OFF-LINE THERMOCHEMOLYSIS-GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) USING SOLID-PHASE MICROEXTRACTION (SPME): PHENOLIC ACID ANALYSIS

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Off-Line Thermochemolysis-Gas Chromatography/ Mass Spectrometry (GC/MS) Using Solid-Phase Microextraction (SPME): Phenolic Acid Analysis.

By

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ABSTRACT

An important class of compounds within the plant and animal kingdom are phenolic acids (i.e. hydroxyl substituted benzoic acids). Traditional methods used for the determination of these compounds are very time-consuming, involving procedures that include extraction, derivatization and finally GC analysis. An alternative method, called Pyrolysis/Methylation-GC (or thermochemolysis), applies the use of a derivatizing reagent, such as tetramethylammonium hydroxide (TMAH) for direct and rapid determination of these compounds in one analytical step. The polar functional groups (i.e. carboxylic and phenolic acid groups) are rapidly methylated and the products thermally desorbed onto the GC/MS column. Most analyses are on-line, where the pyrolysis unit is directly connected to the GC. However, there are serious drawbacks to on-line technique including the introduction of the methylating reagent onto the column and the requirement of a dedicated GC/MS.

This study investigated method development for off-line thermochemolysis using solid-phase microextraction (SPME) for the analysis of phenolic acids using syringic acid as a model compound. The parameters investigated included pyrolysis temperature, SPME adsorption temperature and time, fibre size/phase, and split flow required for GC. Other thermochemolysis reagents investigated were tetramethylammonium acetate (TMAAc) and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). The effect of the solvent (i.e. methanol and water) used to dissolve the different reagents was also investigated. It was determined that aqueous TMAH was the most suitable reagent for phenolic acid analysis. The SPME off-line method was successfully optimized for highest quantity of methylated product. It was also determined that reagents BSTFA and TMAAc did not give reproducible results due to volatility of BSTFA and the insufficient basicity of TMAAc.

The above method was applied to the analysis of phenolic acids present in white pine needles *(Pinus strobus)*. Although most phenolic acids were successfully methylated, it was found that the SPME step was too selective as an extraction technique, that is, the phenolic acids observed in the chromatogram were dependent on the SPME adsorption temperature during headspace analysis. Future work should be focused on the use of SPME in aqueous solution of methylated products.

DEDICATION

This thesis is dedicated to my parents, Garland and Classie Hilliard, my sisters Laurie-Ann, Ellen, and Melanie, my brother Garland Jr., my nephews Kyle, Coady and Cameron, and my niece Kristen.

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GLOSSARY

GC	Gas Chromatography
MS	Mass Spectrometry
m/z	Mass to Charge Ratio
TMAH	Tetramethylammonium hydroxide
TMAAc	Tetramethylammonium acetate
BSTFA	N,O-Bis (trimethylsilyl) trifluoroacetamide
TMS	Trimethylsilyl derivatization
HMDS	Hexamethyldisilazane
TBAH	Tetrabutylammonium hydroxide
TMPAH	Trimethylphenylammonium hydroxide
TMTFTH	(m-trifluoromethylphenyl)-trimethylammonium hydroxide
SPME	Solid-phase microextraction
eV	Electron Volts
RT	Retention Time
sd	Standard Deviation
Mm	Molar mass
SPM	Simultaneous Pyrolysis/Methylation
THM	Thermally-assisted Hydrolysis Methylation
TAC	Thermally-Assisted Chemolysis
PY/GC/MS	Pyrolysis/Gas Chromatography/Mass Spectrometry

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Chapter 1

Introduction

1.1 Overview of pyrolysis and thermochemolysis

1.1.1. Introduction to analytical pyrolysis

Analytical pyrolysis is a thermal degradation technique that breaks larger molecules into smaller volatile and semi-volatile fragments for easier identification and study which is often coupled with gas chromatography/ mass spectrometry (GC/MS) (1,2,3). The first application of analytical pyrolysis was for the structural identification of natural rubber, in 1860 (2). More recently, in the last thirty years, pyrolysis/MS was improved by the combination of the pyrolysis unit with GC/MS (2,4). For example, Anderson et al. (5) showed that structural identification of natural resins and resinites with PY/MS could only be determined when combined with other spectral data. However, with the combination of PY/GC/MS, the structural identification of natural resins was no longer restricted by the limitations of MS, instead, chemical structures of pyrolysis products could now be determined directly from the pyrolysis of those resins using PY/GC/MS (5). This advancement has also led to the structural analysis of many types of natural macromolecules at the molecular level, without any sample pre-treatment and with very small sample quantities (4, 6). However, conventional PY/GC/MS has been found to lead to decarboxylation (4, 6) of underivatized carboxyl groups, such as benzenecarboxylic acids and large fatty acids. Also, structures of molecules may be modified by unwanted thermal reactions, which may lead to misinterpretation of those structures (6). The major

limitation of conventional PY/GC/MS is that of molecules which contain polar components (i.e. COOH, OH^-), because these compounds are difficult to transfer from the pyrolysis unit to the GC and are also difficult to chromatograph due to their higher boiling points (2,4).

A relatively new technique created in the last 20 years by Challinor, known as Pyrolysis/ Methylation (high temperatures in the presence of tetramethylammonium hydroxide (TMAH)) has been shown to eliminate some of the limitations due to the presence of polar functional groups (2). This approach was shown to yield methyl esters of carboxylic acid groups and the methyl ethers of hydroxyl groups of phenolic acids, rendering many of the polar compounds volatile enough for GC (6), through the production of non-polar compounds. This technique used for determining the structural components of polar compounds has had one important implication towards the identification of underivatized carboxylic acids found in natural resins. For example, prior to Challinor's Pyrolysis/Methylation technique, carboxylic acids were known to be problematic in their analysis, such as poor chromatographic behaviour on most stationary phases of the chromatographic column, and the formation of decarboxylation products, which may or may not be readily related to the original structure. Thus, production of methyl esters and ethers for the respective carboxylic acid and hydroxyl groups has contributed to easier identification of many polar compounds (5).

Today, many researchers have used this concept (Pyrolysis/Methylation) under titles such as Simultaneous Pyrolysis Methylation (SPM), Thermally Assisted Hydrolysis Methylation(THM), Thermally Assisted Chemolysis (TAC), or thermochemolysis. To avoid confusion, this report uses thermochemolysis to describe the technique created by Challinor (4,7,8,9,10).

1.1.2. TMAH/ thermochemolysis

The mechanism of methylation in thermochemolysis involves two sequential reactions as shown in figure 1.1 (13). The mechanism provides a method for the analysis of molecular components of polar macromolecular substances, where basic hydrolysis of weak linkages along with subsequent methylation occurs (10). TMAH, a highly alkaline ionic compound, acts as a derivatizing reagent that replaces the acidic proton (hydrogen) of carboxylic and hydroxyl groups to form methyl esters and methyl ethers, respectively (3,6,13,14). The methodology behind TMAH/thermochemolysis has led to its applications to alkyd resins, natural polyester cutins, aromatic polyesters, and other units bearing carboxyl groups in humic substances, lignins, and most importantly, phenolic acids (7).

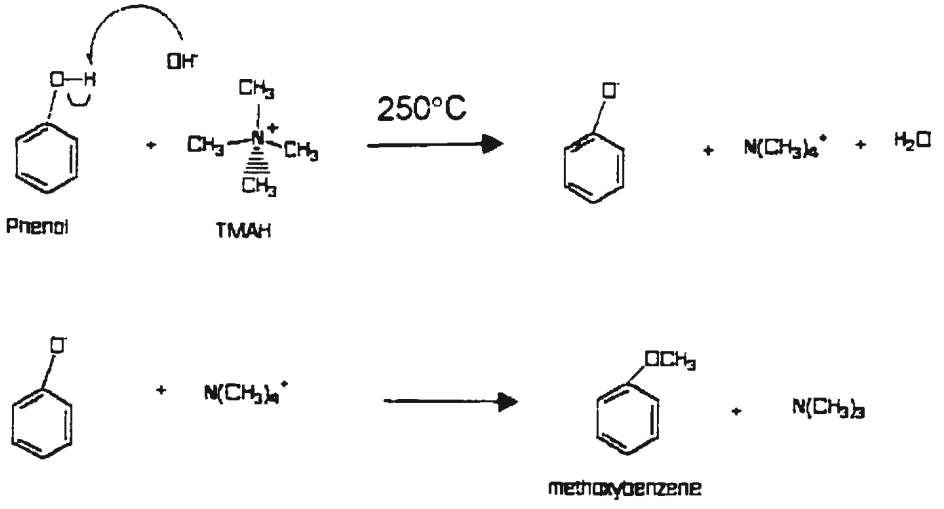


Figure 1.1. The mechanism of TMAH/ thermochemolysis

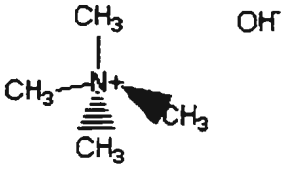
Thermochemolysis has been considered an appropriate term for Challinor's Pyrolysis/Methylation technique because it has been shown, by a number of researchers such as Challinor himself, that the reaction involved is a chemolysis reaction (8). In other words, the use of a base-derivatizing reagent allows ester and ether bond breakage to occur at both relatively low (i.e.≤ 400°C) and intermediate temperatures (i.e. up to 700 °C) compared to conventional pyrolysis, which requires elevated temperatures (i.e. > 800 °C) (11,12,13).

