One interesting observation was the relative absence of chlorinated compounds, especially in the SF extracts. Sequiera[63] reported the presence of chlorinated compounds in all extracts, regardless of pulping or bleaching method. This could be indicative of improvements in the rinsing processes, or the relatively smaller amount of pulp extracted for this study. The greater abundance of chlorine containing compounds in the Soxhlet extract could be attributed to the greater polarity of the methylene chloride than the SFE fluids.

In fact, the Soxhlet extraction with CH_2Cl_2 appears to extract more polar materials than the SF extractions, regardless of the polarity of the SF, especially of the earlier eluting compounds.

There are several different hydroxy carboxylic acids identified in both the Soxhlet and SF extracts (retention times 12.62, 13.65 and 24.17 min). These compounds are theorized to cause processing difficulties of the wood pulp and their quantification, if in sufficient concentration, would be of great interest.

5.2.4 Comparison of Methylation Techniques

Since fatty acids and FAMEs were identified as being the major components of the extracts, a series of extractions was performed on a bleached softwood pulp to determine the amount of C_{10} to C_{18} FAMEs resulting from various methylation techniques. These results are presented in **Table 5-3**. The first column represents the quantification of these FAMEs when no additional derivatization technique was used in conjunction with the SFE. In this instance, only small amounts of the C_{12}, C_{14}, and C_{16} FAMEs were present in the extract. However, if that same extract was treated with BF_3/methanol, the presence of the C_{10} and C_{18} FAMEs were noted (third column of **Table 5-3**), as well as an increase in C_{12} and C_{16} FAMEs. A paired t test indicated a significant difference

[63] Anna Sequiera, Ph.D. dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA, 1989.

Table 5-3: Comparison[a,b] of Methylation Techniques Used on a Bleached Softwood Pulp Extract.

	SFE No Derivatization	**SFE *In-situ* Derivatization**	**SFE Off-line Derivatization**
C_{10} FAME	-	2.4 (8)	2.9 (12)
C_{12} FAME	0.6 (14)[c]	1.7 (9)	1.5 (15)
C_{14} FAME	0.6 (15)	2.7 (6)	0.4 (13)
C_{16} FAME	0.3 (17)	4.7 (5)	1.0 (10)
C_{18} FAME	-	4.3 (8)	1.1 (11)

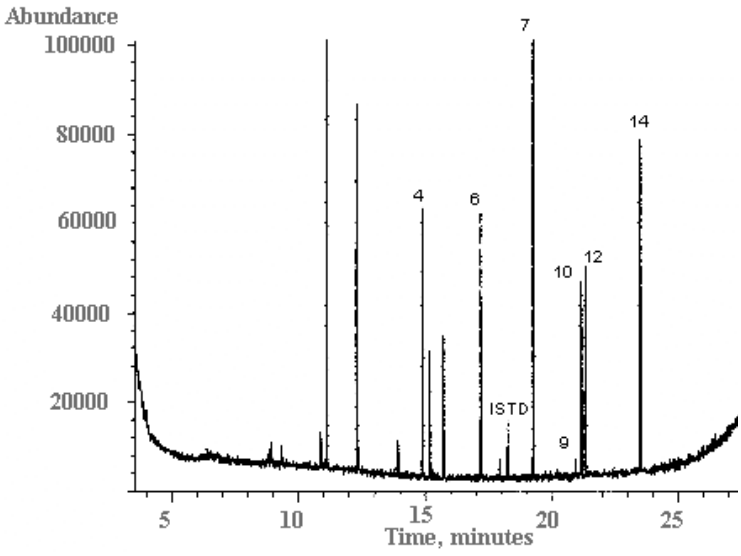
[a]Concentration in µg/10g sample.
[b]Sample was cut into strips.
[c]Numbers in parentheses represent relative standard deviation, n=3.

at the 95% confidence level between these sets of values. The free fatty acids are probably not seen in the chromatograms due to the high activity of the chromatographic system. Additionally, an *in-situ* methylation (BF_3/methanol added to the extraction thimble prior to SFE) was performed, with these results being presented in the second column of **Table 5-3**. Paired t testing again found a significant difference between this method and each of the other two. The most profound difference is noted for the higher molecular weight (C_{16} and C_{18}) FAMEs, with a 4-5 fold increase resulting when comparing *in-situ* to off-line derivatization. This increase can be attributed to the FAMEs being easier to remove from the matrix than the corresponding fatty acids. This difference is most pronounced at the higher molecular weights because of decreasing solubility (of both the FFA and FAME) in the fluid. Once the highly polar carboxylic acid functionality is replaced with the more lippophilic ester moiety, overall solubility in the fluid is increased, thus allowing extraction with the relatively nonpolar extraction fluid. In addition to the increase in the extracted FAMEs detailed above, a comparison of other (non-quantitated) extracted components was performed for the off-line versus *in-situ* derivatizations and is shown in **Table 5-4**. These results indicated that only mono- carboxylic acids are extracted during the SFE, but that diacids are derivatized to their corresponding diesters that are then easily extracted by the same extraction conditions. An identical experiment was performed with a hardwood sample which yielded the same trend of extraction of the diesters of diacids. **Figure 5-6** presents the chromatograms for a bleached softwood pulp extract from which these data were taken.

