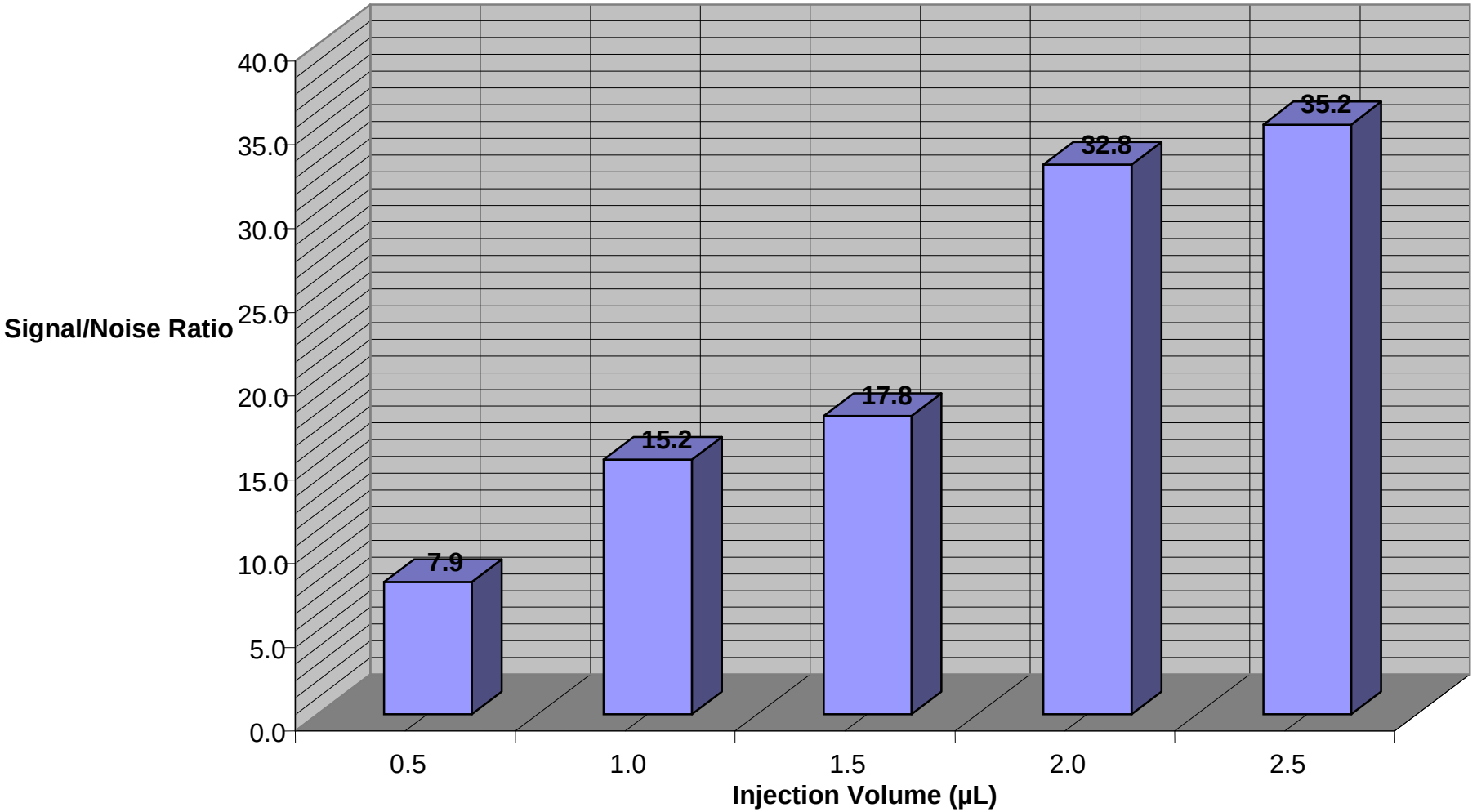
Figure 5.7: Split Vent Flow



6. Sample Injection Volume

The volume of sample contained in each injection to be analyzed was varied in this series of tests. Five volumes ranging from 0.5 μL to 2.5 μL were tested. Results indicated that the signal-to-noise ratio increased significantly when the injection volume was increased from 1.5 μL to 2.0 μL, suggesting 2.0 μL as the minimal injection volume to be considered. An injection volume of 2.5 μL provided the highest S/N ratio (35.2). It was decided however, that an injection volume of 2.0 μL (S/N ratio of 32.8) would be used for this study rather than the 2.5 μL volume. This decision was made primarily in an effort to avoid sample overload in the column. Sample overload occurs when a sample volume is introduced onto the GC column which exceeds the linear range of the detector or the capacity of the column. Exceeding these conditions due to sample overload may result in flat-top peaks, peak fronting or peak tailing in the chromatogram as well as significant or unacceptable band-broadening. Due to the use of splitless injection mode in this work, it was decided that an injection volume of 2.0 μL would be sufficient for accurate sample representation, while avoiding the risk of sample overload.

