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NATIONAL COUNCIL OF THE PAPER INDUSTRY FOR AIR AND STREAM IMPROVEMENT, INC., 260 MADISON AVENUE, NEW YORK, N.Y. 10016

# PROCEDURES FOR THE ANALYSIS OF RESIN AND FATTY ACIDS IN PULP MILL EFFLUENTS

TECHNICAL BULLETIN NO. 501

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NATIONAL COUNCIL OF THE PAPER INDUSTRY FOR AIR AND STREAM IMPROVEMENT, INC.
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PROCEDURES FOR THE ANALYSIS OF
RESIN AND FATTY ACIDS IN PULP MILL EFFLUENTS

The continued refinement and improvement of analytical methods for the analysis of organic compounds of interest to the forest products industry is an important part of the National Council program. The attached technical bulletin summarized recent studies on methodology for the analysis of resin acids, fatty acids and chlorinated resin acids in pulp industry wastewaters and describes both GC/FID and GC/MS analysis procedures.

The methodology improvements incorporated in the new procedures include a refined extraction protocol which allows for better recovery of acid labile resin acids. Derivatization of the extracts is accomplished using triethyloxonium tetrafluoroborate rather than the toxic and hazardous diazomethane used previously. Improved selectivity is accomplished by capillary column chromatography and optimum storage conditions for both wastewater samples and extracts are described.

The GC/FID and GC/MS analysis procedures complement each other; the GC/FID providing a routine analysis procedure and the GC/MS providing a confirmation procedure. Thus, the combination of the two methods should provide useful tools for the monitoring of pulp industry wastewaters and in the evaluation of the environmental significance of these compounds.

The laboratory investigation of these procedures and preparation of the technical bulletin was carried out by Lawrence E. LaFleur, Organic Analytical Programs Manager. He was assisted by Mr. Kenneth Ramage, Research Associate on the West Coast Regional Center staff and Ms. Theresa M. Bousquet, formerly on the West Coast Regional Center staff.

Your comments and questions on the contents of this bulletin are solicited and should be directed to this office or to Mr. LaFleur, NCASI West Coast Regional Center, P.O. Box 458, Corvallis, OR 97339, telephone (503) 754-2015.

Yours very truly,

Russell O. Blosser
Technical Director

ROB:mh
Attach.

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# PROCEDURES FOR THE ANALYSIS OF RESIN AND FATTY ACIDS IN PULP MILL EFFLUENTS

## I INTRODUCTION

The effective evaluation of the environmental significance of resin acids in industrial effluents requires the development of a quality data base. This in turn creates the need for accurate, sensitive and reliable analytical methods. The inevitable complexity of environmental matrices in which these compounds can occur makes this objective difficult to achieve. The analyst attempting to develop analytical methods has a wide choice of isolation, derivitization and chromatographic options available. This has resulted in a number of different approaches to overcome the problem associated with resin acid analyses being reported in the literature. To date, there are no standardized methods for the analysis of resin and fatty acids in pulp mill effluents.

The two main isolation procedures which have been reported include extraction with various organic solvents or absorbtion on XAD resin. Several studies comparing these two approaches have been reported (1,2,3,4) and a discussion of the findings was presented by Voss and Rapsomatiotis (5). The results of these studies indicated certain advantages and limitations to each procedure and neither has emerged as being unambiguously superior.

The next option the analyst has is the selection of derivitization procedure. Zinkel, Lathrop, and Zank (6) have described the preparation of trimethylsilyl esters, but report that these derivatives are susceptible to hydrolysis and are unstable on polyester or polyethylene glycol liquid phases. Holmbom (7) has described their use in pulp mill effluent analyses for confirmations of identification on an SE-30 column. The advantage of this approach is that one can simultaneously analyze for chlorinated phenols, guaiacols and catechols as well as some non-chlorinated phenols along with the resin and fatty acids; however, Holmbom recommended that the resin and fatty acids be determined as methyl esters on a BDS column. Turner and Wallin (8) reported a similar procedure using TMS derivatives of chlorinated phenolics, fatty and resin acids analysis on a SE-S4 fused silica column. They describe losses of the TMS derivatives of 30 to 73 percent over a period of one month.