Table 5-4: Comparison of Extracted Components Resulting from Off-line and *In-situ* Methylation Reactions

Retention Time, min	Off-line Derivatization	*In-situ* Derivatization
6.56	---	Pentanoic acid, 4-oxo-, methyl ester
10.58	---	Hexanedioic acid, dimethyl ester
13.36	---	Octanedioic acid, dimethyl ester
14.34	Dodecanoic acid, methyl ester	
14.63	---	Nonanedioic acid, dimethyl ester
16.69	Tetradecanoic acid, methyl ester	
18.80	Hexadecanoic acid, methyl ester	
19.77	---	Heptadecanoic acid, methyl ester
20.42	9,12-Octadecadienoic acid, methyl ester	
20.49	9-Octadecenoic acid, methyl ester	
20.53	---	11-Octadecenoic acid,methyl ester
20.72	Octadecanoic acid, methyl ester	
22.47	---	Eicosanoic acid, methyl ester
23.08	Tetracosane	
25.62	---	Tetracosanoic acid, methyl ester

A



Peak Identification

1. Pentanoic acid, 4-oxo-methyl ester
2. Hexanedioic acid, dimethyl ester
3. Octanedioic acid, dimethyl ester
4. Dodecanoic acid, methyl ester
5. Nonanedioic acid, methyl ester
6. Tetradecanoic acid, methyl ester
7. Hexadecanoic acid, methyl ester
8. Heptadecanoic acid, methyl ester
9. 9,12-Octadecadienoic acid, methyl ester
10. 9-Octadecenoic acid, methyl ester
11. 11-Octadecenoic acid, methyl ester
12. Octadecanoic acid, methyl ester
13. Tetracosane
14. Tetracosanoic acid, methyl ester

B

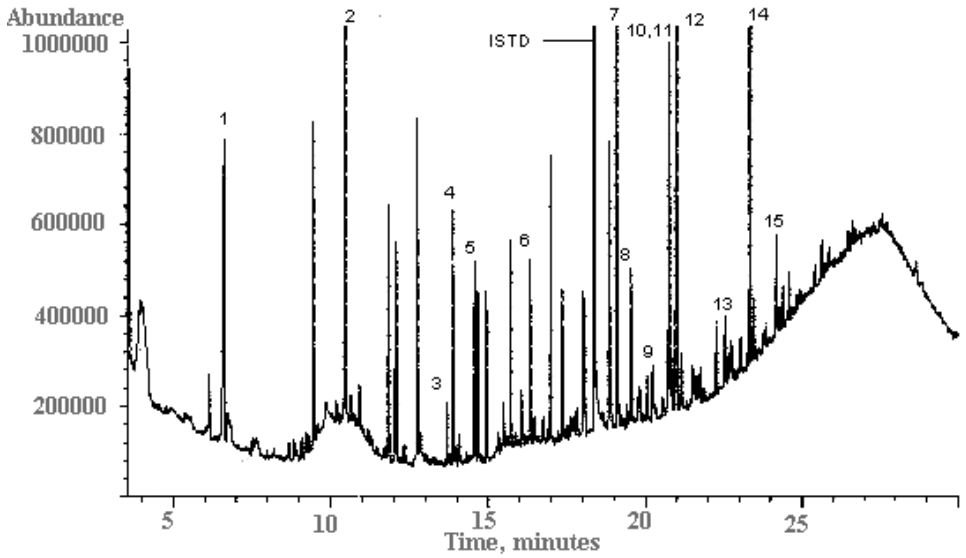


Figure 5-6: Total ion chromatograms of bleached softwood pulp extract. A: Off-line methylation; B: *In-situ* methylation. Chromatographic conditions in text.

5.2.5 Effect of Sample Preparation Method

Most of the extractions discussed to this point were performed on sample strips instead of ground samples. Since the extraction profile indicated a diffusion controlled process, increasing the relative surface area of the pulp by grinding was investigated. It was necessary to frequently tamp the sample into the extraction thimble in order to place approximately the same amount of sample in the thimble as when using wood pulp strips. The results of these experiments, shown in **Table 5-5**, indicated that, for the FAMEs quantitated, grinding the sample resulted in an approximate ten fold increase in the amount of FAME extracted. Paired t testing indicated a significant difference at the 95% confidence level in the amount of material extracted, though the reproducibility expressed as percent relative standard deviation, did not improve. This appears
to be another indication that the extraction is highly diffusion controlled, and that exhaustive extraction has not been approached.

5.2.6 Comparison of Trapping Methods

Because of the extremely low concentrations of extractable materials seen in the bleached softwood pulp extracts, three separate extraction thimbles had to be extracted and the resultant extracts combined into a single sample. The use of several extraction thimbles, versus larger sample size, was necessary because of instrumental constraints when using liquid trapping. (The maximum thimble size for the SFX 3560 is 10 mL.) An alternative method of extraction, using solid phase trapping in tandem with liquid trapping was also employed, as described in Section 5.1.5. The results of these analyses, shown in **Table 5-6**, indicate that the liquid trapping alone results in higher

Table 5-5: The Effect of Surface Area on the Extraction of Fatty Acid Methyl Esters from a Bleached Softwood Pulp Sample[a]

	STRIPS	GROUND
C_{10} FAME	2.4 (8)[b]	21 (9)
C_{12} FAME	1.7 (9)	14 (7)
C_{14} FAME	2.7 (6)	32 (8)
C_{16} FAME	4.7 (5)	45 (8)
C_{18} FAME	4.3 (8)	51 (6)

[a]Concentration in μg /10 g sample.
[b]Numbers in parentheses are % relative standard deviation, n=3.