1.1.3. Other derivatizing reagents

TMAH/ on-line thermochemolysis (i.e. the direct connection between the pyrolysis unit and the GC) allows larger and more derivatized fragments of polymeric material to reach the chromatographic column. However, in time, these compounds (including the reagent TMAH) tend to lead to column degradation and often show higher background noise in chromatograms of the pyrolysates (2).

TMAH (figure 1.2), with high alkalinity, has been shown to result in unfavorable cleavage of substructures in some polymers, such as lignins, through thermally assisted hydrolysis (15,18). Another group of compounds affected by the alkalinity of TMAH is triglycerides (i.e. Lipids). The rapid conversion into their fatty acid methyl esters has been shown to be successful for fats that have saturated fatty acid components such as butter fat. However, additional isomerization products are obtained in triglycerides such as linseed oil which have significant polyunsaturated fatty acids (PUFAs). Thus, in fatty acid TMAH/thermochemolysis, the alkalinity of TMAH results in a based-catalyzed isomerization of the unsaturated polyunsaturated fatty acids (PUFAs) (17). This has led to research into less

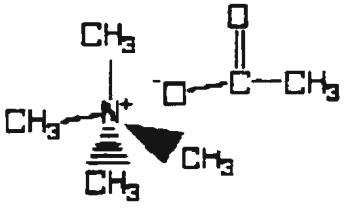
harsh derivatizing reagents such as tetramethylammonium acetate (TMAAc) and N.0-bis(trimethylsilyl) trifluoroacetamide (BSTFA).



TMAH

Figure 1.2. Tetramethylammonium hydroxide (TMAH).

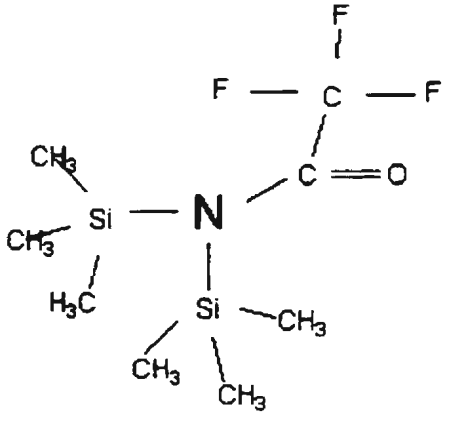
TMAAc (figure 1.3) is a weaker base compared to TMAH. It has been shown to offer an alternative methylating method for the analysis of free fatty acids found in wood extracts and wood pulps. Research showed that when free unsaturated fatty acids were heated in the presence of TMAAc no isomerization was observed, in contrast to that occurring when TMAH was employed (16,17,18). The TMAAc methylating mechanism results in the same formation of methyl esters and methyl ethers for carboxylic and hydroxyl groups, respectively, as TMAH (2,4).



TMAAC

Figure 1.3. Tetramethylammonium acetate (TMAAc).

BSTFA (figure 1.4) is another alternative reagent that provides a less harsh environment compared to TMAH. It involves a trimethylsilyl (TMS) derivatization of hydroxyl groups followed by thermolysis of the derivatized TMS-polymer (17.18). Advantages of BSTFA include producing thermally stable TMS derivatives, which do not easily degrade and are transferable to the injector port and separated by GC. No sample preparation is needed and the analysis can be operated under milder conditions (18). Research has shown that BSTFA was successfully applied to the thermochemolysis of lignin in place of TMAH (15). However, the volatility of BSTFA is high, which makes its effectiveness dependent on the equipment used in thermochemolysis. The reagent also hydrolyzses in the presence of moisture and this is a problem for wet samples. For example, research completed on a bulk dehydrogenative polymer of coniferyl alcohol (G-DHP) was determined to be successful in obtaining the TMS derivatives only when a Bio-Probe was used which enclosed the sample along with BSTFA and prevented the escape (or decomposition) of the reagent (15).



BSTFA

Figure 1.4. N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA).

Other reagents investigated and found in the literature, for use in thermochemolysis, include hexamethyldisilazane (another silylating reagent) (HMDS) (18), other quaternary n-alkyl ammonium hydroxides such as tetrabutylammonium hydroxide (TBAH) , trimethylphenylammonium hydroxide (TMPAH) and (m-trifluoromethylphenyl)-trimethyl ammonium hydroxide (TMTFTH) (2). In most thermochemolysis studies, alternative reagents are investigated to determine their effectiveness compared to TMAH, which is highly alkaline. In other words, to acquire reagents that may produce stable pyrolysates as those obtained from TMAH/thermochemolysis, but without the use of a highly alkaline environment (2,18).

This type of research is crucial for the analysis of fatty acid compounds present in lipid materials. For example, Chiavari et al. (2001) showed that the use of TMAH resulted in side reactions such as the isomerization of double bonds of unsaturated fatty acids (called base-catalyzed isomerization) and methylation of the carbon atom in the 2-position (18). The major concern is that this type of alteration is that it may result in incorrect identification of the fatty acid structure (18). This problem was minimized by replacing TMAH with a milder reagent, such as, hexamethyl- disilazane (HDMS) (18).

Regardless of the reagent used, thermochemolysis has offered a method for the analysis and identification for both phenolic acids and fatty acid compounds, through the production of less polar compounds for rapid sample analysis. This study further investigates the use of TMAH, TMAAc, and BSTFA for the off-line thermochemolysis of samples that contain phenolic acids.

1.2. Solid-phase microextraction (SPME)

Solid-phase microextraction (SPME) utilises a short, thin, solid fibre of fused silica (typically 1cm long and 0.111mm outer diameter) coated with an absorbent polymer. The fibre is composed of the same chemically inert fused silica used to make capillary columns and is very stable at high temperature (22) (figure 1.5). The development of many fibre coatings was important for its use in thermochemolysis because different types of sorbents will extract different groups of analytes. The different fibre coatings available range from non-polar (e.g. polydimethylsiloxane) to polar (e.g. polyacrylate) phases. The theory behind SPME is the same as that of GC column, where " like dissolves like". For example, when the volatile compound is non-polar then a non-polar fibre would be used (20).

SPME techniques include direct immersion of the fibre into an analyte solution or exposure of the fibre to the headspace of the sample, where the target analytes are subsequently extracted from the vial into the fibre coating. After a pre-determined adsorption time, the fibre is withdrawn back into its sheath, which is then pulled out of the vial and immediately transported to the injector liner of the GC where the concentrated analytes are thermally desorbed onto a GC column, as shown in figure 1.6 (22). The particular SPME extraction technique used here for off-line thermochemolysis was headspace extraction. In this report, the analyte produced by thermochemolysis condenses on the walls of the vial. The SPME coating absorb the analyte when the vial is heated (see section 2.2.1)

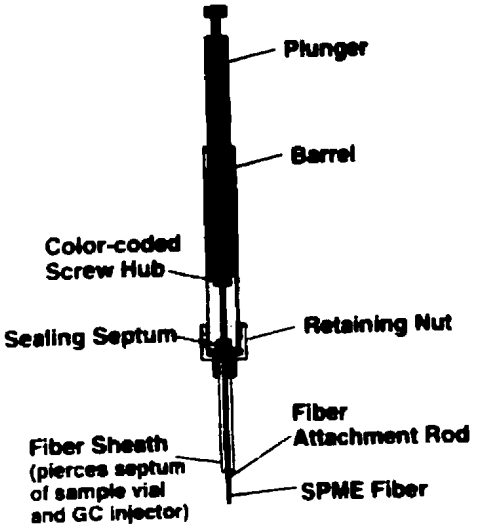


Figure 1.5. Solid-phase microextractiom (SPME) device

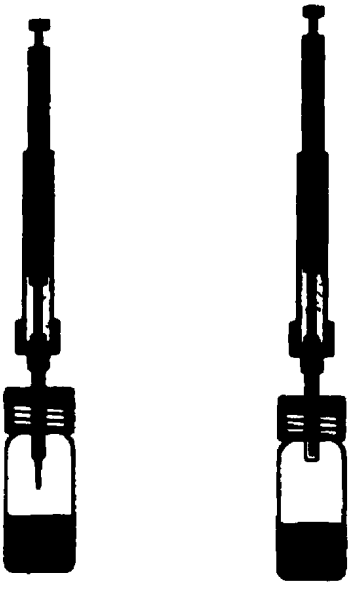


Figure 1.6. Extraction method for SPME (i.e. Headspace)

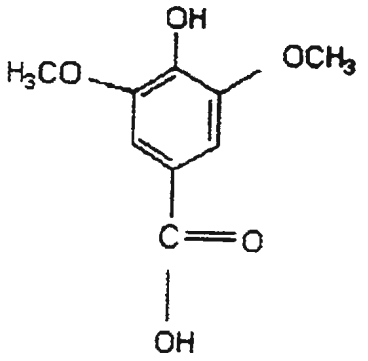
Many parameters affect the successful use of SPME. For example, increasing the thickness of the fibre coating has been shown to increase the amount of analyte that may be adsorbed onto the fibre. Determination of the appropriate coating polarity that would be most suitable for an aqueous sample must be done, however, this is more of a trial and error process. Finally, the most important parameter that must be controlled is the optimisation of the sample temperature. This is because an increase in sample temperature has been shown to result in faster extraction times because a higher concentration of the analyte is present within the headspace. Also, an increase in temperature allows higher boiling point analytes to be adsorbed onto the fibre. However, this may be considered a limitation because low volatility components have been shown to be adversely affected. Thus, when SPME is used in analysis it is important that optimisation of conditions is performed in order to obtain the most effective result (22).