Methylation is by far the most common derivitization procedure currently being used. Diazomethane is usually the reagent used for derivitization, but suffers from the disadvantage of being both explosive and toxic thus making it hazardous to handle or store. Other resin acid derivitization procedures which have been reported include tetraalkyl ammonium salt catalysis or pyrolysis (9-12), the use of dimethylformamide-dialkyl acetals (10,13) or, for HPLC analysis, imidazolides via

carbonyldiimidazole (14-18). These latter methods have not seen widespread use.

Capillary column gas chromatography provides the analyst with a powerful tool to resolve the components in the complex matrices typically encountered in environmental analyses and its utility in this respect has been widely recognized. Improvements in instrument design and the advent of fused silica column technology has made this technique more accessible to non-research laboratories.

Capillary chromatographic techniques have been applied extensively to the analysis of fatty and/or resin acids in wood extractives, tall oil, rosin and pulp mill effluents (2,3,5,7,and 19-27). Most of these methods use glass capillary columns and the columns which provide the best separation of all the compounds likely to be encountered are not commercially available. Utilization of these procedures would require the allocation of time and resources to the preparation of the columns, thereby substantially decreasing the convenience. This approach may prove practical for large or research laboratories but probably would not find widespread use in laboratories set up for routine analyses.

In reviewing the available resin acid analytical methods, several shortcomings were identified which could be improved. The ideal procedure for resin acid analysis should: (a) utilize commercially available columns, (b) require the smallest sample volume possible to provide a detection limit of one to two parts per billion, (c) provide a safe yet reliable alternative to diazomethane derivitization, (d) use isolation, derivitization and clean-up procedures which would provide an extract suitable for either FID or GC/MS analysis, and (e) incorporate an in-sample quality control check. The objective then became the development of a routine analysis procedure which satisfied these criteria and provided accurate and reliable data.

The following summarizes efforts to achieve this objective. Data on method precision, linearity, detection limits and recoveries are presented. Once the details of the analytical procedure were suitably refined, studies on the optimum storage and preservation techniques for both samples and extracts were undertaken and the results are reported.

## II METHODS

### A. Materials

Redistilled U.S.P. Grade diethyl ether was used for extractions. Cyclohexane, n-hexane and dichloromethane were purchased from Burdick and Jackson; methanol was redistilled reagent grade (J.T. Baker). Diazomethane was prepared from Diazald (Aldrich) using the procedure described by the supplier (28). Triethylox-

onium tetrafluoroborate (Fluka) was used as supplied with no further purification. Diisopropylethylamine was obtained from Aldrich and was redistilled prior to use. Potassium chloride was baked at 400°C overnight before use. Woelm 100-200 μ silica-gel (activity grade 1) (K & K) and powdered sodium sulfate (J.T. Baker) were stored at 130°C prior to use. Azulene (Aldrich) and Sudan I (MCB) were used as supplied.

B. Reference Standards

Isopimaric, sandracopimaric, neoabietic, palustric, levopimaric, dichlorodehydroabietic, chlorodehydroabietic (mixture of 12- and 14-chloro isomers) and 7-oxodehydroabietic acids were obtained from B.C. Research (Vancouver, British Columbia). Pimaric acid was obtained from Chemical Procurement Labs (College Point, New York). Dehydroabietic acid was obtained from Pfaltz & Bauer (Stanford, Connecticut) and was purified via its 2-aminoethanol salt as described by Halbrook and Lawrence (29). Abietic acid was prepared by isomerizing levopimaric acid by the method of Schuller et al., (30). Oleic and linoleic acids were obtained from Nu-Check Prep, Inc. (Elysian, Minnosota). n-Heptadecanoic acid was obtained from J.T. Baker. O-Methylpodocarpic acid was prepared by the saponification of methyl-O-methylpodocarpate (Aldrich) as described in Appendix A. n-Propyl dehydroabietate was prepared from dehydroabietic acid by first making the acid chloride in a manner analagous to that used by Campbell and Todd (31). The acid chloride was then reacted with anhydrous n-propanol. Details are described in Appendix B.