Table 5-6: Comparison[a] of Saturated Fatty Acid Methyl Ester Concentrations in a Bleached Softwood Pulp Extract When Using Liquid Trapping and Tandem (Solid/Liquid) Trapping

	Liquid Trapping	**Tandem Trapping**
C_{10} FAME	21 (9)[b]	-
C_{12} FAME	14 (7)	-
C_{14} FAME	32 (8)	-
C_{16} FAME	45 (8)	43 (14)
C_{18} FAME	51 (6)	32 (16)
C_{20} FAME	40 (12)	21 (5)

[a]Concentrations in µg of FAME/10 g sample.
[b]Numbers in parentheses represent % relative standard deviation, n=3 for liquid trapping, n=2 for tandem trapping.

concentrations of the targeted saturated FAMEs in the extracts. Most pronounced is the absence of the C_{10}, C_{12}, and C_{14} FAMEs in the tandem trapping extract. This probably is a result of the higher temperature (ambient) of the liquid methanol trap in comparison to the thermostatted trap on the SFX 3560. In the tandem trapping experiment the only additional pressure on the trap results from a wax film seal surrounding the inlet tubing. The pressurization on the SFX 3560 has been shown (Chapter 3) to decrease losses of even relatively non-volatile compounds such as tetracosane, especially at near ambient conditions.

If paired t-testing is performed on the mean concentrations of the C_{16}, C_{18}, and C_{20} FAMEs, there is not a statistically significant difference between the two trapping methods at the 95% confidence level.

5.2.7 Acetylation of Wood Pulp Extract

Because of the appearance of hydroxy carboxylic acids in the original, non-derivatized SF extracts (retention times of 12.62, 13.65, and 24.17 minutes in **Table 5-2**), attempts to perform *in-situ* acetylations were made. As previously mentioned, the presence of these highly polar acids is theorized to hinder further processing of the wood pulp into cellulose esters, especially cellulose acetate. When *in-situ* acetylations were performed subsequent to the *in-situ* methylations no additional peaks of quantifiable nature were noted, but various siloxanes, attributed to chromatographic column degradation, were noted. However, on several separate occasions, when analyzing both softwood and hardwood extracts, peaks identified as β-D-glucopyranose, pentaacetate (match quality =86) and α-D-glucopyranoside, tetraacetate (match quality=91) (shown in **Figure 5-7**) were evident, as seen in the chromatogram and mass spectra in **Figures 5-8 and 5-9**. These results were noted when the acetylation