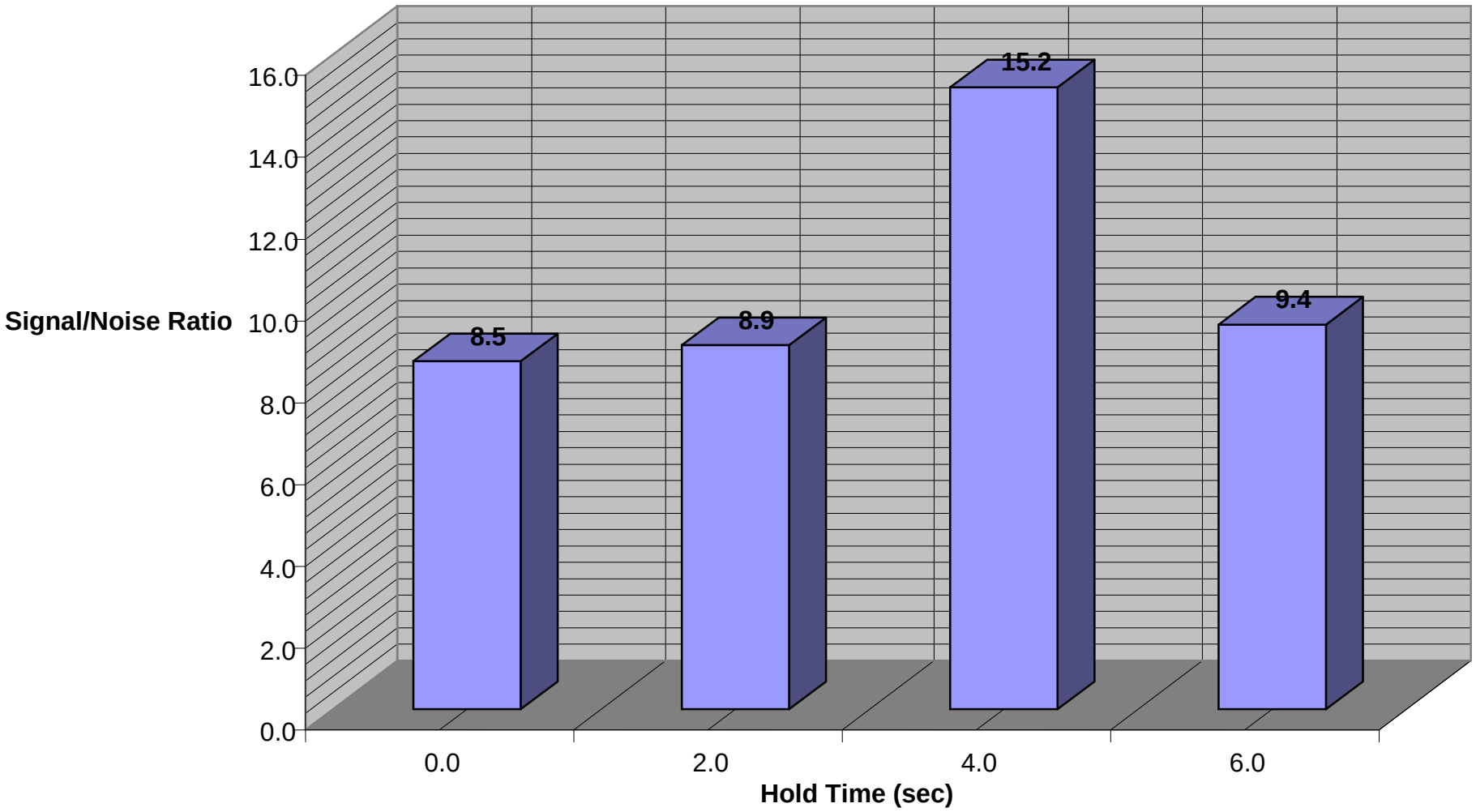
Figure 5.8: Sample Injection Volume



7. Needle Residence

Needle residence is the amount of time, in seconds, the syringe needle resided in the injection port following injection of a sample is called the needle residence. When a needle residence is too short, vaporized sample may be dragged out of the injection port along with the needle as it exits. This would leave a smaller portion of sample in the injection port to be introduced onto the head of the column for analysis. The result was a decrease in sensitivity, particularly during a splitless injection. As with the pre-injection hold time, fractions of a second could not be tested using the Finnigan MAT A200S autosampler. Four separate times in a range from 0.0 to 6.0 seconds were tested. The experiment results showed that a needle residence time of 4.0 seconds resulted in a S/N ratio of 15.2. No other time period resulted in a S/N ratio of greater than 9.4. Therefore, a needle residence time of 4.0 seconds was chosen for use in this work.

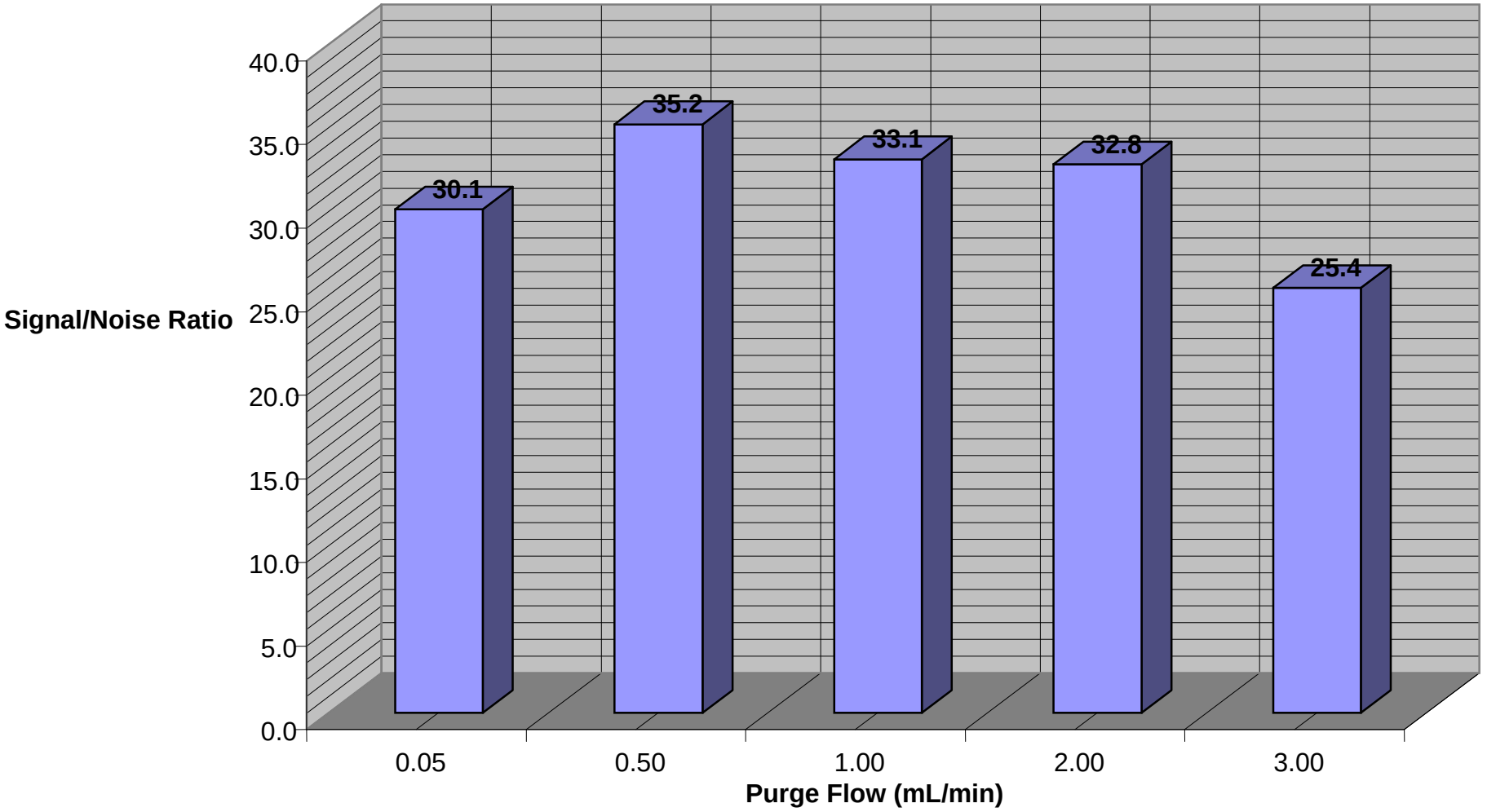
Figure 5.9: Needle Residence



8. Septum Purge Flow

Septum purge flow was the rate the carrier gas (helium) flowed from the carrier gas inlet through the septum purge vent. This small secondary flow of carrier gas bathed the underside of the injector's septum to prevent hot vaporized sample gases from interacting and possibly sticking to the septum, resulting in problems with peak shape and reproducibility. Septum purge flow rates were measured using a Varian Intelligent Digital Flowmeter at the septum purge outlet valve. Five different flow rates ranging from 0.05 mL/min to 3.00 mL/min were tested. As expected, the results showed little difference in the signal-to-noise ratios between the range of 0.05 mL/min to 2.00 mL/min (30.1- 35.2). The flow rate of 3.00 mL/min was significantly lower (25.4) than the other rates, which may have been due to excess sample being swept into the septum purge vent. Based on these results, the septum purge flow rate was kept at a constant 0.5mL/min., which showed the highest S/N ratio (35.2), for all injections.