SPME can be used in conjunction with off-line analysis of thermochemolysis products whereby the SPME coating adsorbs the products and these are subsequently transferred to the GC. The advantage of SPME is that it prevents non-volatile compounds from reaching the GC, thereby producing chromatograms with less background noise, which unlike on-line thermochemolysis permits all material to reach the chromatographic column resulting in higher background noise over time (2). Before SPME can be an effective extraction technique, the optimisation of SPME conditions is crucial. Thus, this study optimizes SPME using a syringic acid standard for the subsequent analysis of phenolic acids of white pine needles *(Pinus strobus)*.

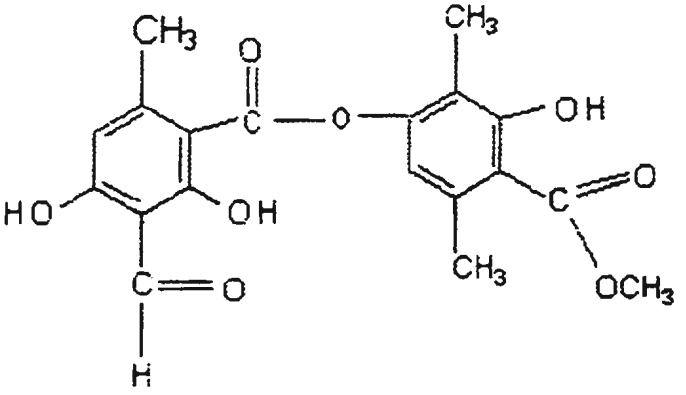
1.3. Use of pure phenolic acids as models for optimisation of off-line thermochemolysis

Until TMAH was included in pyrolysis, the analyses of polar components (e.g. phenolic acids) were limited. TMAH/on-line thermochemolysis offers an alternative method for the analysis of polar compounds. This study used off-line thermochemolysis with reagents TMAH, TMAAc, and BSTFA for the analysis of phenolic acids such as syringic acid, atranorin and usnic acid, shown in figure 1.7, to determine the effectiveness of off-line thermochemolysis in combination with SPME.

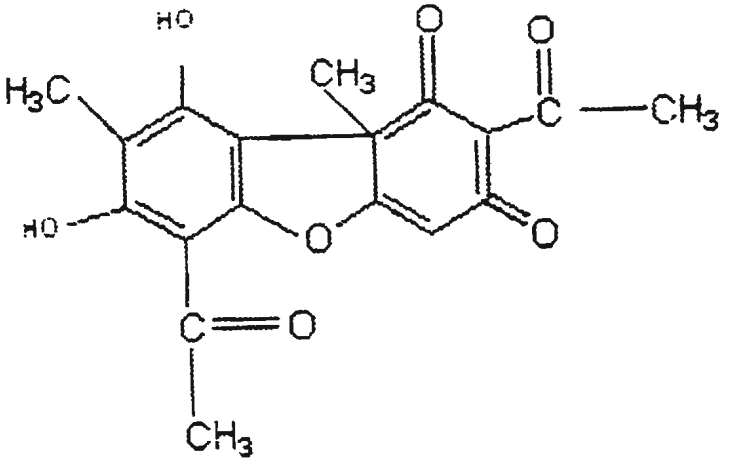
All model phenolic acids contain hydroxyl and carboxylic acid groups that form methyl derivatives with TMAH and TMAAc, and silane derivatives with BSTFA. Syringic acid was used as a model compound to optimize the parameters of off-line TMAH/thermochemolysis using SPME.



Syringic Acid



Atranorin



Usnic acid

Figure 1.7. The phenolic acids used as models for optimisation in off-line thermochemolysis.

1.4. Model study: Plant phenolics in white pine needles

1.4.1. Plant phenolics

Plant phenolics are important to the survival and evolution of land plants. These compounds play an important role at the molecular level where they are components of the structure of plants which screen against irradiation, help regulate the nutrient cycle, and in the defence against pathogens (21). Some compounds found in plants that have phenolic acid components include lignins, anthraquinones, phenylpropanoids, and condensed tannins (21). For example, the two major groups of phenolic acids that usually occur in the conjugated and esterified forms commonly found in leaves include a range of substituted benzoic acid derivatives and those derived from cinnamic acids. An important benzoic acid, syringic acid, combines with other acids such as p-hydroxyl benzoic and vanillic acid to form components of lignins, which are highly branched polyphenolic compounds with complex structures that play an important role in the rigidity of cell walls (20).

Thermochemolysis is important in determining the chemical structures found in plants and towards the quantification of their changed abundances as a result of exposure to environmental stresses such as pollution, and disease (20). The research completed on plants using thermochemolysis has or involves determination of the structure of the pyrolysates in order to recreate the original molecule. Thus, analysis of plant chemistry through thermochemolysis ideally requires a thorough knowledge of the structures that are present within a plant.

Up to now, applications of thermochemolysis of plants has been completed using TMAH/on-line, in which the pyrolysis unit and the GC are connected. This method is not without problems. For example, large quantities of unchromatographable sample reaches the chromatographic column

which, after time may lead to column degradation. Thus, this study of off-line thermochemolysis offers an alternative method to on-line thermochemolysis for plant structural analysis (of white pine needles *(Pinus strobus)* or any other plant species).

1.4.2. Phenolics in white pine needles (*Pinus strobus)*

When analyzing wood by thermochemolysis, it is important to be specific about the species. This is because different species of wood have been shown to have different structures and quantities of phenolics (3). Clifford et al. (12) showed that on-line TMAH/ thermochemolysis was very selective for lignin and the predominant peaks obtained were guaiacyl derivatives, which are primarily monomethylphenol units found in Gymnosperm lignin (13).

Other research completed by Zhang (1993) using on-line thermochemolysis of white pine needles *(Pinus strobus)* showed the presence of small amounts of hydrolysable and condensed tannins (20). A major peak was determined to be 3,4-dimethoxy benzoic acid methyl ester (Mm= 196), figure 1.8a, and a minor peak 3-methoxy benzoic acid methyl ester (Mm= 166), figure 1.8b. The work completed by Zhang is important in this study for the comparison of on-line to off-line thermochemolysis of the same white pine needles (*Pinus strobus)*, in relation to the above compounds (20).

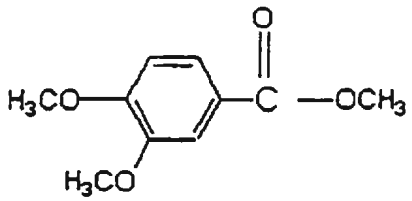


Figure 1.8a. 3.4-Dimethoxy benzoic acid methyl ester (Mm=196)

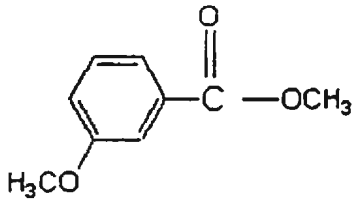


Figure 1.8b. 3-Methoxy benzoic acid methyl ester (Mm = 166)

1.5. Objectives of this study

There has been much research on the structural identification of plant phenolic acids using TMAH/thermochemolysis. However, much of this research entails the use of on-line thermochemolysis (limitations discussed in section 1.4.1), thus, the objectives of this study include:

1) The optimisation of off-line thermochemolysis/SPME using syringic acid as a model phenolic acid.

2) The effectiveness of alternative reagents TMAAc, and BSTFA, including the solvent in which the reagent should be dissolved (i.e. methanol or water).

3) Application of the developed method to the analysis of the phenolic acids present in white pine needles *(Pinus strobus)*.

Chapter 2

Experimentation: Approach and Results

2.1. Materials and Solutions

Derivatizing reagents

Tetramethylammonium hydroxide (TMAH) as a 25 mass % solution in methanol. TMAH-pentahydrate (97%) and tetramethylammonium acetate (TMAAc; 95%) were purchased from Aldrich. N,O- bis(trimethylsilyl) trifluoroacetamide (BSTFA + tms. 99:1) was purchased from Supelco. TMAH and TMAAc were dissolved in either nanopure water or spectrograde methanol. Fresh solutions were prepared weekly.

The BSTFA/TMS was shipped in sealed vials and only opened when required for analysis. Any unused reagent was discarded if not consumed during the day.