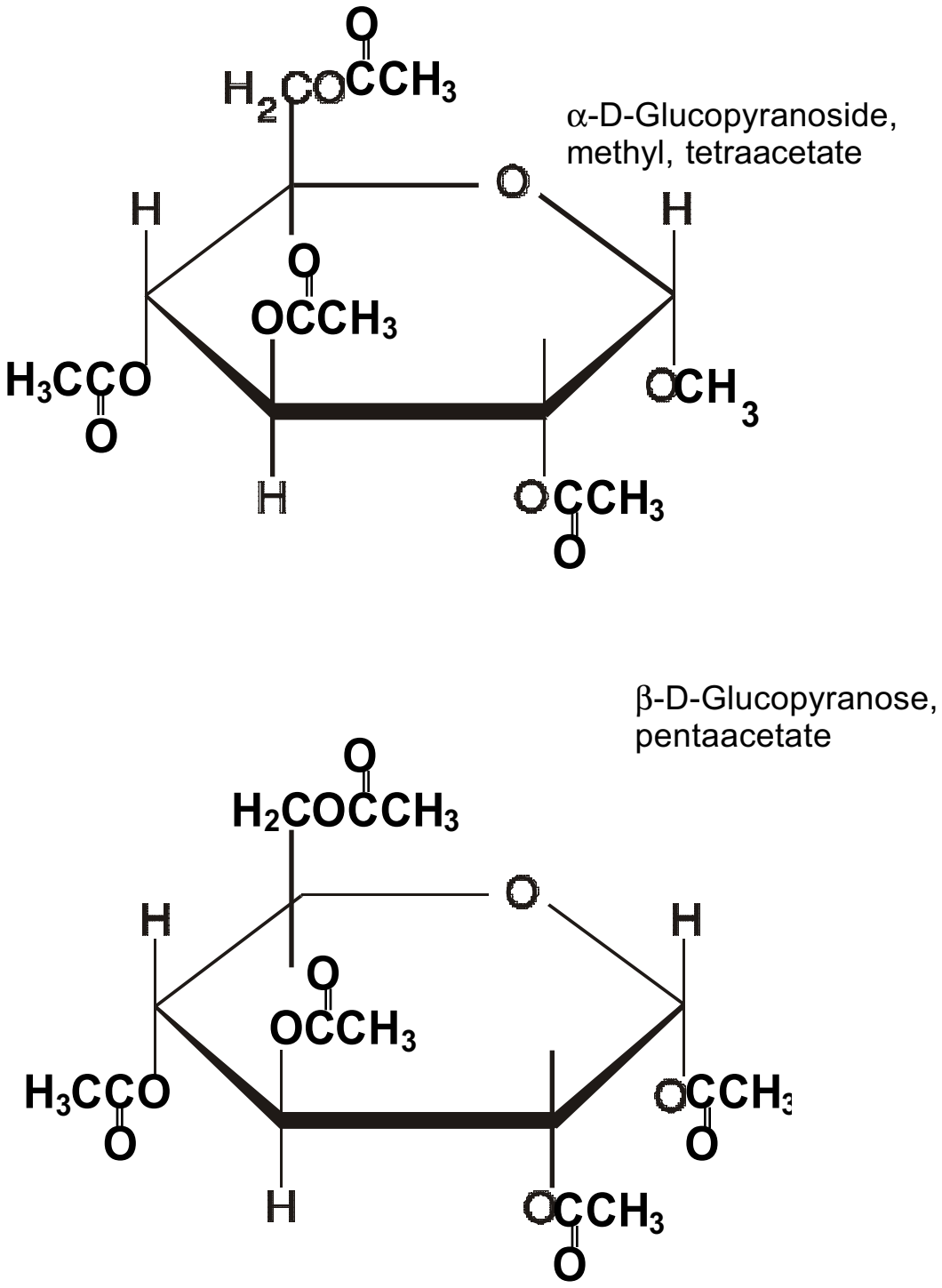


Figure 5-7: Structures of acetylated products identified in wood pulp extracts following *in-situ* methylation and acetylation when the acetylation products were added only to the top of the extraction thimble.

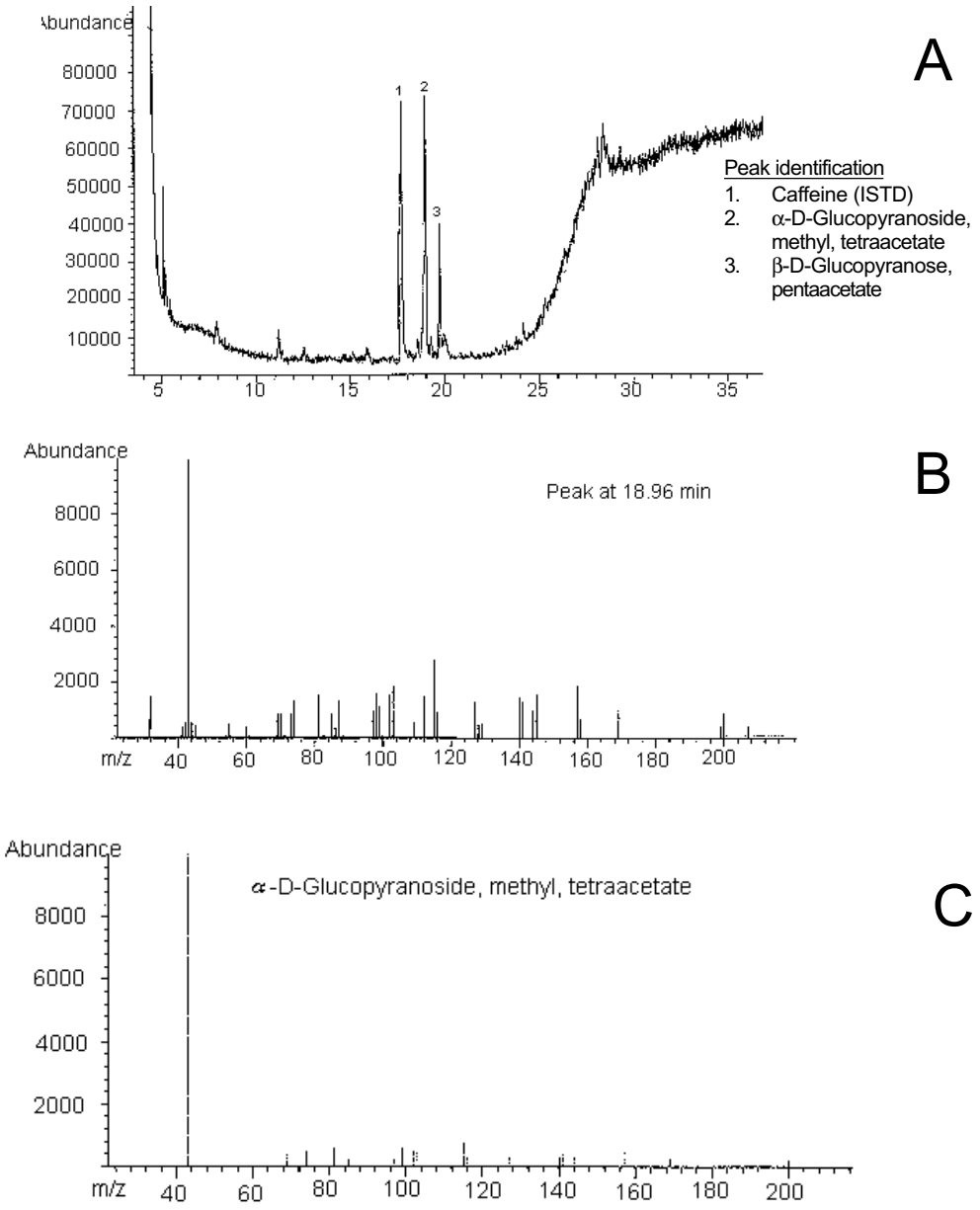


Figure 5-8: A.Total ion chromatogram of a softwood pulp extract following *in-situ* acetylation; B. Mass spectrum of the chromatographic peak eluting at 18.96 minutes; C. Standard mass spectrum of α-D-glucopyranoside, methyl, tetraacetate Chromatographic conditions in text.