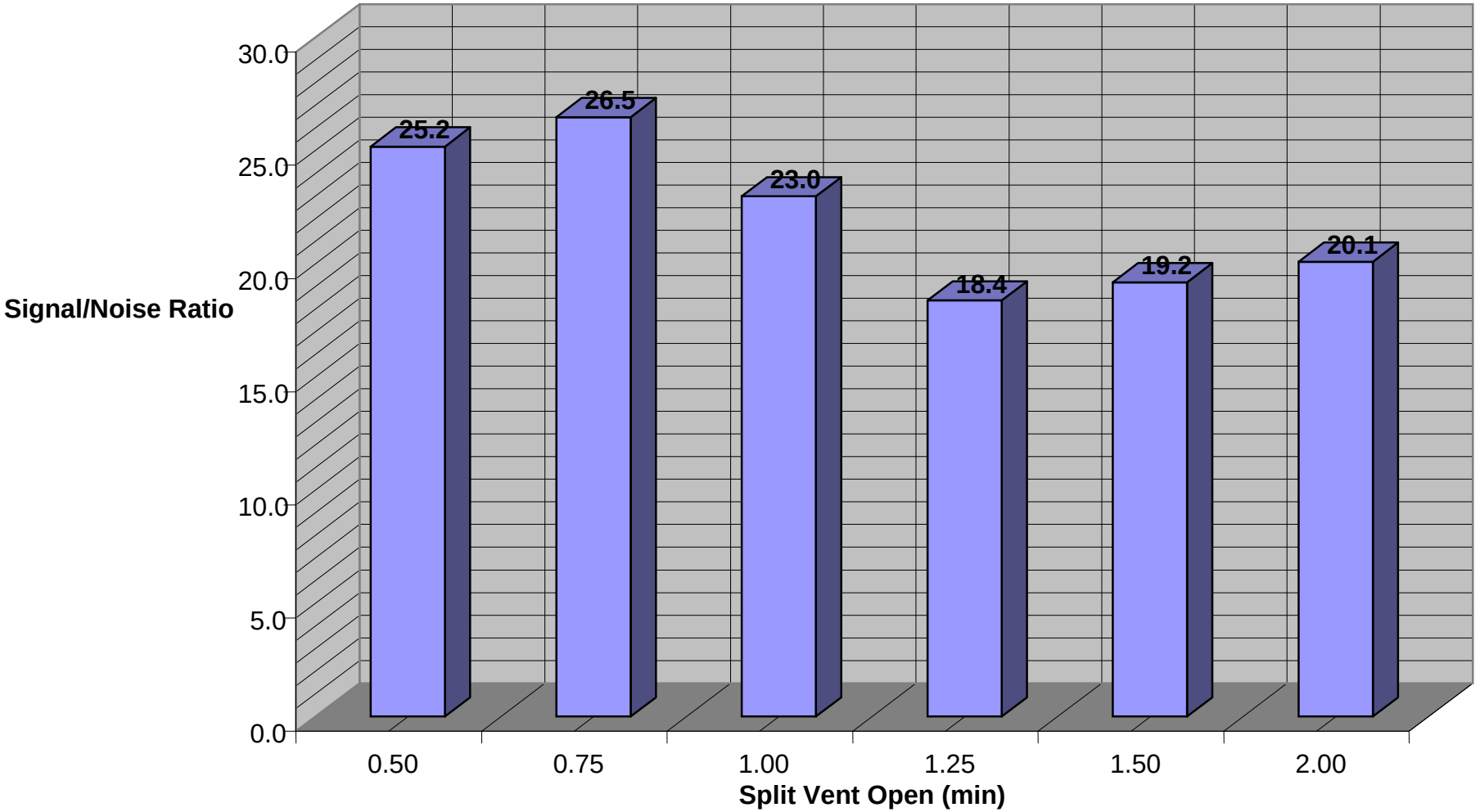
Figure 5.10: Septum Purge Flow



9. Split Vent Open Time

In a splitless injection, as was used in this work, the split vent was closed at the time of injection via a computer controlled split valve. After a short amount of time the split valve was opened and the sample purged. "Split vent open time" is the amount of time that passes, in minutes, following the injection of a sample into the injector port and before the opening of the split vent. Enough time must pass before the opening of the split vent to allow a representative portion of the sample to elute onto the column head or sensitivity and detection limits will suffer. Likewise, too long a period of time before the split vent was opened would result in column overload, which could lead to poor resolution on the chromatogram. A range of six times from 0.5 minutes to 2.00 minutes was tested in this experiment. The highest signal-to-noise ratios were recorded in the time periods shorter than 1.00 minutes and displayed a fairly consistent decline up to the 2.00 minute time period. The time period showing the highest S/N ratio (26.5) was 0.75 minutes. Based on these results, the split vent was opened at 0.75 minutes after all injections in this work.

Figure 5.11: Split Vent Open Times



C. Method Validation

1. Linearity

Linearity in an analytical method shows that the instrument response is linearly proportional to the concentration of the analyte within a desired concentration range. Standard samples of 2-aminoacetophenone, 2,6-dichloroaniline, ethyl anthranilate, indole, methyl anthranilate and skatole were prepared in seven separate concentrations ranging from 0.05 ppm to 1.0 ppm. The samples were then analyzed via GC/MS in triplicate and the average peak height of the three trials for each analyte was determined. The average peak height of each analyte was then plotted against the range of concentrations listed previously (see Figures 5.13 – 5.18) and the consequent plots were then analyzed for linearity (see Table 5.1). The average peak height of the instrument background noise for varying levels of analyte concentration was also analyzed to ensure a relatively uniform response independent of the changing analyte concentrations (see Figure 5.19).

Table 5.1 Linearity Data

Analyte	Equation of the Line	R^2
2-AAP	y = 354265x + 3298.7	0.9985
2,6 Dichloroaniline	y = 512958x + 12343	0.9919
Ethyl anthranilate	y = 534625x + 9986.3	0.9951
Indole	y = 337144x + 7702.4	0.9961
Methyl anthranilate	y = 378646x + 7025.7	0.9954
Skatole	y = 487711x + 8914	0.9948
Noise	y = 103.28x + 595.77	0.6418