Standards and pine needles

Syringic acid and atranorin were purchased from Sigma while usnic acid (98%) was purchased from Aldrich. The standards were used as is and methanolic solutions of each were prepared weekly. For accuracy, known quantities of standards used for experiments were delivered volumetrically (e.g. 0.2 μg/ μl) because of the small quantities needed.

The white pine needles, I.D. 38 (Pinus Strobus) were obtained from work completed by Forestry Canada, Maritimes region and used in a previous study (20). Powdered samples (200 μg ± 5 μg) were directly weighed onto a quartz boat using a microbalance.

2.2. Off-line thermochemolysis GC/MS using SPME

2.2.1. Optimisation of solid-phase microextraction (SPME) of syringic acid

Solid-phase microextraction (SPME) was optimized using syringic acid. A schematic of the SPME method used for this study is shown in figure 2.1 (2).

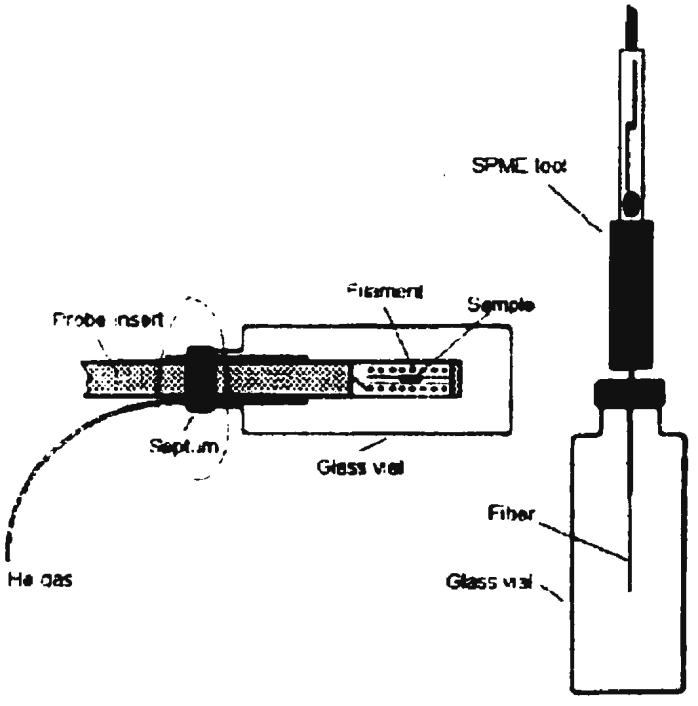


Figure 2.1. Off-line thermochemolysis GC/MS using solid -phase microextraction (SPME) where by the SPME device is placed into vial.

The amount of analyte and reagent used varied throughout the study. Their amounts are either given in the text or along with the figures and tables. Initially, solid syringic acid was weighed out onto quartz pyrolysis tubes, but it was very difficult to accurately weigh these small quantities (i.e. 5, 10 μg). Solutions of syringic acid were therefore used in optimisation experiments.

Syringic acid (a known amount dissolved in methanol) was added to a quartz boat containing a small piece of glass wool. To this, a known amount of derivatizing reagent (i.e. TMAH, TMAAc, or BSTFA dissolved in methanol) was added. The quartz boat was then placed within the platinum coil filament of a model 120 pyroprobe from Chemical Data Systems, and enclosed by a vial. Syringic acid was added to the quartz boat and then the reagent to ensure an accurate amount of the acid used could be maintain throughout the experiment. Also, placement of the analyte into the quartz boat allowed the regent to be placed directly on to the sample helping to ensure that there was adequate time for the analyte and reagent to react.

All chromatograms of off-line thermchemolysis of syringic acid gave only one chromatographic peak, that of the fully derivatized compound. The identity of the product was confirmed by comparing its mass spectrum with that in the NBS mass spectra library. All mass spectral data is listed in the appendix.

The optimal pyrolysis temperature used for syringic acid was determined to be 500°C for 10 seconds, as shown in table 2.1, with a relative peak area of 100 %. When thermochemolysis was completed, the SPME syringe was inserted into the vial (see figure 2.1) and placed into a pierce Reacti-Therm Stirring/Heating module. The optimal temperature determined for

adsorption onto the SPME fibre was 90°C, as shown in table 2.2, which gave a relative peak area of 98 %. The optimum heating time for the SPME fibre was determined to be 10 minutes, as shown in table 2.3, which gave a relative peak area of 100 %. Finally, the size and type of fibre chosen for SPME was 100 μm diameter coated with polydimethylsiloxane, as shown in table 2.4. Although the 30 μm diameter fibre had a slightly higher relative peak area, the 100 μm fibre was chosen for thermochemolysis because of its ability to handle more sample (20).

Temperature (°C)	Relative peak height (%) relative to 500°C, run 1	
	Run 1	Run 2
300	76	72
400	92	89
500	100	101
600	105	107
700	90	87

Conditions: 10 μl of syringic acid (0.2 μg/ μl) with
10 μl of 25% methanolic TMAH. SPME: 90°C for 10 min.

Table 2.1. Optimization of pyrolysis temperature for syringic acid using solid-phase microextraction (SPME): duplicate analysis.

Temperature (°C)	Relative peak height (%) relative to 120°C, run 1	
	Run 1	Run 2
30	5	8
50	20	22
70	85	79
90	98	95
120	100	105

Conditions: 10 μl of syringic acid (0.2 μg/ μl) with
10 μl of 25% methanolic TMAH. SPME: for 10 min
Pyrolysis temperature: 500°C.

Table 2.2. Optimization of solid-phase microextraction (SPME) adsorption temperature for syringic acid; duplicate analysis.

Time (min)	Relative Peak height (%) relative to 10 min, run 1	
	Run 1	Run 2
4	70	62
6	80	82
8	85	88
10	100	102
12	103	105
14	110	107

Conditions: 10 µl of syringic acid (0.2 µg/ µl) with
10 µl of 25% methanolic TMAH. SPME: 90°C
Pyrolysis temperature: 500°C.

Table 2.3. Optimization of solid-phase microextraction (SPME) adsorption time using a heating module: duplicate analysis.

Stationary Phase	Description	Relative peak height(%) relative to 30µm
100µm polydimethylsiloxane	Non-bonded	97
30µm polydimethylsiloxane	Non-bonded	100
7µm polydimethylsiloxane	Bonded	87
85µm poly acrylate	Partially crossed-linked	76

Conditions: 10 µl of syringic acid (0.2 µg/ µl) with
10 µl of 25% methanolic TMAH. SPME: 90°C for 10 min
Pyrolysis temperature: 500°C.

Table 2.4. Optimization of coat thickness and type.

The optimisation shown in tables 2.1-2.3 was performed twice to determine reproducibility and to determine the optimum parameters for use in the off-line thermochemolysis of white pine needles *(Pinus strobus)*. The reproducibility between runs was shown to be within 10-15 %, which indicate that the conditions optimised for syringic acid were effective in obtaining results that could be quantified. Most importantly, optimisation of the SPME conditions improves the ability of the device to acquire sample that is to be introduced into GC/MS. The SPME also eliminates non-volatile compounds before they reach the chromatographic column, thus improving the sensitivity of GC/MS. The optimisation of the SPME coating thickness and type (table 2.4) indicates the choice of coating thickness most beneficial to off-line to be the non-bonded polydimethylsiloxane. This 100μm fibre was selected in part due to its ability to hold more sample compared to other smaller coating thicknesses .

2.2.2. Optimization of gas chromatography/mass spectrometry (GC/MS)

For qualitative analysis, a Hewlett-Packard 5890 series Gas Chromatograph was interfaced to a Hewlett-Packard 5971 Series Mass Sensitive detector. Both instruments were controlled by Hp-Chem software, which also collected the mass spectral data. The optimization was carried out again using syringic acid.

The GC injector port temperature was 250°C and the oven temperature program was initially set at 150°C and held for 2 minutes before ramping up to 250°C at 16°C/min. The final temperature was held for 2 minutes. The GC column pressure was 18.05 psi. The optimal split flow, of helium gas, was determined, through duplicate analysis, to be at 18.0 ml/min, as shown in table 2.5. The duplicate analysis showed the variation to be better than 10% for the chosen

split flow (i.e. 18 ml/min). The GC column was a DB-5 capillary column (Chromatographic Specialities) length 28 m. i.d. of 0.25 mm. and a film thickness of 0.25 μm. The film consisted of 5% phenyl. and 95% methyl groups on a siloxane backbone.

Split flow (ml/min)	peak area ($x10^3$)	
	Run 1	Run 2
9.0	9.1	8.9
18.0	25.1	25.4
40.0	2.2	2.4

Conditions: 10 μl of syringic acid (0.2 μg/ μl) with
10 μl of 25% methanolic TMAH. SPME: 90°C for 10 min
Pyrolysis temperature: 500°C.