C. Extraction Procedure

After allowing the sample to equilibrate to room temperature and shaking thoroughly to resuspend any solids which may have settled during storage, a 250 mL aliquot was withdrawn. The sample was then spiked with a methanolic solution of the surrogate compounds, n-heptadecanoic acid and O-methylpodocarpic acid. This portion was then adjusted to pH 2 $\pm$ 0.2 with 6M $H_2SO_4$ and was transferred to a 500 mL separatory funnel and extracted with diethyl ether (1x75 mL, 3x50 mL). One minute of vigorous shaking was followed by 15 to 20 minutes settling time. The emulsions which formed were broken by centrifugation in screw capped centrifuge tubes to minimize evaporation of the diethyl ether.

The combined diethyl ether phases were concentrated to approximately 2 to 4 mL in Kuderna-Danish (K-D) evaporators fitted with 3-ball Snyder columns. Diethyl ether washes (2x2 mL) of the Snyder column and K-D apparatus were combined with the sample extract in the receiving tube and were reconcentrated to 2 to 3 mL after fitting the tube with a Micro-Snyder column.

D. Derivitization

Prior to analysis, the ethyl ester derivatives of the resin

and fatty acids had to be prepared. Thus, the extracts were transferred to 16 mm x 125 mm screw capped culture tubes with Teflon-lined caps and concentrated to 200 to 300 µL with a stream of dry nitrogen. If the extract contained methanol, sufficient cyclohexane was added prior to concentration in order to form a mixture which would allow for the removal of the methanol by azeotropic distillation. Approximately 1 mL of a 1M solution of diisopropylethylamine in dichloromethane was added to the extract followed by approximately 1 mL of a 1M solution of triethyloxonium tetrafluoroborate in dichloromethane. This mixture was immediately shaken for 30 seconds. One milliliter of a saturated KCl solution was added and the tube was again capped and shaken. One milliliter of hexane was added and the tube was capped and shaken for an additional minute. If emulsions formed the tube was centrifuged. The organic phase was withdrawn and the hexane extraction step was repeated twice with 1 mL portions of hexane. The organic phases were combined and concentrated to the desired volume with a stream of dry nitrogen. The derivatized extract thus prepared was suitable for capillary GC/MS analysis.

E. Silica-Gel Clean-Up

If the sample was to be analyzed by capillary GC/FID, a silica-gel clean-up as described in Appendix D of Technical Bulletin No. 281 (32) was required. Thus, the column was constructed by joining a 10 cm length of 6 mm i.d. glass tubing to the bottom of a 10 cm test tube and tapering the end of the tube to a small orifice. A small plug of analytical filter pulp (Schleicher & Scheull, Inc. No. 289) was gently pushed to the bottom of the glass column to retain the silica-gel. The column was then packed with 4 cm of activated silica-gel with gentle tapping of the side to help settling. A 1 cm layer of anhydrous powdered sodium sulfate was placed on top of the silica-gel. The columns were stored in a drying oven at 130°C for a minimum of 8 hours until just prior to use.

The clean-up of an ethylated extract involved concentrating to ca. 200 to 300 µL with a stream of dry nitrogen and then spiking with 20 µL of a solution consisting of 500 ng/µL Sudan I and 7.5 µg/µL azulene in n-hexane. The prepacked column was removed from the oven and allowed to cool to room temperature. The column was then prewashed with 1 mL of n-hexane. When the n-hexane reached the top of the sodium sulfate layer, the sample was added to the column. The sample tube was rinsed with a 200 to 300 µL wash of n-hexane and this was added to the column when the original sample drained to the top of the sodium sulfate. The sample tube was further washed with 200 to 300 µL of 95:5 n-hexane:diethyl ether and this was also added to the column. When the solvent from this final rinse reached the top of the sodium sulfate, the column was filled with 95:5 n-hexane:diethyl ether and was kept above the sodium sulfate throughout the remainder of the procedure.