Figure 5.13: 2-Aminoacetophenone

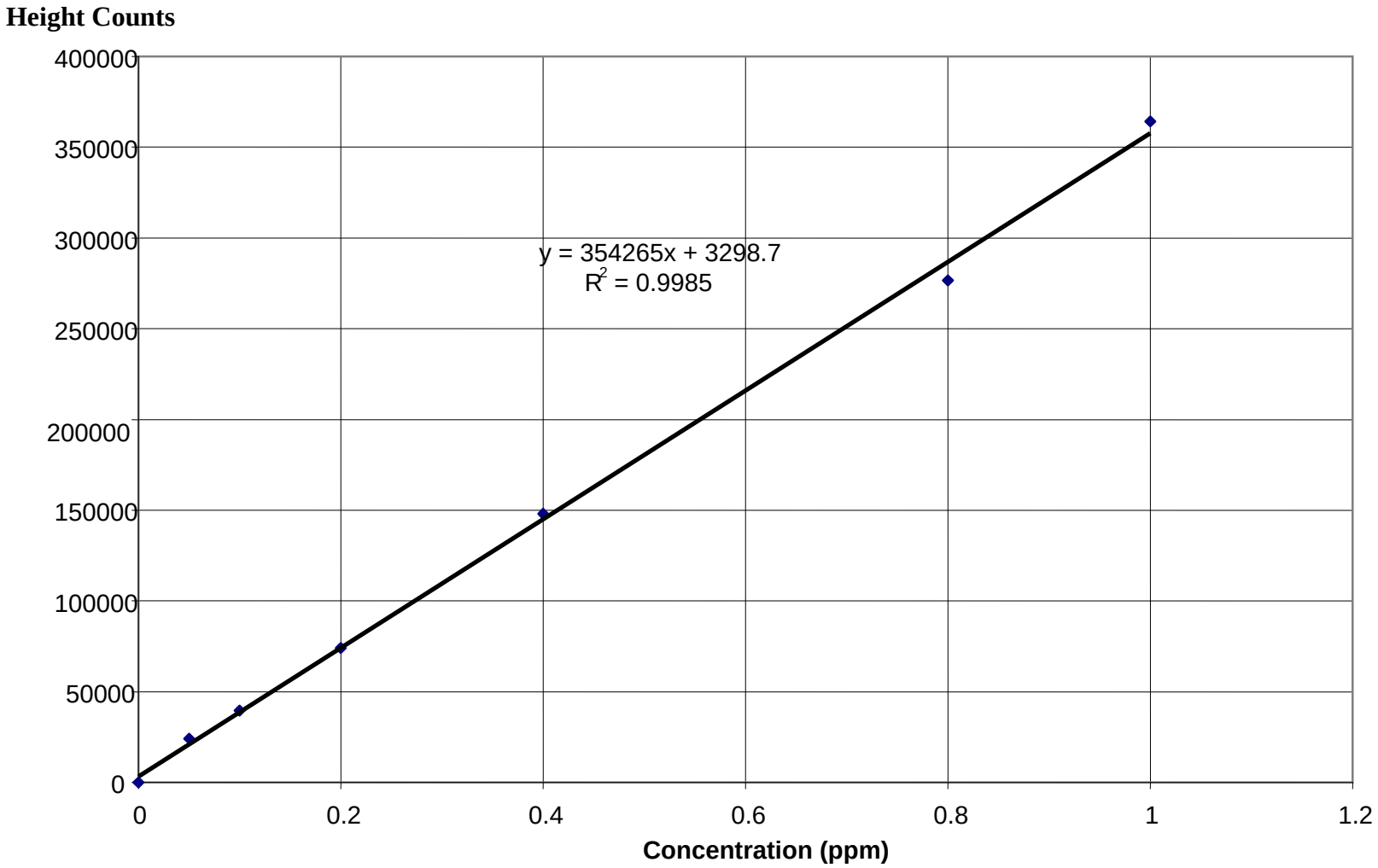
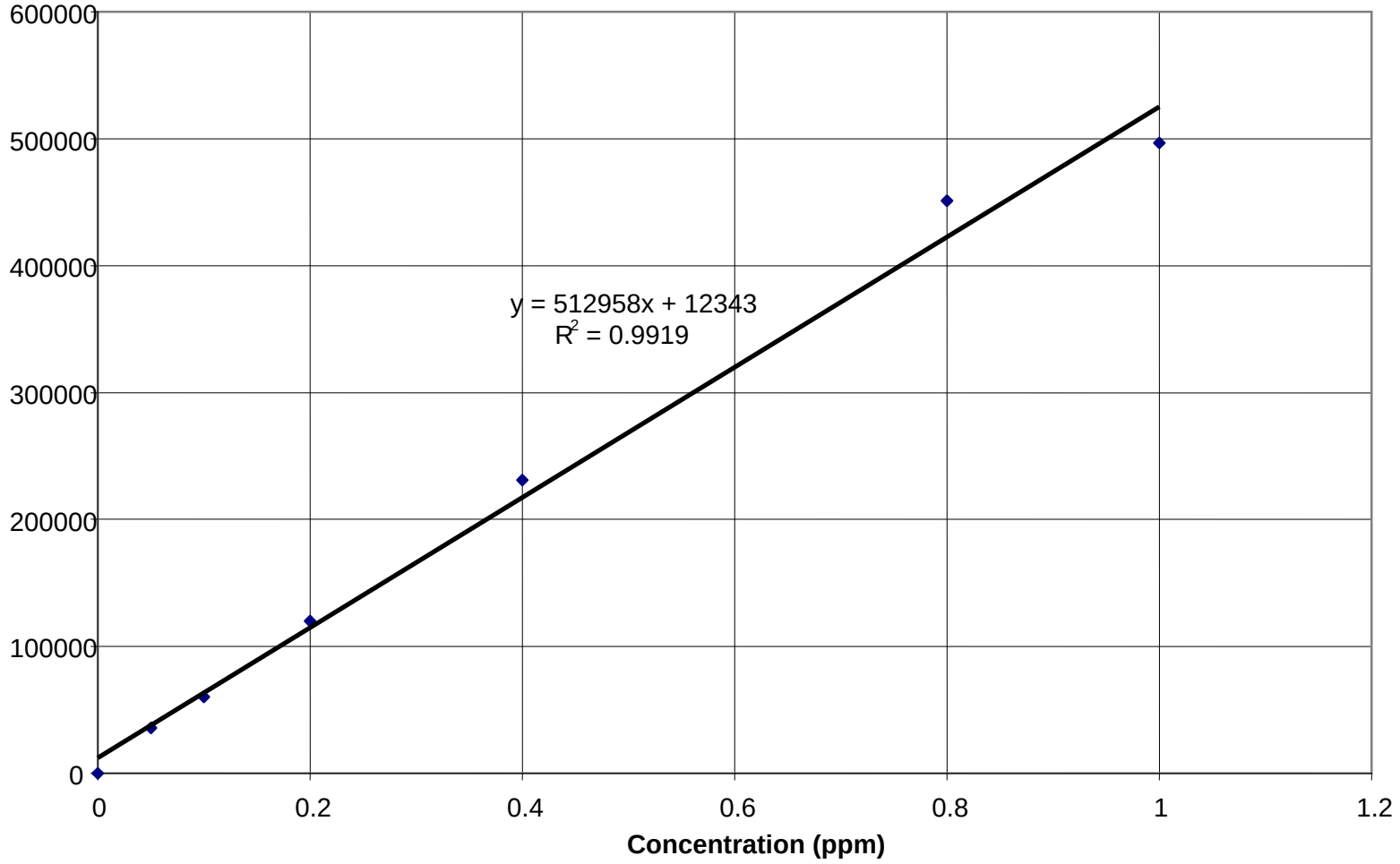


Figure 5.14: 2,6 Dichloroaniline



Height Counts

Figure 5.15: Ethyl Anthranilate (Ethyl 2-Aminobenzoate)