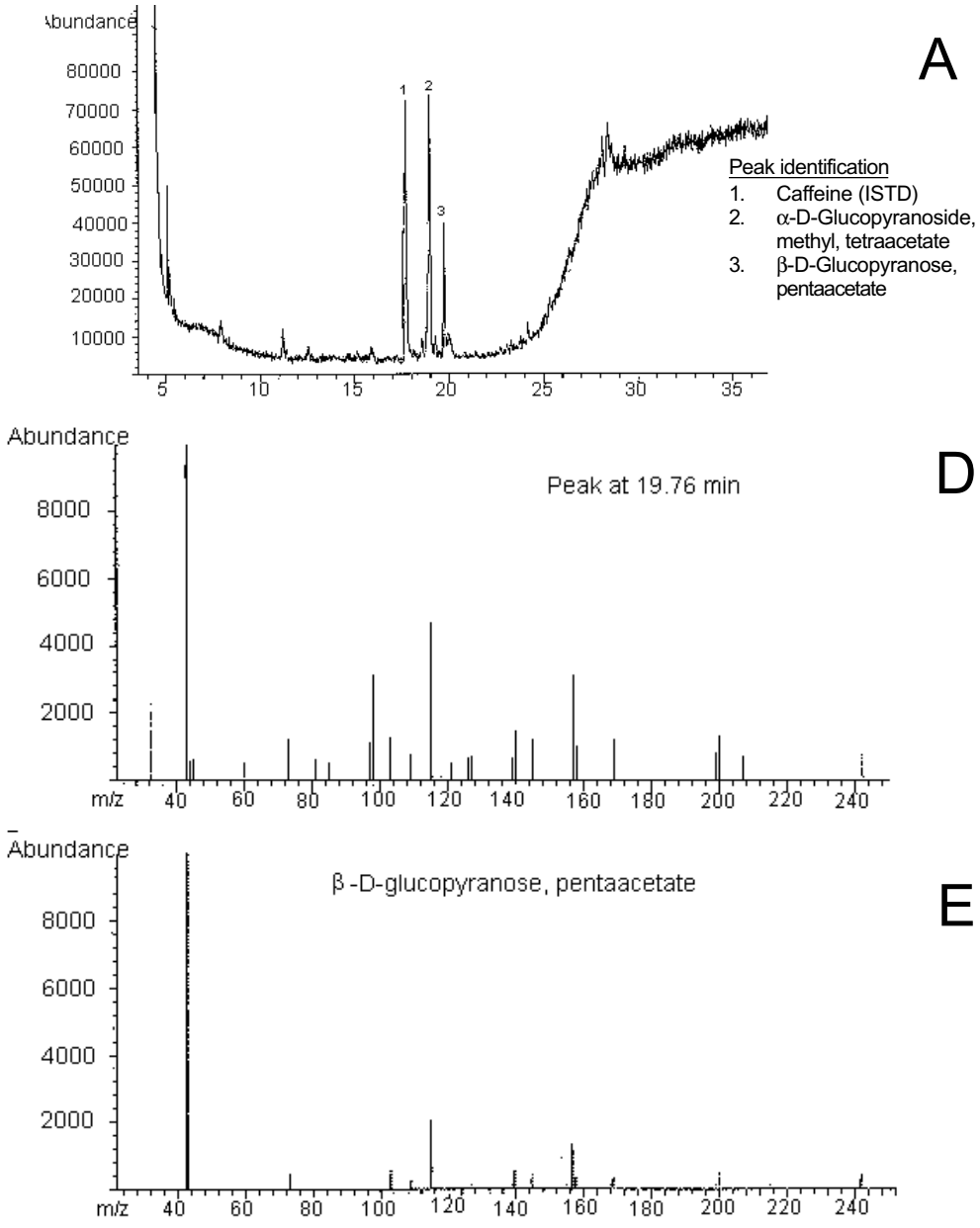


Figure 5-9: A.Total ion chromatogram of a softwood pulp extract following *in-situ* acetylation; D. Mass spectrum of the chromatographic peak at 19.76 minutes; E. Standard mass spectrum of β-D-glucopyranose, pentaacetate. Chromatographic conditions in text.

reagents had been added to the top of the extraction thimble(s) as a plug, instead of distributed throughout. This could easily result from the base catalyzed decomposition of cellulose into glucose units, which were then acetylated. Very noticeable in these chromatograms was the evidence of pyridine and column degradation products, since no clean-up steps were taken after the acetylations were performed. When acetylations were performed off-line column degadation products were minimized, but again no acetylated products were evident in the extract.

Because of the higher degree of column degradation when performing *in-situ* acetylation versus *in-situ* methylation, and the relative absence of any pertinent information in the resultant chromatograms, *in-situ* acetylation was abandoned and only the *in-situ* methylations were used for the comparison of wood pulp extracts.

5.2.8 Quantitation of FAMEs in Various Wood Pulp Samples

At this point, attempts were made to compare the amounts of extracted saturated FAMEs in several softwood and hardwood samples. The results for both the softwood and the hardwood samples are presented in **Table 5-7**. Results for the softwood samples indicate that the C_{16} and C_{18} FAMEs are the most prevalent in all of the samples. Sample SWD-1 has the greatest amount of the C_{10}, C_{12}, and C_{14} FAMEs, and the highest level of overall extractable FAMEs. Only samples SWD-1 and SWD-2 contained the C_{10} FAME, and none of the samples showed the presence of either the C_{22} or C_{24} FAMEs. The hardwood sample results showed the presence of the higher molecular weight FAMEs C_{22} and C_{24}) in all four extracted samples. The most striking result from these analyses was the large difference in the amount of methyl tetracosanoate between the A hardwood samples and the B samples. The A samples averaged about 30 µg/10 g sample, while the B samples averaged almost ten times that amount. The overall level of these extractable materials