The eluant was collected in 15 mL centrifuge tubes. The

receiving tube was changed just prior to the elution of the blue azulene band. Collection of the eluant was continued until just prior to the orange colored Sudan I band. This fraction which contains the ethyl esters was typically between 6 and 7 mL for a properly packed and activated column. The fraction was then concentrated to the desired volume with a stream of dry nitrogen.

F. GC/MS Analysis

The GC/MS analyses were performed on a Hewlett-Packard Model HP-5993 equipped with a Model 18740B capillary injection port and a specially designed direct coupled capillary interface (designed and constructed by NCASI). The component separation was accomplished using a 30 m x 0.25 mm i.d. fused silica DB-5 column with a 0.25 µ film thickness (J & W Scientific) and helium as the carrier gas. The injection port temperature was 280°C and the oven was programmed from 140°C after a 1 minute hold at 4°C/min to a final temperature of 280°C. A Grob type splitless injection technique with a 30 second purge activation delay was used for all injections. The MS was operated in the repetitive scan mode, scanning from m/z 50 to 400 at a rate of 162.5 AMU/sec and using 70 eV electron impact ionization. The GC/MS was calibrated daily by first tuning the MS on perfluoro-tri-n-butylamine and then running a calibration standard using the conditions described above. Figure 1 shows the total ion chromatogram of a typical resin and fatty acid ethyl ester calibration standard.

Upon completion of the GC/MS analysis, the extracted ion chromatograms for the quantitation ions listed in Table 1 were plotted. Relative response factors for the characteristic ions of each compound were calculated as follows:

$$\text{Relative Response Factor (RRF)} = (A_{IS})(ng_{ai})/(A_{ai})(ng_{IS})$$

where: $A_{ai}$ = area of the characteristic ion of the analyte
$A_{IS}$ = area of the 239 ion of the Internal Standard
$ng_{ai}$ = nanograms of the analyte injected
$ng_{IS}$ = nanograms of the Internal Standard injected.

Sample extracts were analyzed under conditions identical to those described above. Following GC/MS analysis, the extracted ion chromatograms of the quantitation ions were plotted and when a peak occurred at the same relative retention time as observed for the standard compound in the daily calibration standard, the peak was integrated and the area recorded. The concentration of each component detected was calculated as follows:

$$\text{Concentration } (\mu g/L) = (A_{ai}/A_{IS})(RRF)(\text{Conc. IS})$$

where: $A_{ai}$ = area of the characteristic ion of the analyte
$A_{IS}$ = Area of the 239 ion of the Internal Standard
RRF = Relative Response Factor for the ion
Conc. IS = Concentration of the Internal Standard in µg/L