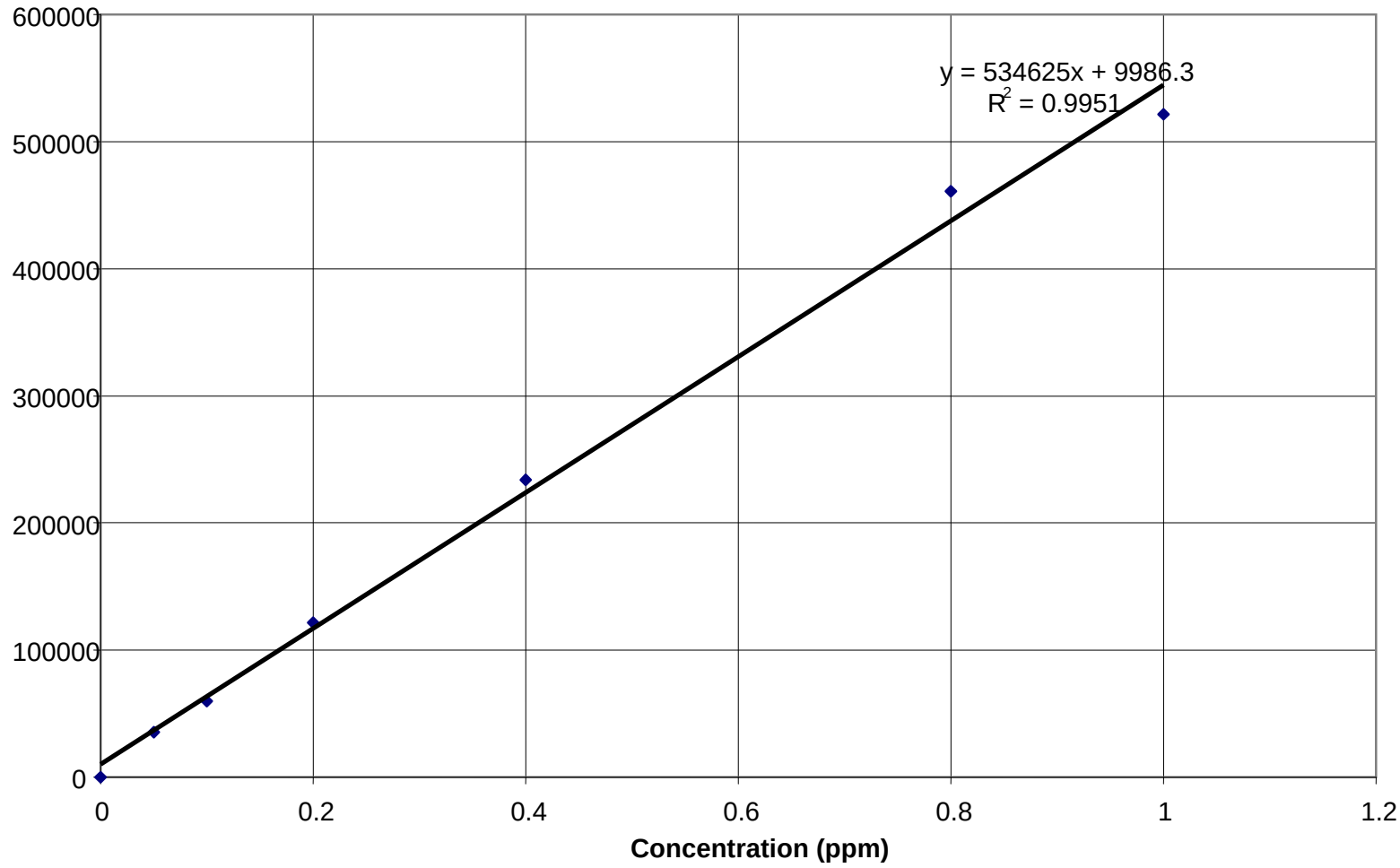
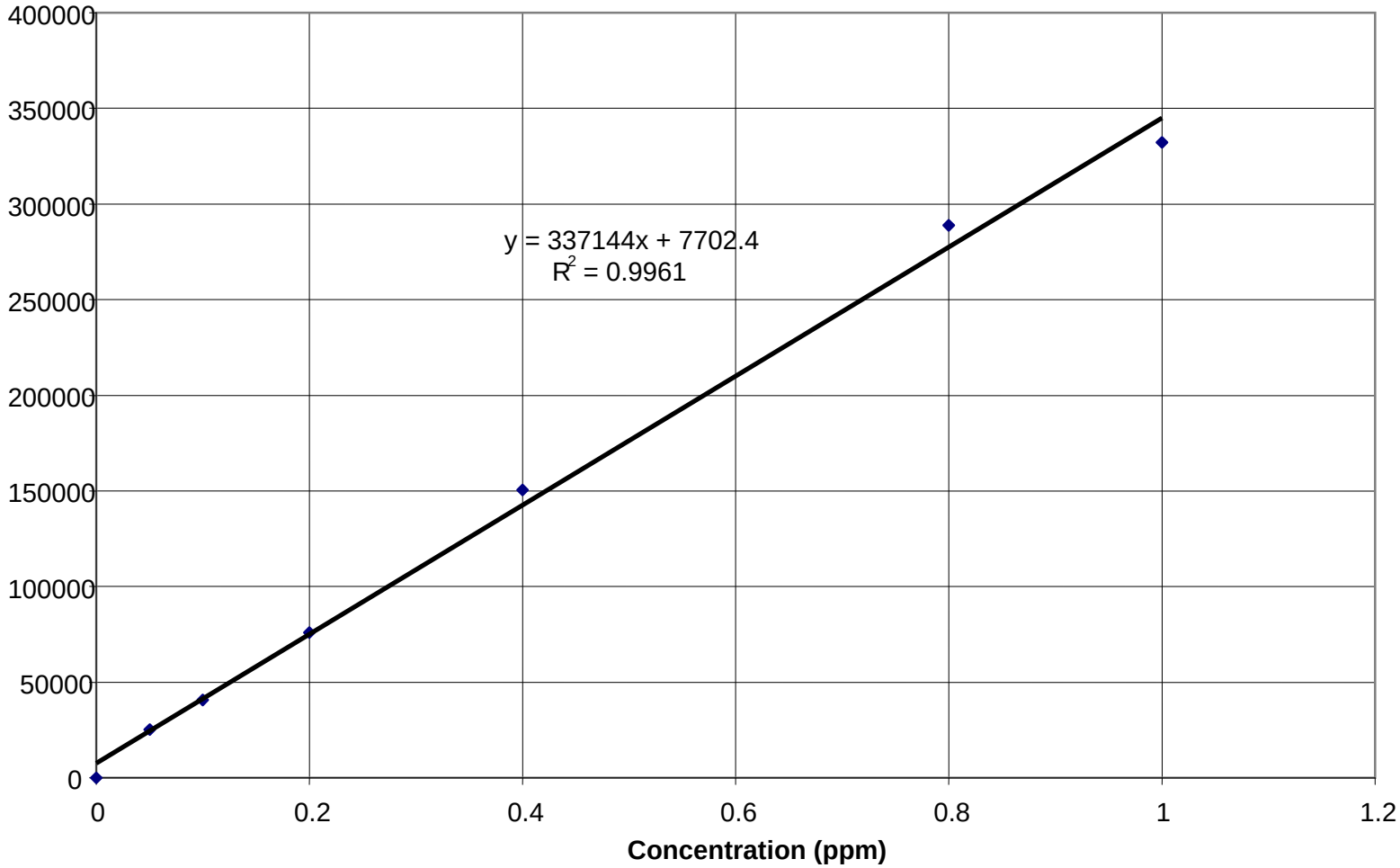


Figure 5.16: Indole (2,3-Benzopyyrole)



Height Counts

Figure 5.17: Methyl Anthranilate (Methyl 2-aminobezoate)

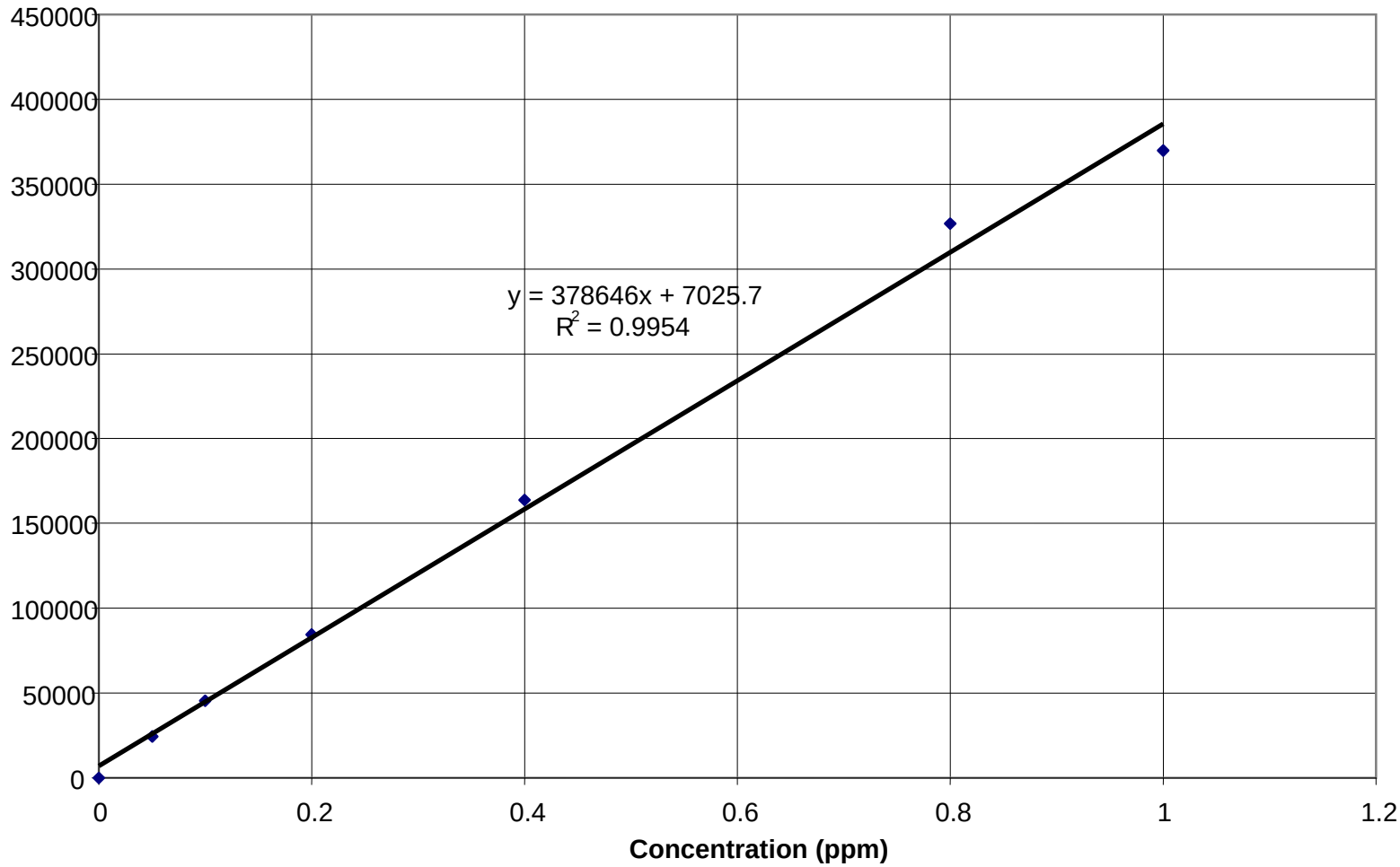


Figure 5.18: Skatole (3-Methylindole)