Table 5-7: Concentration[a] of Saturated Fatty Acid Methyl Esters (FAMEs) Extracted from Bleached Softwood and Hardwood Pulp Samples

Softwood Samples

	SWD-1	SWD-2	SWD-3	SWD-4
C_{10} FAME	21 (9)[b]	1 (10)	-	-
C_{12} FAME	14 (7)	3 (14)	10 (9)	4 (12)
C_{14} FAME	32 (8)	10 (9)	14 (8)	21 (8)
C_{16} FAME	45 (8)	39 (5)	28 (7)	46 (6)
C_{18} FAME	51 (6)	31 (6)	28 (5)	32 (9)
C_{20} FAME	23 (12)	20 (11)	16 (10)	21 (12)
C_{22} FAME	-	-	-	-
C_{24} FAME	-	-	-	-

Hardwood Samples

	AHWD-1	AHWD-2	BHWD-1	BHWD-2
C_{10} FAME	1 (18)	-	1 (23)	-
C_{12} FAME	2 (18)	3 (12)	2 (11)	1 (18)
C_{14} FAME	2 (15)	3 (15)	2 (14)	2 (9)
C_{16} FAME	15 (9)	21 (7)	35 (8)	40 (8)
C_{18} FAME	15 (13)	15 (10)	15 (9)	14 (7)
C_{20} FAME	6 (9)	4 (15)	12 (10)	11 (10)
C_{22} FAME	12 (12)	8 (12)	72 (7)	62 (8)
C_{24} FAME	35 (10)	29 (9)	314 (8)	278 (6)

[a]Concentrations in µg/10 g sample.
[b]Numbers in parentheses represent % relative standard deviation, n=3.

was about five times higher in the B samples than in the A samples. Whether these differences in the extracted material led to any differences in processing of the pulp samples was unknown.

5.3 Conclusions

The objectives of this work were 1) to develop an SFE method to determine extractive material in wood pulp samples, 2) to compare results with those obtained by Soxhlet extraction with methylene chloride, and 3) to separate and quantitate as many extracted compounds as practical. A reproducible SFE method was developed, though exhaustive extraction of the wood pulp samples was not achieved by this method. It was found that SFE produced methyl esters of fatty acids, not seen in the Soxhlet extraction, but that these FAMEs reverted to the FFAs when the solvent was evaporated to dryness. Reconstitution of the extract in methanol did not yield the FAMEs, but instead the FFAs, more than likely due to the reversible nature of the methylation reaction and the removal of the acid catalyst (CO_2) from the solution. The Soxhlet extraction tended to extract more polar materials than the corresponding SFE method. From the early work indicating the majority of the extractable material was fatty acids, both off-line and *in-situ* methylations were performed on the samples. Off-line methylation of the extract allowed detection of greater amounts of C_{10}, C_{12}, C_{16} and C_{18} saturated FAMEs. However, when compared to *in-situ* derivatizations a four-fold increase in the C_{16} and C_{18} FAMEs was seen. This is more than likely due to both the decrease in polarity (and higher solubility in the extraction fluid) of the FAME as compared to the FFA, and a modification of the sample matrix, decreasing sample-matrix interactions and allowing extraction into the bulk fluid. The *in-situ* methylation

also allowed dimethyl esters of the diacids to be extracted, which was not seen in the off-line methylations.

Sample preparation of the wood pulp was found to be important, with roughly a ten-fold increase in the amount of saturated FAMEs extracted when comparing ground samples to sample strips for a bleached softwood pulp. The trapping method was also seen to affect the amount of FAMEs in the extract. A tandem trapping method utilizing a solid phase trap followed by an ambient, non-pressurized liquid trap resulted in lower recoveries of the lower molecular weight (C_{10}, C_{12}, and C_{14}) FAMEs. This is more than likely due to the lack of temperature and pressure control of the liquid portion of the tandem trap. There were no significant differences in the C_{16}, C_{18}, and C_{20} FAMEs recoveries.

In-situ acetylation of the previously extracted (and derivatized with BF_3/methanol) samples resulted in extraction of the penta-acetylated pyranose and tetra-acetylated pyranoside. Off-line acetylation of the same samples showed no additional components being extracted. The *in-situ* method did cause degradation of the chromatographic column, and would not really be suitable for routine use. Finally, the amount of saturated FAMEs in several softwood and hardwood pulp samples was determined. Rather large differences were noted between the A and B hardwood samples, especially for the C_{22} and C_{24} FAMEs. Lesser differences were noted for the different softwood samples. It is unknown whether there were any processing difficulties with any of these wood pulp samples.

CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

6.0 Introduction

It was the objective of this body of work to attempt to elucidate the process of liquid trapping with direct restrictor immersion. Several different studies addressed the role of the physical properties of both pure and modified collection solvents upon the trapping efficiencies of analytes of similar solubilities and polarities, as well as those differing in polarity and solubility. The potential reactivity of liquid solvent traps was also investigated via the formation of fatty acid methyl esters from their corresponding fatty acids, both during the supercritical fluid extraction and trapping processes. Finally, the application of the previous work for the successful SFE and trapping of extractable fatty acids from wood pulp samples was performed. This work compared solid/liquid (tandem) trapping to liquid trapping alone, as well as comparing fatty acid derivatization methods.