Table 2.5. Optimization of GC/MS split flow using syringic acid; duplicate analysis

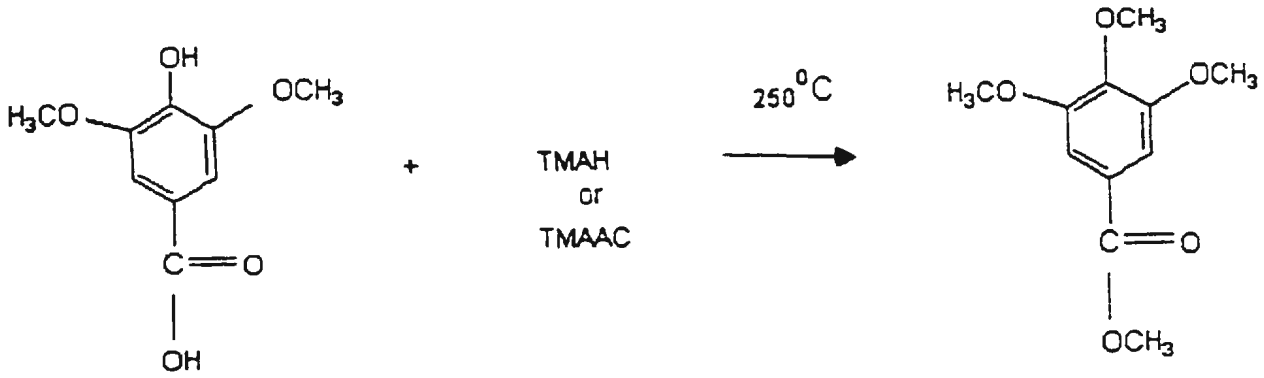
The mass detector was tuned daily using the Autotune program. Ionization occurred by electron impact using electrons with 70 eV. The mass analyzer was a quadrupole used in the scan mode for m/z of 50 to 650.

2.3. Qualitative results using GC/MS

2.3.1. TMAH and TMAAc/ off-line thermochemolysis of syringic acid

The TMAH and TMAAc/ off-line thermochemolysis of syringic acid produced the dimethyl derivative (Mm=226), shown in Figure 2.2.The resulting chromatogram from each experiment gave a peak at the same retention time (RT = 4.500 min) and the same mass spectrum, which was identified as that of the dimethyl derivative. This was in agreement with the reactions of TMAH and TMAAc

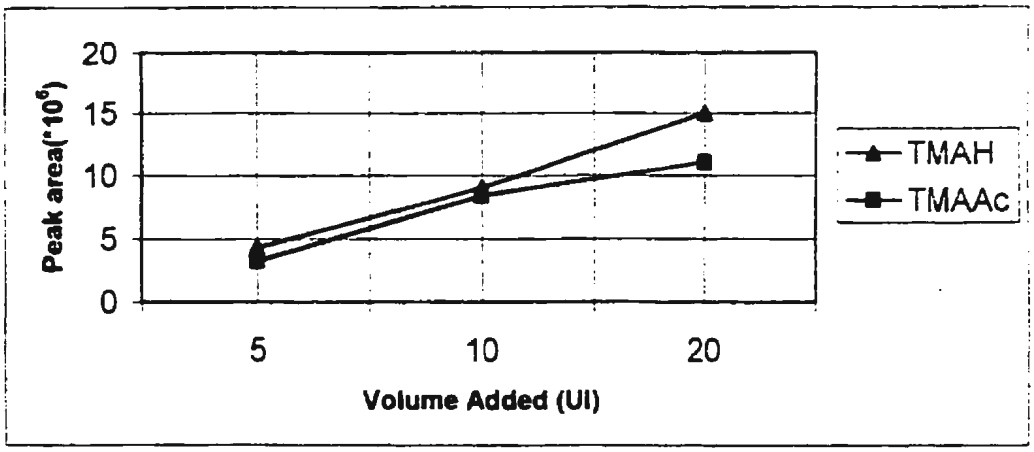
with syringic acid as reported in the literature, which resulted in the formation of the same product (3,4,5-trimethoxybenzoic acid methyl ester) (3,5,12,13).



Syringic Acid

Figure 2.2. The dimethyl derivative of syringic acid (Mm= 226) through off-line/ thermochemolysis with TMAH or TMAAc.

Chromatograms of TMAH and TMAAc-thermochemolysis of syringic acid [0.2 µg/µl] showed a reasonably linear correlation between the volumes of syringic acid and the peak area as shown in figure 2.3.



Conditions: 5,10,20 µl of syringic acid (0.2 µg/ µl) with
10 µl of 25% methanolic TMAH. SPME: 90°C for 10 min
Pyrolysis temperature: 500°C.

Figure 2.3. Correlation between volume of syringic acid (0.2 µg/µl) added to the peak area determined through TMAH and TMAAc/ off- line thermochemolysis.

Additional experiments were also performed to observe the reproducibility of TMAH and TMAAc-thermochemolysis. For TMAH as methylating reagent, the reproducibility was quite reasonable with SD= 4.79 % (table 2.6a). However, the use of TMAAc showed less reproducibility, SD= 44.0 %, and these results are shown in table 2.6b. The plausible reason for this is the weakly basic conditions when the acetate salt is employed.

peak area ($x10^6$)
4.39
4.83
4.60
SD = 4.79%

Conditions: 10µl of syringic acid (0.2 µg/ µl) with
10 µl of 10 % methanolic TMAH. SPME: 90°C for 10 min
Pyrolysis temperature: 500°C. Spilt flow: 18 ml/min

Table 2.6a. Reproducibility of TMAH/off-line thermochemolysis GC/MS for syringic acid [0.2 µg/µl] using SPME

peak area ($x10^6$)
1.35
1.45
3.04
3.22
SD = 44.0%

Conditions: 10µl of syringic acid (0.2 µg/ µl) with
10 µl of 10 % methanolic TMAH. SPME: 90°C for 10 min
Pyrolysis temperature: 500°C. Spilt flow: 18 ml/min

Table 2.6b. Reproducibility of TMAAc/off-line thermochemolysis GC/MS for syringic acid [0.2 µg/µl] using SPME.

2.3.2. BSTFA/ off-line thermochemolysis of syringic acid

The BSTFA/ off-line thermochemolysis of syringic acid produced the silane derivative (Mm = 342), as shown in figure 2.4. The BSTFA/ off-line thermochemolysis of syringic acid produced only one peak, the di-silane derivative (Mm= 342) shown in figure 2.4 (its mass spectra listed in appendix). The resulting chromatograms showed a RT of 6.282 min for the silylated derivative compared to 4.500 min for the methylated derivative of syringic acid. The longer retention time is due to the increased mass of the derivative and hence lower volatility as a result of the larger silyl group compared to the methyl group.

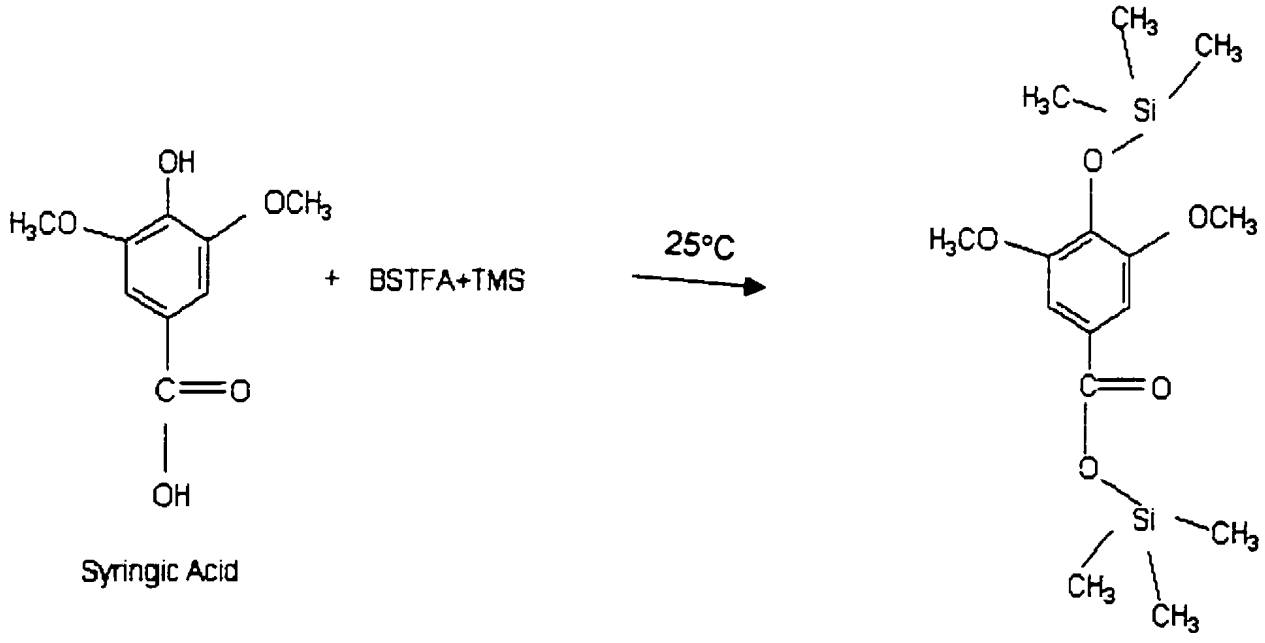


Figure 2.4. The silane derivative from BSTFA/ off-line thermochemolysis with syringic acid [0.2 μg/μl] using SPME.