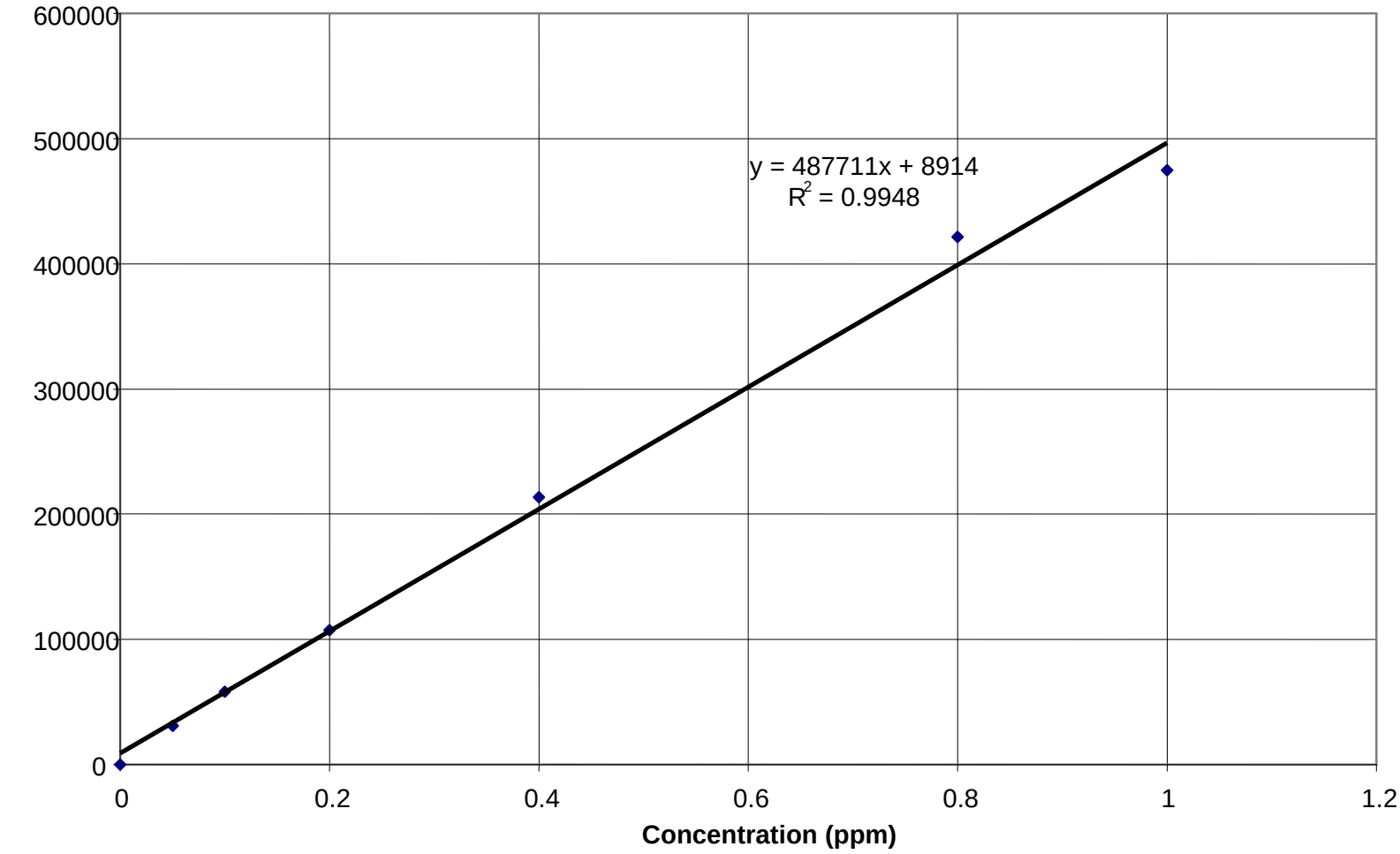
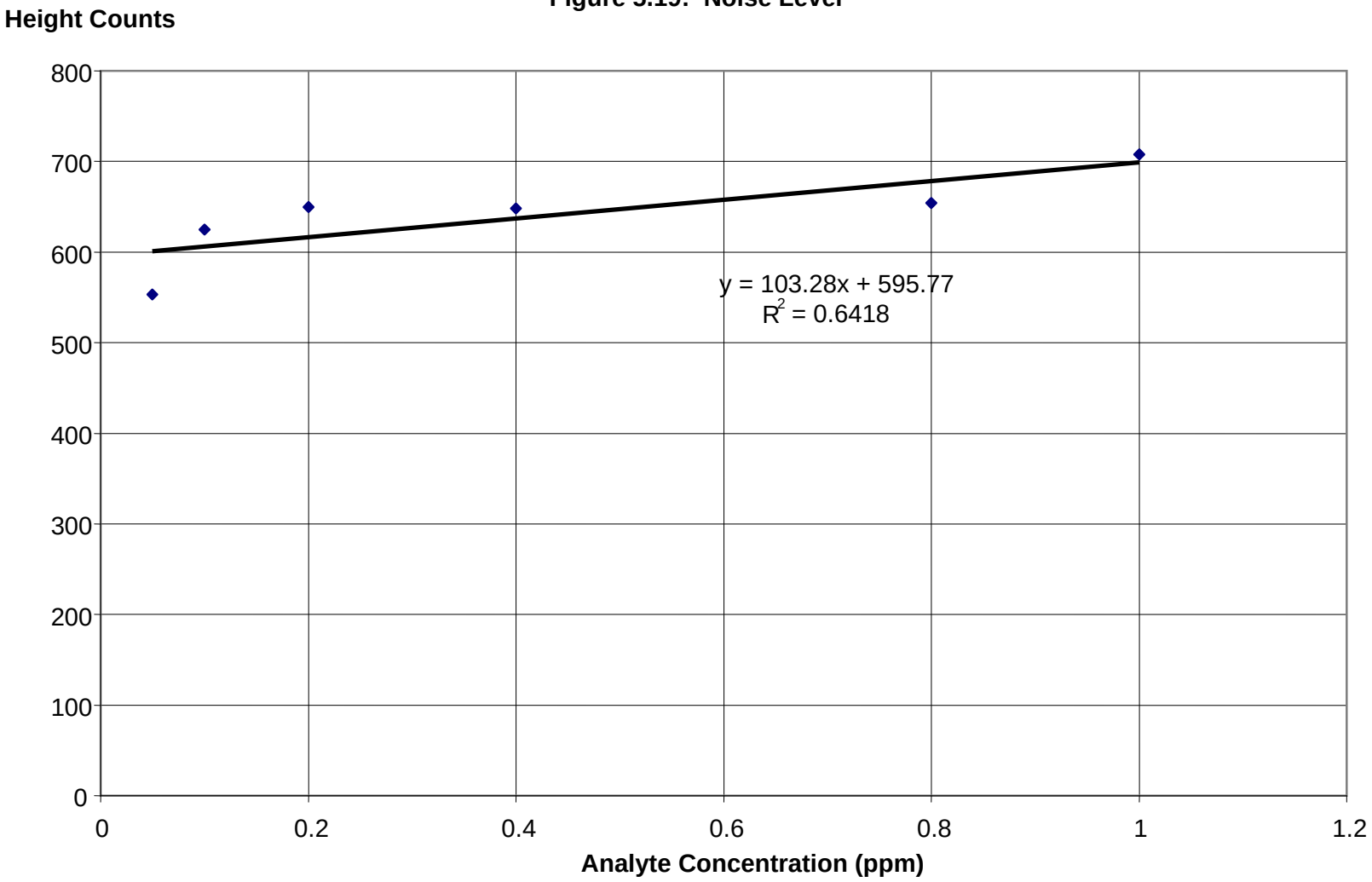
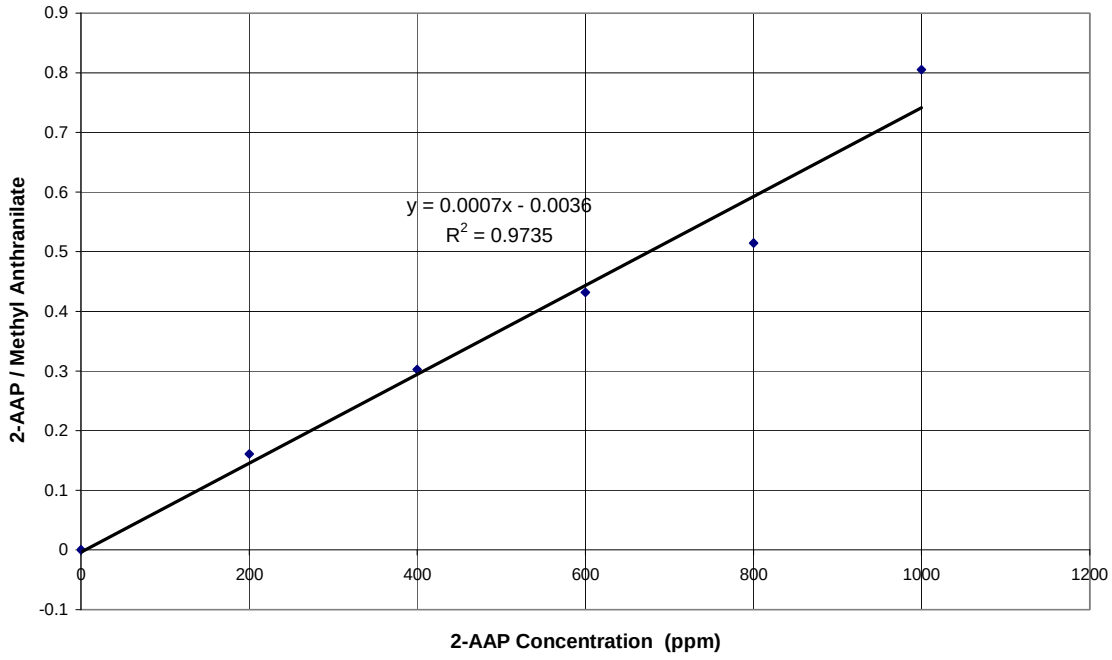