6.1 The Influence of Collection Solvent Physical Properties

This work, presented in Chapter 2, involved four full factorial experiments using pure carbon dioxide as the extraction fluid. A full factorial experiment varies only one parameter at a time. The goal of this study was to investigate the effect of five trapping parameters on the collection efficiencies of several fat-soluble vitamins, given a fixed set of extraction conditions. The use of these vitamins, similar in polarity and solubility, allowed the direct measurement of the effect of the parameters studied.

The major conclusion of this work was that the physical properties of a collection solvent used after supercritical fluid extraction greatly influenced trapping efficiency. In the case of these fat soluble vitamins of similar polarities and solubilities changing the trapping parameters of extraction flow rate, restrictor temperature, collection temperature and collection pressurization had less effect on the trapping efficiencies than did changing the solvent. The major physical properties that influence liquid trapping efficiencies were found to be the viscosity and surface tension as predicted by gas-fluid dynamics. From the results of this study, the influence of modifiers to the collection solvent on the trapping efficiencies of analytes of varying solubilities was undertaken.

6.2 The Influence of Collection Solvent Modifiers

This work, presented in Chapter 3, involved adding a collection solvent modifier to the collection solvent when using pure carbon dioxide as the extraction fluid. The target analytes consisted of a mixture of semi-volatile and non-volatile compounds of varying polarities, commonly used for trapping studies. In this work the goal was to investigate, given a fixed set of extraction conditions, the effect of the addition of a modifier to the collection solvent on the collection efficiencies of these test compounds. The addition of a modifier to the collection solvent induced small changes in the physical properties of the solvent, and allowed prediction of trapping differences that would result from the use of non-modified CO_2 versus modified CO_2. It was also shown that the addition of the collection solvent modifier could help to overcome a common instrumental constraint, lack of collection temperature control, but was unable to compensate for a lack of pressurization.

In agreement with the previous work from Chapter 2, the choice of the collection solvent was found immensely important in achieving effective liquid trapping of analytes. The addition of a modifier to the collection solvent allowed for the use of higher trapping temperatures to trap semi-volatile and non-

volatile analytes. This result indicated that trapping systems cou;d be operated under ambient conditions instead of sub-ambient temperatures. Collection pressurization makes a significant difference at higher collection temperatures, which is most profound when trapping more volatile analytes in a more volatile solvent. This effect unfortunately could not be overcome by the addition of a collection solvent modifier, and thus pressurization was highly recommended.

Changing the viscosity and surface tension of the collection solvent can also change trapping efficiencies. This is an important point that must be considered when trapping in a pure collection solvent after extraction has been performed with a modified fluid. The collection efficiency will change with the addition of the modifier, which can either be beneficial or detrimental to the collection process. This indicates that the choice of SFE trapping parameters can be as important as the choice of extraction parameters.

6.3 Methylation Reactions during the Liquid Trapping Process

The objective of this work, presented in Chapter 4, was to investigate the methylation of decanoic acid occurring during the supercritical fluid extraction process. The methylation was found to occur primarily during the collection process and was greatly enhanced (reaction rate increased almost ten-fold) by the presence of an acid catalyst (i.e. additional to any carbonic acid formed from the CO_2 and residual water.) Increasing reaction time and collection temperature also increased the conversion rate to the methyl ester, but very little in comparison to the catalyzed reaction. This work indicated that extraction and derivatization can be performed simultaneously for subsequent chromatographic analysis.

6.4 The Analysis of Wood Pulp

The objectives of this work, presented in Chapter 5, were to develop an SFE method to determine extractive material in wood pulp samples, to compare results with those obtained by Soxhlet extraction with methylene chloride, and to separate and quantitate as many extracted compounds as practical. A reproducible SFE method was developed, though exhaustive extraction of the wood pulp samples was not achieved by this method. It was found that SFE produced the methyl esters of fatty acids (FAMEs), not seen in the Soxhlet extraction, but that these FAMEs reverted to the FFAs when the solvent was evaporated to dryness. Reconstitution of the extract in methanol did not yield the FAMEs, but instead the FFAs, more than likely due to the reversible nature of the methylation reaction and the removal of the acid catalyst (CO_2) from the solution. The Soxhlet extraction tended to extract more polar materials than the corresponding SFE method. From the early work indicating the majority of the extractable material was fatty acids, both off-line and *in-situ* methylations were performed on the samples. Off-line methylation of the extract allowed detection of greater amounts of C_{10}, C_{12}, C_{16} and C_{18} saturated FAMEsin comparison to the underivatized SF extract. However, when off-line derivatizations were compared to *in-situ* derivatizations, a four-fold increase in the C_{16} and C_{18} FAMEs was seen. This is more than likely due to both the decrease in polarity (and higher solubility in the extraction fluid) of the FAME as compared to the FFA, and a modification of the sample matrix, decreasing sample-matrix interactions and allowing extraction into the bulk fluid. The *in-situ* methylation also allowed the dimethyl esters of the diacids to be extracted, which was not seen in the off-line methylations.

Sample preparation of the wood pulp was found to be important, with roughly a ten-fold increase in the amount of saturated FAMEs extracted when comparing ground samples to sample strips for a bleached softwood pulp.

The trapping method was also seen to affect the amount of FAMEs in the extract. A tandem trapping method utilizing a solid phase trap followed by an ambient, non-pressurized liquid trap resulted in lower recoveries of the lower molecular weight (C_{10}, C_{12}, and C_{14}) FAMEs. These lower recoveries are more than likely due to the lack of temperature and pressure control of the liquid portion of the tandem trap. There were no significant differences in the C_{16}, C_{18}, and C_{20} FAMEs recoveries.