The reproducibility of BSTFA/ off-line thermochemolysis of syringic acid, as shown in table 2.7, was not as good as with TMAH (with SD =4.79%). However, this may be due to the volatility of BSTFA, which will tend to evaporate during sample heating. Thus, care must be taken to ensure that sample has actually reacted with BSTFA before chromatographic analysis. Therefore, for this study, no assumptions may be applied to whether or not BSTFA can be used as an alternative reagent in off-line thermochemolysis until further precautions are taken to ensure BSTFA has reacted only with the sample in question and not lost to evaporization.

methylated peak area ($x10^6$)
21.0
4.3

Conditions: 10µl of syringic acid (0.2 µg/ µl) with
3 µl of BSTFA+tms. SPME: 90°C for 10 min
Pyrolysis temperature: 500°C. Spilt flow: 18 ml/min

Table 2.7. Reproducibility of BSTFA/ off- line thermochemolysis GC/MS with syringic acid [0.2 µg/µl] using SPME.

2.3.3. TMAH, TMAAc, and BSTFA/ off-line thermochemolysis of atranorin and usnic acid

The products that may result from an expected thermochemolysis of atranorin and usnic acid with their individual reagents (TMAH, or TMAAc) are shown in Figures 2.5a, and 2.5b, respectively. For reagent BSTFA, the groups designated by (*) would have silyl groups present. It is important to understand that many researchers have shown that each reagent has a chemical effect on the products obtained. For example, MacGillivary (3) showed that when atranorin was exposed to TMAH, the intact structure was broken down into fragments, and that each fragment was methylated (3).

The experimental conditions used were the same as those used for the analysis of syringic acid. The results of off-line thermochemolysis of atranorin and usnic acid were very poor. No chromatographic peaks were observed in any of the chromaotgrams, not even TMAH-methylated fragments of atranorin observed by MacGillivary (3).

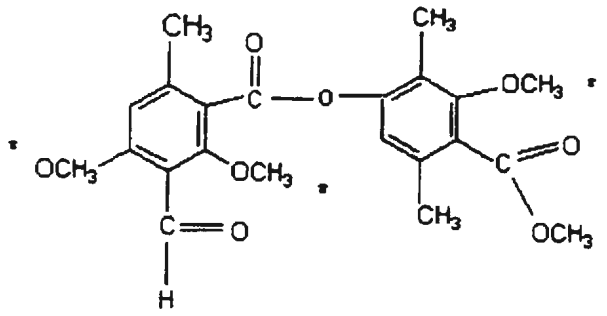


Figure 2.5a. Expected product from TMAH or TMAAc/ thermochemolysis of atranorin with methylated groups designated by (*) (Mm = 416).

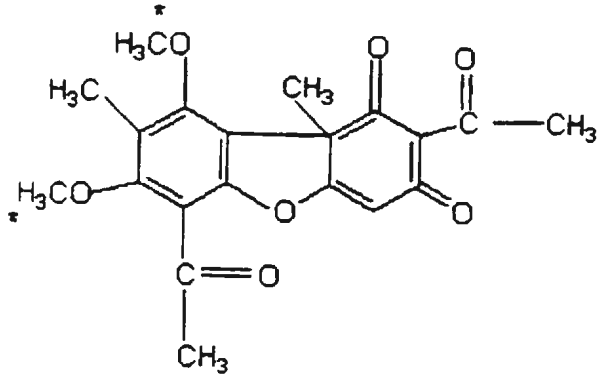


Figure 2.5b. Expected product from TMAH or TMAAc/ thermochemolysis of usnic acid with methylated groups designated by (*) (Mm = 372).

It is possible that atranorin and usnic acid were derivatized by thermochemolysis and condensed on the walls of the collected vials. But the products may not have been able to desorbed onto the SPME fibre because of their high boiling points (or molar mass, (methylated atranorin; Mm= 416; usnic acid; Mm= 372)). SPME temperature as high as 150 °C was tried without success.

For this study, the important aspect was to verify if off-line thermochemolysis has potential in the analysis of phenolic acids in white pine needles *(Pinus strobus)* in comparison to the results obtained from on-line thermochemolysis (i.e. Zhang, 1993) (20). Thus, TMAH/syringic acid was used as an internal standard for the method development of off-line thermochemolysis of white pine needles *(Pinus strobus)* to ensure that optimum methylation conditions were maintained.

2.3.4. TMAH/thermochemolysis/SPME of white pine needles (*Pinus strobus*)

The verification of off-line thermochemolysis potential in white pine needle *(Pinus strobus)* analysis was made possible with the work completed by Zhang (1993). The peaks of interest were peak **46**, 3-methoxy benzoic acid methyl ester (Mm= 166), and peak **55**, 3,4-dimethyl benzoic acid methyl acid (Mm= 196), as shown in figure 2.6.

The chromatograms of TMAH/ off-line thermochemolysis of white pine needles *(Pinus strobus)* showed an easily determined peak to be 3,4-dimethoxy benzoic acid methyl ester (Mm= 196) with retention (RT) 3.290 min, while a minor 3-methoxy benzoic acid methyl ester peak (Mm = 166) was shown to be present only when an extracted ion chromatogram was performed, as shown in figure 2.7. The mass spectra data is given in the appendix

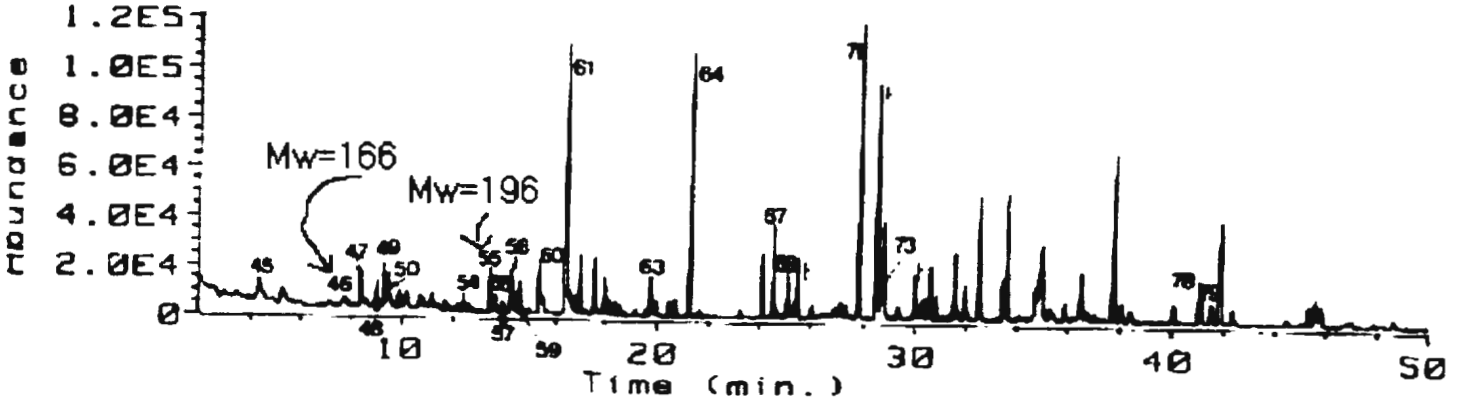
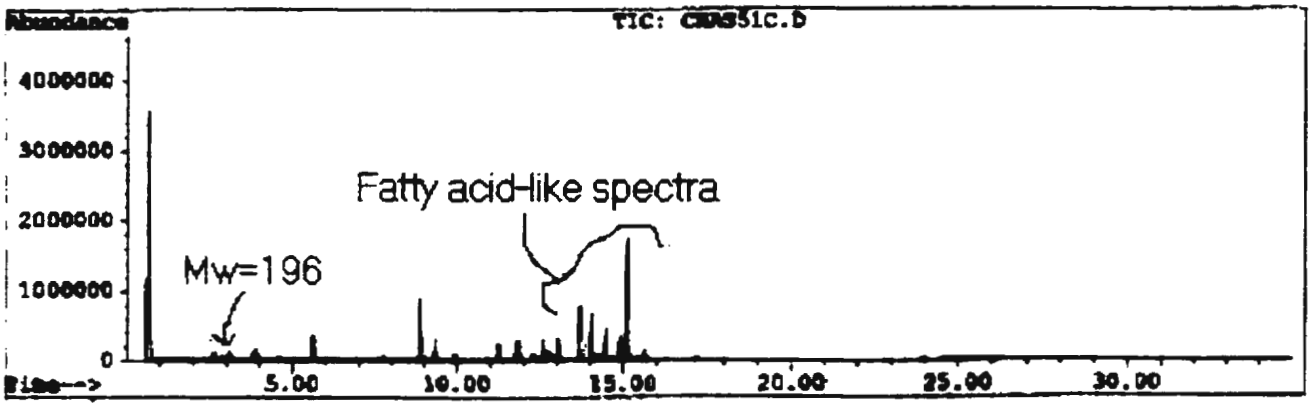


Figure 2.6. TMAH/ on-line thermochemolysis of white pine needles *(Pinus strobus)* completed by Zhang (1993) (20).