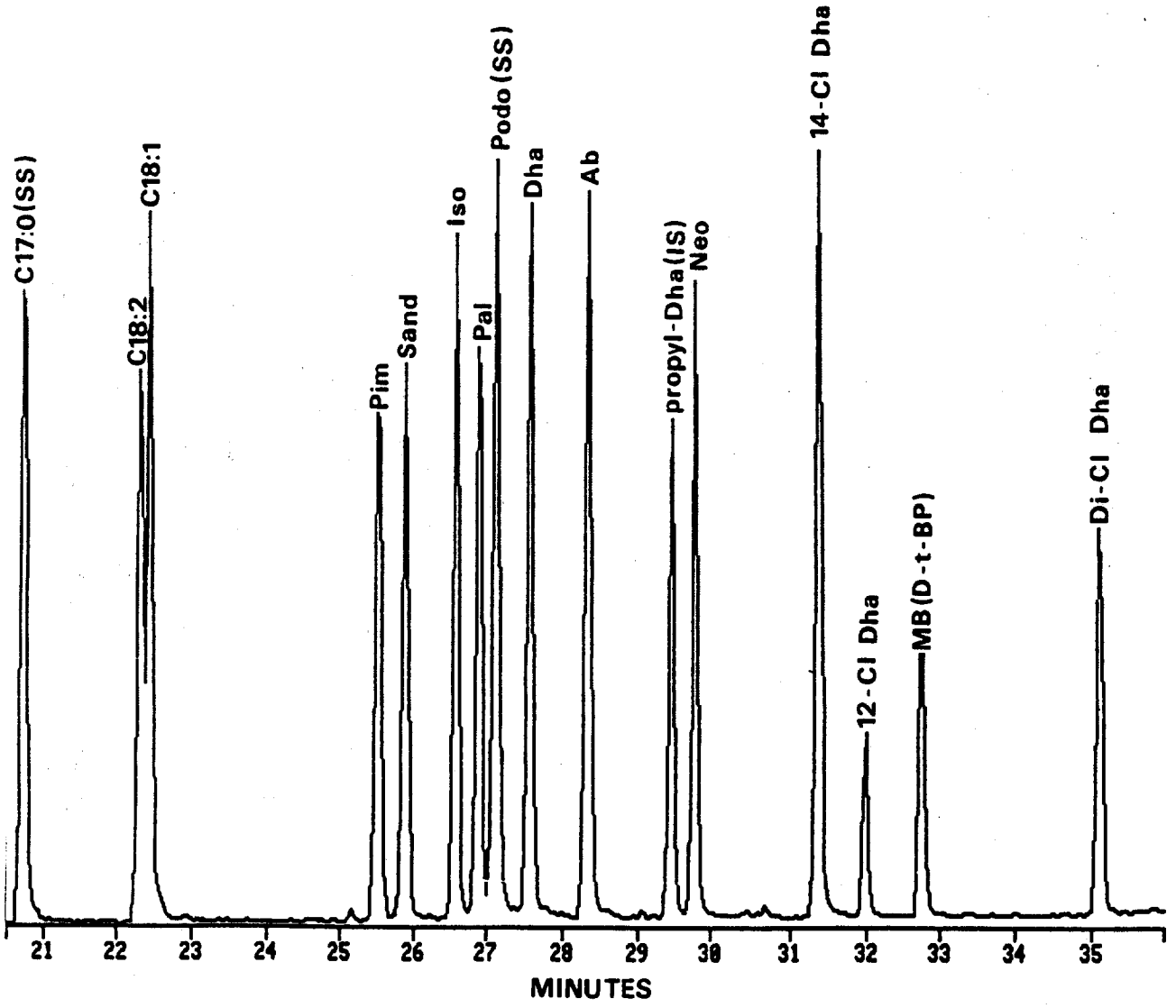



FIGURE 1

GC/MS TOTAL ION CHROMATOGRAM OF RESIN AND FATTY ACID ETHYL ESTERS. CONDITIONS: 140°C (1 MIN) $\xrightarrow{4°/MIN}$ 280°C, INJECTION PORT TEMPERATURE - 280°C, MASS SCAN RANGE m/z = 50 to 400, SCAN SPEED 162.5 AMU/Sec

TABLE 1 GC/MS QUANTITATION IONS

| Compound | Quantitation Ions | Relative Retention Time |
|---|---|---|
| Ethyl Oleate | 55, 264, 310 | .760 |
| Ethyl Linoleate | 55, 67, 308 | .753 |
| Ethyl Pimarate | 121, 330 | .863 |
| Ethyl Sandracopimarate | 121, 330 | .877 |
| Ethyl Isopimarate | 241, 256, 330 | .897 |
| Ethyl Palustrate | 241, 315, 330 | .907 |
| Ethyl Dehydroabietate | 239, 328 | .935 |
| Ethyl Abietate | 241, 256, 330 | .959 |
| Ethyl Neoabietate | 135, 330 | 1.014 |
| Ethyl 14-Chlorodehydroabietate | 273, 275 | 1.068 |
| Ethyl 12-Chlorodehydroabietate | 273, 275 | 1.089 |
| Ethyl 7-Oxodehydroabietate | 253, 342 | 1.096 |
| Ethyl Dichlorodehydroabietate | 307, 309 | 1.202 |
| Ethyl n-Heptadecanoate (SS) | 88, 101 | .699 |
| Ethyl O-Methylpodocarpate (SS) | 227, 316 | .919 |
| n-Propyl Dehydroabietate (IS) | 239 | 1.0 (29.2 min) |

If the agreement in concentration determined from each of the two or three characteristic ions was judged satisfactory (i.e. within ±20 percent) the reported concentration was the mean of the concentrations calculated from the individual ions. If there was any question about the identification of a compound, a background subtracted mass spectrum was obtained and compared to the spectrum obtained from the analysis of reference standards. Appendix C contains reference spectra for the resin acid ethyl esters.