Figure 5.19: Noise Level



Internal standards were also tested for linearity through the use of internal standard curves. A series of wine samples was prepared and spiked with six different concentrations of 2-aminoacetophenone ranging from 0 ppb to 1000ppb. Each wine sample was also spiked with a single concentration of the internal standard to be tested. The samples were extracted and analyzed via GC/MS. Instrument response to the analyte and the internal standard was recorded as peak height. A calibration curve was then constructed plotting the ratio of instrument response to 2-aminoacetophenone versus internal standard against the concentration of 2-AAP present in the wine extracts. The resulting graphs were analyzed for linearity. Figure 5.20 shows an example of an internal standard curve using methyl anthranilate as the internal standard.

Figure 5.20: Methyl Anthranilate Curve



2. Levels of Detection and Quantitation

The detection limit of a method, also known as the level of detection (LOD), is defined as the lowest analyte concentration that can be resolved within a given degree of confidence, typically three times the adjacent noise level. To measure the detection limit of a method for an analytical instrument, the relative noise level of the instrument must first be determined. Noise level is defined as that signal which is produced by the instrument when a sample which contains no analyte (a blank sample) is analyzed with the instrument. The ratio between the signal produced by an analyte and that which is produced by a blank is called the signal/noise ratio (S/N).

Similar to the limit of detection, the limit of quantitation (LOQ) or quantitation limit of a method is the lowest concentration of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. Typically, the limit of quantitation is the concentration of analyte that produces an instrument response that is 10 times that of the adjacent noise level. The limits of detection and quantitation in a sample wine matrix for the five standards selected and 2-aminoacetophenone were each analyzed. Table 5.2 summarizes this data.

Table 5.2 Levels of Detection and Quantitation

Compound	LOD (ppb)	LOQ (ppb)
2,6-Dichloroaniline	1.25	12.48
Indole	1.9	18.98
2-AAP	1.81	18.07
Methyl Anthranilate	1.69	16.9
Skatole	1.31	13.12
Ethyl Anthranilate	1.2	11.97

3. Recovery

The accuracy of an analytical method can be defined as the proximity of the measured value to the true value of the sample being analyzed. Recovery is one method that may be used in assessing accuracy. One method of determining recovery is to spike a blank sample matrix with a known concentration of analyte, extract the sample and then analyze the spiked sample extract using the analytical method in question. Recovery is then determined by comparing the instrumental response of the extract to the instrumental response of the analyte dissolved in pure solvent. Percent recoveries were calculated using the following equation:

$$\%R = [PH\ Wine\ Extract / PH\ Standard] \times 100$$

where *PH* is the average peak height of an analyte at a given concentration. Average peak height measurements for both the pure standards and the spiked wine extracts were calculated after each had been run in triplicate for six separate concentrations ranging from 0.05 ppm to 0.5 ppm. The mean percent recoveries of the five standards and 2-aminoacetophenone over the entire range of concentrations are summarized in Table 5.3.

Table 5.3 Recovery Results

Compound	Avgerage Percent Recovery	RSD
2,6-Dichloroaniline	78.4 %	3.67
Indole	89.3 &	1.58
2-AAP	81.9 %	1.88
Methyl Anthranilate	83.8 %	2.54
Skatole	92.3 %	0.95
Ethyl Anthranilate	87.4 %	1.66

CHAPTER VI

Conclusions

This research has demonstrated the development of a method for the gas chromatographic/mass spectrometric separation, identification, and quantitation of the chemical compound 2-aminoacetophenone (2-AAP) in a wine matrix. Although its role is not completely understood at present, 2-AAP is believed to be a key component in connection with the wine defect known as "atypical aging" (ATA) or "untypical aging" (UTA), sometimes found in white wine varietals. The development presented here includes a complete method for liquid-liquid extraction of wine and the development and optimization of a GC/MS method for the separation and identification of 2-AAP. Gas chromatography parameters have been described and individually tested for optimal sensitivity and detectability of the compound. Several internal standards were used in this work. Based on the relative isolation of the peak in the wine extract chromatogram plots and a low level of detection and quantitation, methyl anthranilate appears to be a good choice for an internal standard, although another internal standard could be used with little or no modification necessary.

Originally the presented method was intended to be used to test for 2-AAP in ATA affected wines. However, there has been some controversy in the determination of what constitutes an ATA affected wine. Wine samples from the Placido Domingo and Wagner vineyards, suspected of being ATA affected, showed no detectable concentration of 2-aminoacetophenone using the method described in this work. Figure 6.1 shows a sample chromatogram of an extracted 1998 Wagner Vineyards Riesling that was thought to be affected by atypical aging. The wine sample shown in the figure was spiked with

10 ppb each of the internal standards ethyl anthranilate and methyl anthanilate. The peaks of the internal standards are indicated in the chromatogram, as is the area where 2-aminoacetophenone should appear.

Selected-ion monitoring was also used to scan extracts of wine samples suspected of being ATA affected. Figure 6.2 represents a sample chromatogram of a wine sample extract scanned in total-ion current in comparison with a single-ion scan at m/z 135 (the base peak of 2-AAP). The chromatogram shows no significant peak in the single-ion scan mode.

The results of the tests of the Wagner and Placido Domingo wine samples were independently corroborated by researchers at Cornell University (Geneva, NY) using a different method of extraction, separation and detection (*14*). The above method was effective however, in detecting spiked 2-AAP in a wine matrix at the levels reported to exist in ATA affected wines.