In-situ acetylation of the previously extracted (and derivatized with BF_3/methanol) samples resulted in extraction of the penta-acetylated glucose. Off-line acetylation of the same samples showed no additional components being extracted. The *in-situ* method did cause degradation of the chromatographic column, and would not really be suitable for routine use. Finally, the amount of saturated FAMEs in several softwood and hardwood pulp samples was determined. Rather large differences were noted between the A and B hardwood samples, especially for the C_{22} and C_{24} FAMEs. Lesser differences were noted for the different softwood samples.

6.5 Future Work

Despite years of study, the approach to trapping, either solid, liquid or tandem, after supercritical fluid extraction appears to be one of a best guess, with little understanding of the factors involved in the decompression. However, successful trapping is frequently achieved for all three trapping methods. Future work could focus on the actual descriptive process of liquid trapping, along with quantitation of the factors involved using gas-fluid dynamics.

Since the level of methylation seen as a result of liquid trapping in a methanol-containing solvent is low, this should present very little experimental problems, but a study to correlate pK_a to the methylation rate or degree would be interesting and informative.

Lastly, in the case of wood pulp extraction and analysis, the major area to be addressed should be that of sample size, so that additional extracted components could be accurately quantified.

APPENDIX ONE

STATISTICAL CALCULATIONS

In Chapters 2 and 5 statistical treatments of data are reported. Calculations were performed with Microsoft Excel 97©, with the assumption made that all tested samples were normally distributed.

1. Analysis of Variance (ANOVA)

Analysis of variance is a powerful statistical tool that can be used to separate and measure different causes of variation[64]. The null hypothesis states that each source of variance is equal to the other (For Chapter 2 work: $\sigma^2_{\text{trapping method}} = \sigma^2_{\text{collection solvent}} = \sigma^2_{\text{overall variance}}$). The alternate hypothesis is that all sources of variance are *not* equal. In two-way ANOVA, each measurement is classified according to two factors, *i* and *j*, which correspond to the trapping method and collection solvent, respectively. There are N measurements, divided between r trapping methods (or blocks) and c collection solvents (or treatment levels). Column totals and row totals are also needed for the calculations. The general calculation form for this testing is as follows:

[64] *Statistics for Analytical Chemistry;* J. C. Miller and J. N. Miller; Ellis Horwood Limitedl; West Sussex, England (1993) ,3rd edition.

	TREATMENT						
	1	2	….	*j*	….	C	Row Total
Block 1	$X_{1,1}$	$X_{1,2}$	….	$X_{1,j}$	….	$X_{1,c}$	$T_{row\ 1}$
Block 2	$X_{2,1}$	$X_{2,2}$	….	$X_{2,j}$	….	$X_{2,c}$	$T_{row\ 2}$
.	….	….	….	….	….	….	.
.	….	….	….	….	….	….	….
Block *i*	$X_{i,1}$	$X_{i,2}$	….	$X_{i,j}$	….	$X_{i,c}$	$T_{row\ i}$
.	….	….	….	….	….	….	….
Block r	$X_{r,1}$	$X_{r,2}$	….	$X_{r,j}$	….	$X_{r,c}$	$T_{row\ r}$
Column Total	$T_{column\ 1}$	$T_{column\ 2}$	….	$T_{column\ j}$	….	$T_{column\ c}$	T=grand total

To determine the F statistic, the sums of squares, degrees of freedom and mean squares must all be calculated. (The mean squares are the estimates of the variances.)

Source of Variation	Sum of Squares	Degrees of freedom	Mean square
Between treatment	$\sum_{j=1}^{c} T_{.j}^2/r - T^2/N$	c – 1	Sum of squres/c-1
Between block	$\sum_{i=1}^{r} T_{i.}^2/c - T^2/N$	r – 1	Sum of squares/r-1
Residual	By subtraction	By subtraction	Sum of squares/(N-c-r-1)
Total	$\sum_i \sum_j x_{ij}^2 - T^2/N$	N – 1	

The F statistic is then calculated from the following and compared to the tabulated value for F. If the F_{calc} is greater than the F_{table} then the null hypothesis is rejected and the alternate hypothesis accepted.

$$F_{treatment} = \frac{\text{mean square}_{\text{between treatment}}}{\text{mean square}_{\text{residual}}}$$

and

$$F_{block} = \frac{\text{mean square}_{\text{between block}}}{\text{mean square}_{\text{residual}}}$$

2. Paired t-testing

After determining, from ANOVA testing, that there is a difference in the variances, paired t-testing was performed to determine if the means differed significantly. In this test the null hypothesis is that the means are equal (that is, their difference is zero), while the alternate hypothesis is that the means are not equal. The t value is calculated from the following equation[65]:

$$t_{calc} = \frac{\overline{x_d} - 0}{s_d \sqrt{n}}$$

where $\overline{x_d}$ = the difference between corresponding means, $\overline{x_1} - \overline{x_2}$

s_d = the standard deviation of the differences

n = the number of pairs of data

[65] *Introduction to Probability and Statistics*; W. Mendenhall; Duxbury Press; North Scituate, MA (1979) 5th edition.

If the $|t_{calc}|$ is greater than the t_{table}, the null hypothesis is rejected and the alternate hypothesis is accepted. The sign of the t_{calc} indicates whether data set 1 (positive t_{calc}) or data set 2 (negative t_{calc}) had the higher mean value.

VITA

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