GC conditions: injector port temperature: 290°C: Oven temperature: 100°C (held for 2 min) to 280°C (held for 3 min) at 32 °C/min.
Other conditions: 100µg white pine needles; 20 µl (25%) TMAH

Figure 2.7. TMAH/ off-line thermochemolysis of white pine needles at SPME adsorption of 90°C for 10 minutes.

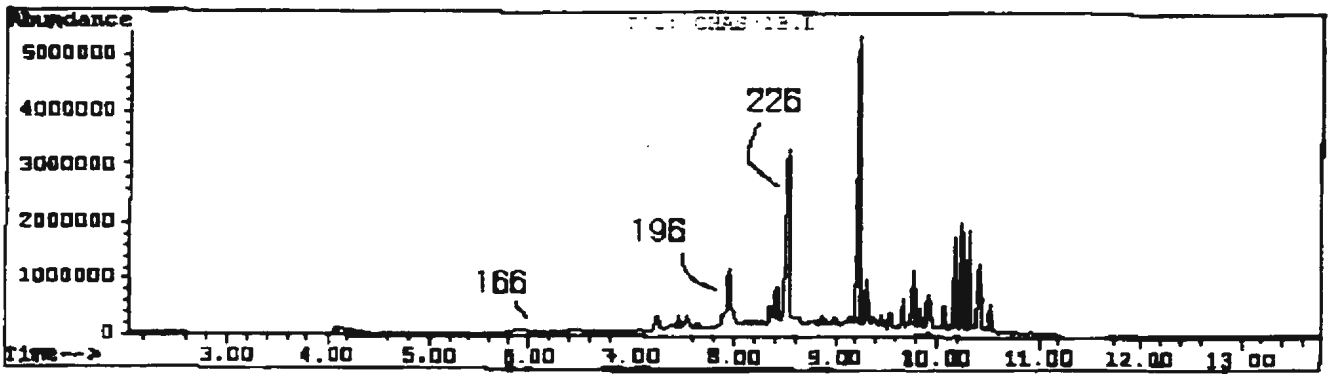
The comparison of chromatograms (figures 2.6, and 2.7) was important for determining the potential of off-line thermochemolysis in white pine needle analysis. However, to obtain the above chromatogram (figure 2.7), the injector port temperature was increased to 290°C. Also, the oven temperature program was changed to 100 °C, held for 2 minutes, and increased to 280 °C at 32 °C/ min and held for 3 minutes, in order to resemble the operation of on-line thermochemolysis performed by Zhang (20), in figure 2.6. The peaks obtained from the chromatogram (figure 2.7) are relatively different from the chromatogram obtained by Zhang (figure 2.6) because only the phenolic acid components are shown (RT less than 10 minutes), along with a fatty acid- like spectrum. This is due to the use of SPME, which allowed only volatile compounds to reach the GC column, while figure 2.6 results from the complete introduction of all pyrolysates onto the column due to the direct connection between the GC and the pyrolysis unit.

One major difference in off-line thermochemolysis is the presence of the solvent in which the derivatizing reagents TMAH and TMAAc are dissolved. Prior to off-line thermochemolysis of white pine needles, all optimised conditions of syringic acid were completed with the reagents dissolved in methanol solution. However, a comparison was made between the affect of water and methanol as the solvent. It was determined that when dealing with the real sample of white pine needles *(Pinus strobus)*, the use of water appeared to produce a chromatogram that showed a higher abundance of 3-methoxy benzoic acid methyl ester (Mm = 166) and 3,4-dimethoxy benzoic acid methyl ester (Mm = 196) peaks. However, the methanol solvent seemed to suppress the 3-methoxy benzoic acid methyl ester peak. Thus, for off-line thermochemolysis of white pine needles *(Pinus strobus)*, water was used as the solvent for the TMAH and TMAAc reagents.

2.3.5. Internal standard (syringic acid)/ TMAH in water for off-line thermochemolysis of white pine needles (*Pinus strobus*)

Syringic acid was used as an internal standard in off-line thermochemolysis of white pine needles *(Pinus strobus)* to ensure that the reproducibility of GC/MS was maintained. Reproducibility is important in GC analysis so that quantitative results may be determined.

The optimisations made to off-line thermochemolysis for syringic acid were discussed in section 2.3.4. However, for white pine needles, the pyrolysis temperature needed to be optimized to ensure that all the products are observed in good quantity. Using pyrolysis temperatures of 300 and 500 °C the chromatogram results indicated that the methylated product (Mm= 196) was present but only in small quantities, which was not satisfactory. But a pyrolysis temperature of 750 °C produced some 3-methoxy benzoic acid methyl ester (Mm = 166) peak, a major 3,4-dimethoxy benzoic acid methyl ester (Mm=196) peak, and a methylated syringic acid (Mm = 226) peak (see figure 2.8). The SPME adsorption temperature used was 90°C for 10 min.



GC Conditions: injector port temperature: 250°C; Oven temperature: 150°C (held for 2 min) to 250°C (held for 2 min) at 16 °C/min; Split flow:18.0 ml/min

Other conditions: 200μg white pine needles; 10 μl (25%) TMAH; 5μl syringic acid (0.2 μg/μl)

Figure 2.8.TMAH (in water)/ off-line thermochemolysis of white pine needles *(Pinus strobus)* using the internal standard, syringic acid, at pyrolysis temperature of 750°C and SPME at 90°C.

Triplicate samples of white pine needles (200μg) pyrolysed at 750°C were taken to determine the reproducibility of off-line thermochemolysis using a SPME adsorption temperature of 90°C for 10 minutes, as shown in table 2.8. From this table, the reproducibility of syringic acid (Mm = 226) was best although lower than the standard by itself (< 5 %). The two peaks at Mm = 166 and 196 had a much worse reproducibility. There was indication that the SPME adsorption temperature was affecting the recovery of these two products to a greater extent than the methylated syringic acid. To determine the limitation of this method, the adsorption temperature was changed to 25 °C, 55 °C, and 75 °C, as shown in table 2.9. Chromatograms of the different SPME adsorption temperatures are shown in figures 2.9-2.11, respectively.

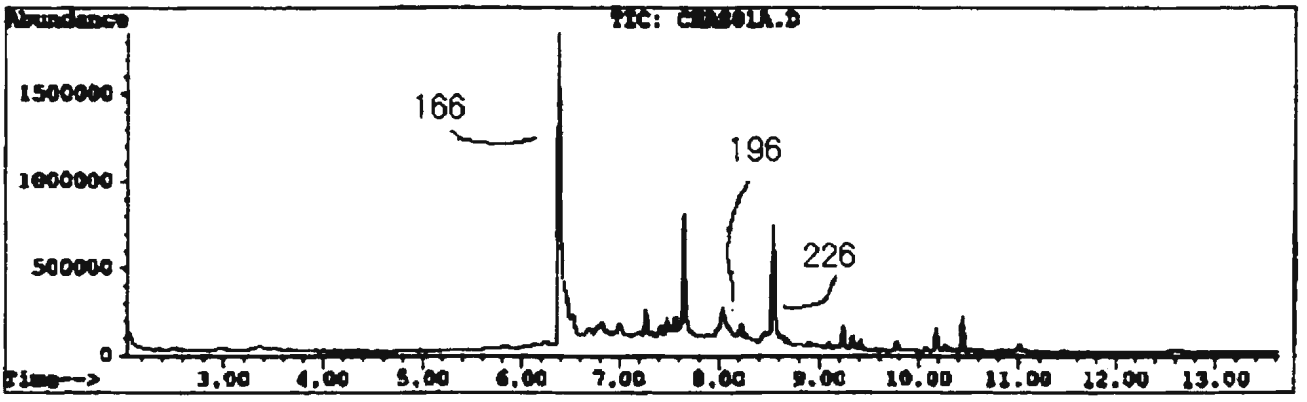
Molar mass	Peak area ($x10^7$)
166	1.17
166	4.04
166	9.54
SD = 86.4 %	
196	2.87
196	7.26
196	11.7
SD = 60.6 %	
226	9.03
226	8.28
226	11.2
SD = 16.0 %	

Table 2.8. Triplicate samples of white pine needles (*Pinus strobus*) with syringic acid internal standard, for TMAH/water-thermochemolysis at 750°C for 20 seconds with a SPME adsorption temperature of 90°C.