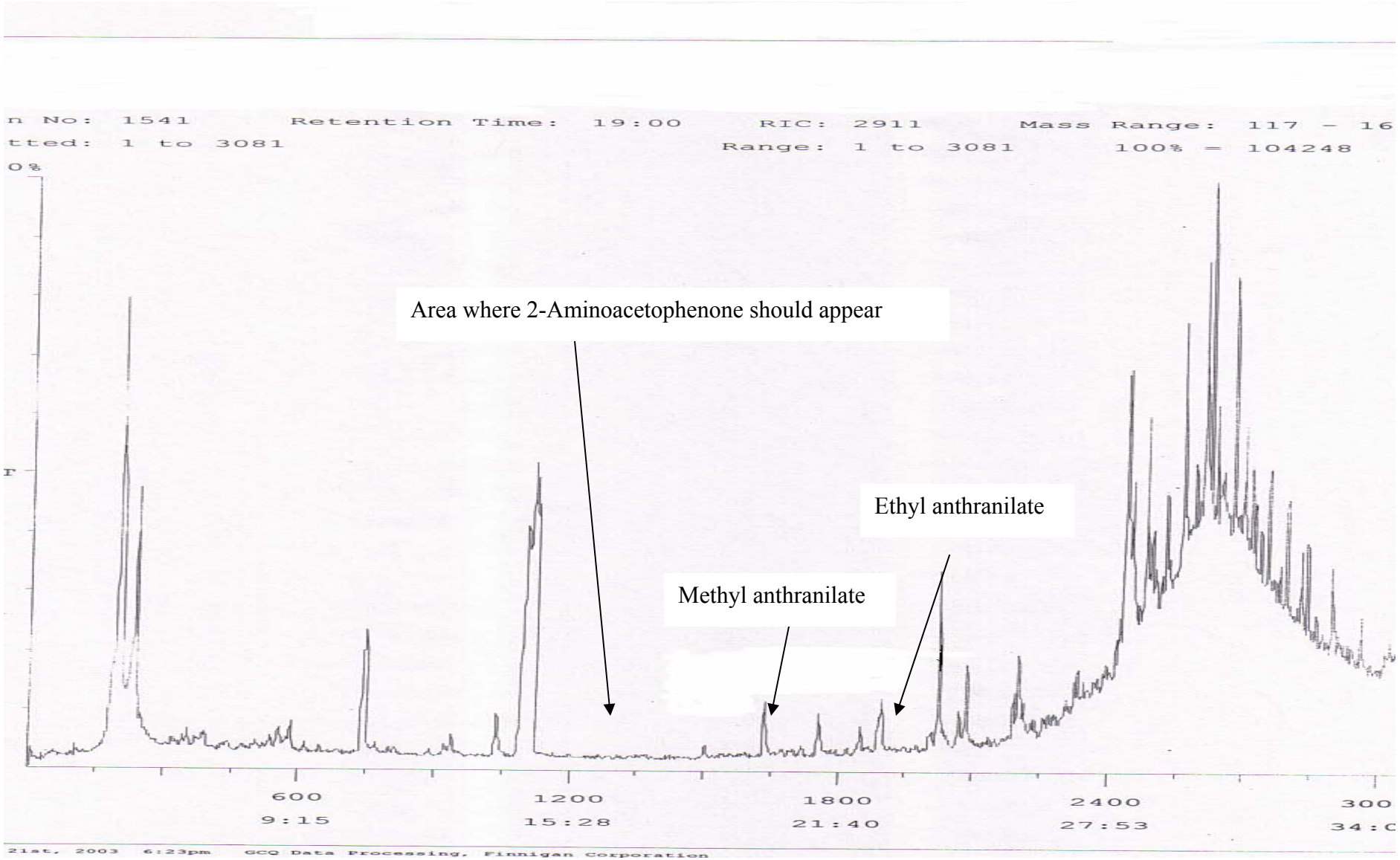


Figure 6.1: Wagner Reisling Extract Spiked With Anthranilates

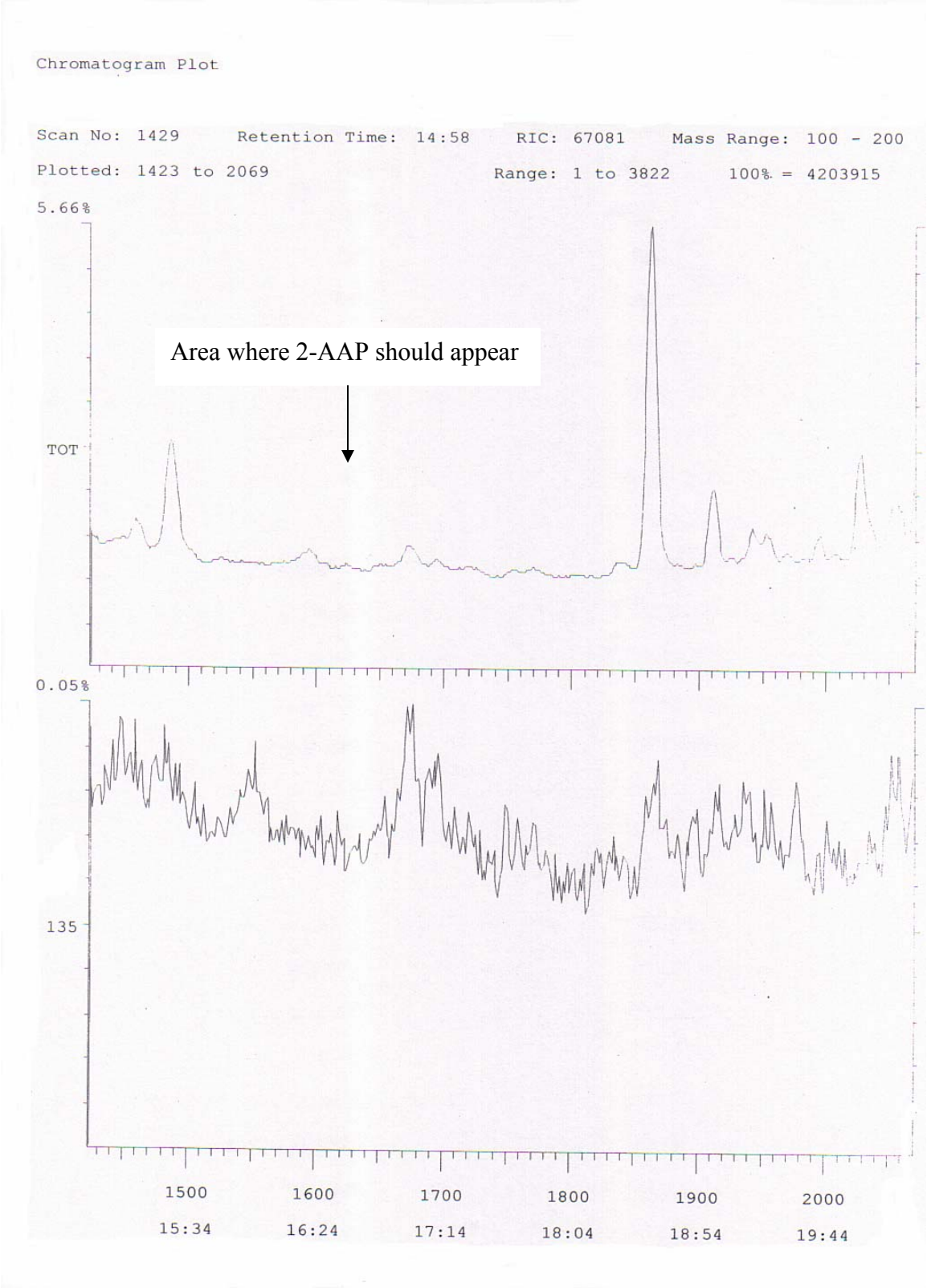


Figure 6.2: Single Ion Monitoring for 2-AAP in Wine Extract

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