G. GC/FID Analysis

The GC/FID analyses were performed on a Hewlett-Packard Model HP-5840 gas chromatograph using a 30 m x 0.25 mm i.d. fused silica DB-5 column with a 0.25 μ film thickness (J & W Scientific). Hydrogen was used as the carrier gas at a linear velocity of 42 cm/sec (measured at 200°C). The injection port temperature was 280°C, the detector temperature was 320°C and the oven was programmed from 130°C after a 1 minute hold at 6°C/min for 6 minutes, then 2°C/min to a final temperature of 280°C. A Grob type splitless injection technique with a 30 second purge activation delay was used for all injections. Figure 2 shows a chromatogram of a typical calibration standard.

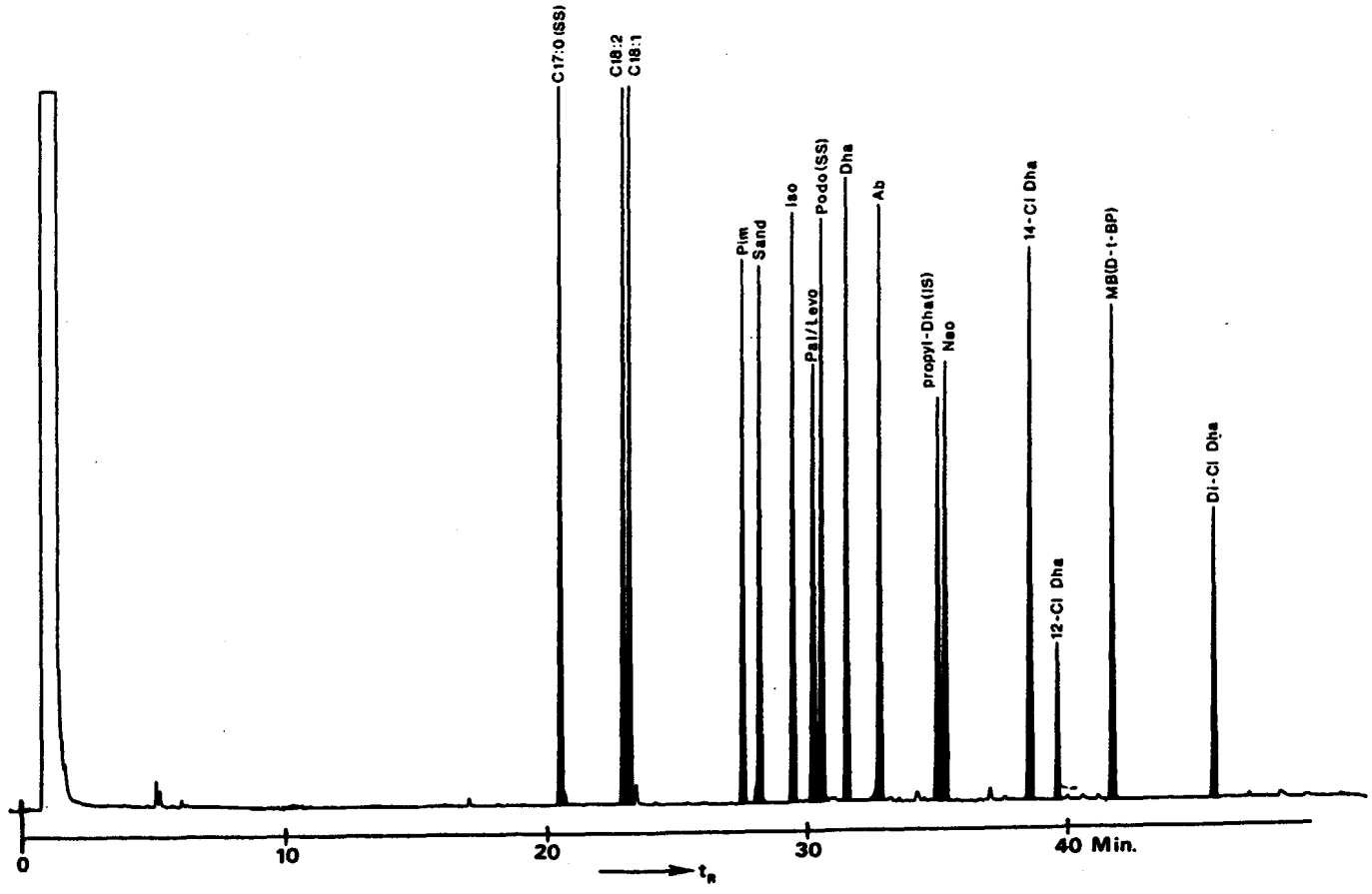



FIGURE 2

FID CHROMATOGRAM OF RESIN AND FATTY ACID ETHYL ESTERS

CONDITIONS: 130°C (1 MIN) 6°C/MIN (6 MIN) 2°C/MIN 280°C

INJECTION PORT 280°C, HYDROGEN CARRIER GAS

The instrument was calibrated prior to use by running a standard using the conditions described above. Relative response factors (RRF) were calculated as follows:

$$RRF_a = (A_{IS})(ng_a)/(A_a)(ng_{IS})$$

where: $A_a$ = area of the analyte peak
$A_{IS}$ = area of the Internal Standard peak
$ng_a$ = nanograms of the analyte injected
$ng_{IS}$ = nanograms of the Internal Standard injected.

The relative response factors were then compared to the criteria summarized in Table 2 to determine if they were acceptable.

TABLE 2 ACCEPTANCE CRITERIA FOR RESIN/FATTY ACID FID RELATIVE RESPONSE FACTORS