Molar mass	Temperature (°C)	peak area ($x10^7$)
166	25	5.49
	55	4.94
	75	5.60
196	25	0.20
	55	0.30
	75	7.14

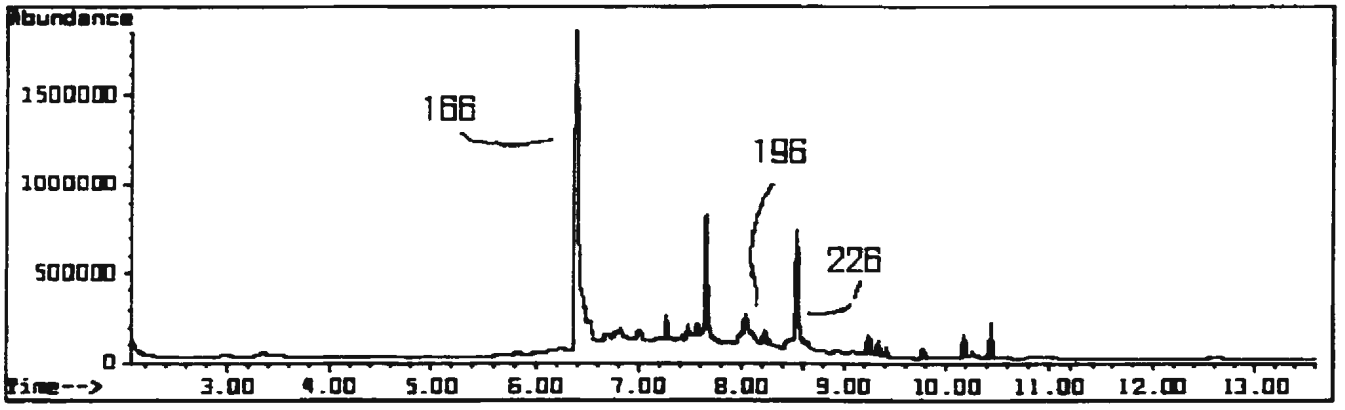
Table 2.9. The TMAH/off-line thermochemolysis of white pine needles with internal standard of syringic acid, done at different SPME adsorption temperatures of 25, 55, and 75°C.

An analysis of table 2.9 indicates that SPME temperatures affected the methylated peaks of 3.4-dimethoxy benzoic acid methyl ester (Mm = 196), but not 3- methoxy benzoic acid methyl ester (Mm = 166). This is also shown in figures 2.9-2.11. Also, the change in SPME temperatures altered the abundance of the internal standard, syringic acid (Mm= 226) as can be clearly observed in the chromatogram. This is a major problem for using syringic acid as an internal standard



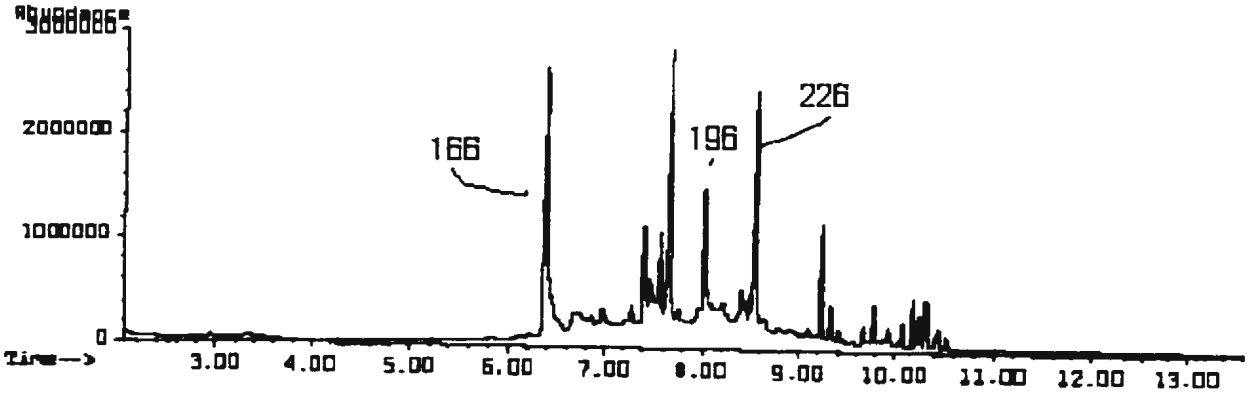
GC Conditions: injector port temperature: 250°C; Oven temperature: 150°C (held for 2 min) to 250°C (held for 2 min) at 16 °C/min; Split flow:18.0 ml/min
Other conditions: 200μg white pine needles; 10 μl (25%) TMAH; 5μl syringic acid (0.2 μg/μl)

Figure 2.9 The TMAH/off-line thermochemolysis of white pine needles with internal standard of syringic acid, an adsorption temperature of 25°C



GC Conditions: injector port temperature: 250°C; Oven temperature: 150°C (held for 2 min) to 250°C (held for 2 min) at 16 °C/min; Split flow:18.0 ml/min
Other conditions: 200µg white pine needles; 10 µl (25%) TMAH; 5µl syringic acid (0.2 µg/µl)

Figure 2.10. The TMAH/off-line thermochemolysis of white pine needles with internal standard of syringic acid, an adsorption temperatures of 55°C.



GC Conditions: injector port temperature: 250°C; Oven temperature: 150°C (held for 2 min) to 250°C (held for 2 min) at 16 °C/min; Split flow:18.0 ml/min
Other conditions: 200µg white pine needles; 10 µl (25%) TMAH; 5µl syringic acid (0.2 µg/µl)

Figure 2.11. The TMAH/off-line thermochemolysis of white pine needles with internal standard of syringic acid, an adsorption temperatures of 75°C.

2.4 Future work

The off-line thermochemolysis of white pine needles (*Pinus strobus*) has been shown to have some potential in the analysis of the phenolic compounds found in plants. The method of off-line thermochemolysis still requires a lot of future work aimed towards validating its results with on-line thermochemolysis. Some potential work includes: the use of SPME by directly inserting the fibre into a solution containing dissolved pyrolysates (as in figure 1.6), along with optimisation of the SPME fibre, the use of other reagents such as TMAAc and BSTFA, the investigation of the use of ^{13}C-labelled TMAH, in off-line thermochemolysis, in order to distinguish the difference between reagent methylated carboxylic acid and hydroxyl groups from those methyl groups that may already be present within that compound (23), and finally, off-line thermochemolysis may be explored as a qualitative method for the analysis of fatty acids (figure 2.7).

Chapter 3

CONCLUSIONS

The method development of off-line thermochemolysis using SPME for analysis of white pine needles was completed with use of syringic acid as a model compound. The use of TMAH, TMAAc and BSTFA as derivatizing reagents was applied to syringic acid and it was determined that TMAH was important here since it gave more reproducible results than either TMAAc or BSTFA.

The main requirement for off-line thermochemolysis compared to on-line (the direct connection between the GC and pyrolysis unit) was the use of SPME (Solid-Phase microextraction). The off-line thermochemolysis SPME parameters were determined to be a pyrolysis temperature of 500°C for 20 sec, an SPME adsorption temperature of 90°C for 10 minutes and a GC split flow of 18ml/min using syringic acid. This optimisation of the off-line thermochemolysis was important for the application of syringic acid as an internal standard in white pine needles analysis. The method of TMAH/off-line thermochemolysis using SPME of white pine needles (section 2.3.5), with syringic acid as an internal standard, was shown to be dependent on the adsorption temperature of the SPME apparatus (section 2.2.1) with a change in peak abundances of 3- methoxy benzoic acid methyl ester (Mw=166) and 3,4-dimethoxy benzoic acid methyl ester (Mw=196) (table 2.8).

The chromatogram of the methylated peaks (Mw =166 and 196) determined by off-line thermochemolysis was compared to the chromatogram completed by Zhang (1993) (20) on white pine needles. The above peaks were found to be present in both methods, however the chromatograms for off-line thermochemolysis were not as congested as those of Zhang. This resulted from the use of SPME in off-line thermochemolysis, where only the non-polar compounds are

adsorbed onto the fibre and desorbed to reach the chromatographic column. The comparison of the above peaks, regardless of the different chromatograms for both methods, shows that use of TMAH/ off-line thermochemolysis using SPME has great potential in qualitative analysis of white pine needles.

The solvent in which the reagents were dissolved (i.e. methanol or water) was also investigated. The optimisation of the off-line thermochemolysis method was completed with the reagents dissolved in methanol. The use of water as a solvent was not investigated except for the experiment involving the thermochemolysis of white pine needles. It was found that solvent in which reagents are dissolved alters the chromatograms produced. Water as a solvent was determined to be the most effective for the thermochemolysis of white pine needles in both off-line and on-line methods.

This study was a major development towards a complementary method to on-line thermochemolysis. However with off-line thermochemolysis, there are many parameters that must be investigated to ensure that the chromatograms are realistic for a particular sample. In particular, the adsorption temperature of SPME (i.e. 90 °C) was the most important parameter when analyzing real samples. Regardless of the difference between chromatograms for off-line and on-line thermochemolysis (figures 2.7 and 2.6, respectively,) the identification of peaks with Mm= 166 and 196 in off-line thermochemolysis verify the potential that may exist for this method to be an alternative to on-line thermochemolysis.

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APPENDIX

Mass Spectral Data of Important Chromatographic Peaks used in This Study

NAME	Molar mass	Spectral Data: m/z (relative abundance)
Methylated syringic acid	226	226 (100%), 211(60%), 155 (50%), 195 (35%)
Silylated syringic acid	342	342 (83%), 327 (100%), 312 (85%),297(80%), 283 (35%), 253 (65%), 223 (45%)
3-methoxy benzoic acid methyl ester	166	166 (60%), 135 (100%), 107 (40%), 92 (30%), 77 (35%), 64 (20%)
3.4 methoxy benzoic acid methyl ester	196	196 (100%), 181(12%) 165 (85%), 79 (20%), 51(19%), 15 (15%)