| Compounds | Acceptable Range Minimum | Maximum |
|---|---|---|
| Ethyl Oleate | 0.70 | 0.82 |
| Ethyl Linoleate | 0.77 | 0.95 |
| Ethyl Pimarate | 0.77 | 0.89 |
| Ethyl Sandracopimarate | 0.79 | 0.89 |
| Ethyl Isopimarate | 0.79 | 0.90 |
| Ethyl Palustrate | 0.81 | 0.95 |
| Ethyl Dehydroabietate | 0.84 | 0.94 |
| Ethyl Abietate | 0.80 | 0.95 |
| Ethyl Neoabietate | 0.86 | 1.02 |
| Ethyl 14-Chlorodehydroabietate | 1.05 | 1.24 |
| Ethyl 12-Chlorodehydroabietate | 1.01 | 1.20 |
| Ethyl Dichlorodehydroabietate | 1.08 | 1.36 |
| Surrogate Spikes | | |
| Ethyl n-Heptadecanoate | 0.77 | 0.97 |
| Ethyl O-Methylpodocarpate | 0.88 | 1.00 |

Ethylated sample extracts were analyzed as described above and the concentration of any analytes which were detected were calculated as follows:

$$\text{Concentration } (\mu g/L) = (A_a/A_{IS})(RRF_a)(\text{Conc. IS})$$

where: $A_a$ = area of the analyte peak
$A_{IS}$ = area of the Internal Standard peak
$RRF_a$ = Relative Response Factor for the analyte
Conc. IS = Concentration of the Internal Standard in µg/L

## III DISCUSSION OF THE METHOD

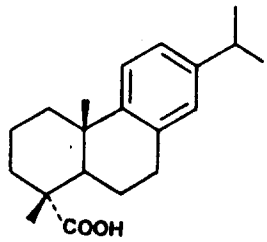
Since the selection of certain elements of the procedure such as column choice and derivitization had a substantial impact on other aspects of the analysis, a decision regarding these choices was required very early in the development of the method. Once this commitment was made, subsequent elements were selected to conform to the needs dictated by the chosen column and derivitization.

Solvent extraction was elected as the first step in the isolation procedure. This was due in part to the fact that most of the EPA approved procedures use solvent extraction and thus most laboratories are familiar with the technique and have the necessary equipment. There was also concern over the ability of porous polymer isolation procedures to reproducibly and quantitatively recover resin acids which are associated with suspended solids (1).

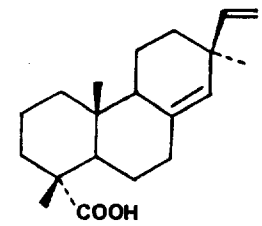
The resin acid stability problems discussed in more detail in Section IV-G precluded the use of dichloromethane as the extraction solvent. Past NCASI experience with diethyl ether (4, 32-34) indicated that diethyl ether extracts were stable and that reproducible and acceptable recoveries could be obtained. Thus, despite the safety problems, it was the preferred solvent. Preliminary studies showed that methyl-t-butylether gives similar results but this substitution has not been thoroughly tested. Voss and Rapsomatiotis (5) recently reported a procedure for the analysis of resin acids in pulp mill effluents which uses this solvent.

n-Heptadecanoic acid was spiked into the samples prior to extraction as a surrogate spike for the fatty acids. O-Methylpodocarpic acid was adopted as a surrogate spike for the resin acids. Although O-methylopodocarpic acid has a methoxyl rather than an alkyl substituent on the C ring and the carboxyl group is epimeric to the normal resin acid configuration, the compound represents a reasonable analogue to the tricyclic diterpene acids, Figure 3. To the best of our knowledge, O-methylpodocarpic acid has not been reported as a constituent of any North American tree species. Thus O-methylpodocarpic acid represents the most readily available model compound for use as a resin acid surrogate.

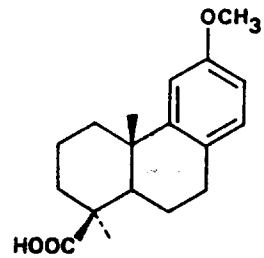
The extraction procedure consisted of acidifying a 250 mL aliquot of the sample to pH 2 and then extracting with diethyl ether. This size sample provided satisfactory detection limits and was quite practical. There was some concern that the acid catalyzed isomerization of neoabietic, palustric and levopimaric acids (35-42) would result in low recoveries but preliminary studies showed that not to be the case. These studies proved to be somewhat optimistic when routine quality assurance recovery spike data developed during the validation of the method (see Section IV-E) indicated that acceptable recoveries of neoabietic




DEHYDROABIETIC ACID




PIMARIC ACID




O-METHYL PODOCARPIC ACID

FIGURE 3

STRUCTURES OF REPRESENTATIVE RESIN ACIDS COMPARED TO THE SURROGATE COMPOUND, O-METHYLPODOCARPIC ACID

and palustric acids could not be consistently achieved, particularly near the detection limit of the method. Although all the method validation data discussed below were developed using the pH 2 extraction procedure, we have since changed the procedure to three extractions at pH 5 followed by two extractions at pH 2. As discussed in Section IV-E, adoption of this modified extraction procedure brought the average recoveries for palustric acid and neoabietic acid up to 79 percent (cv = 9.2 percent) and 81 percent (cv = 10.6 percent) respectively without changing the recoveries of the remaining analytes.

Our objectives included finding a suitable substitute for the use of diazomethane as a derivitizing agent. Consideration of the alternatives led us to select triethyloxonium tetrafluoroborate. As shown in Figure 4, the first step in the derivitization was the formation of the diisopropylethylamine salts of the resin acids by the addition of a 1.0 M solution of the amine in dichloromethane. This enhances the acids reactivity and the excess amine also serves to neutralize the fluoroboric acid formed during the derivitization. Using the highly hindered tertiary amine minimizes its reaction with the derivitizing agent. The esters were then formed by the addition of 1.0 M triethyloxonium tetrafluoroborate in dichloromethane. After shaking for thirty seconds, the reaction was complete and the resin esters were partitioned with saturated potassium chloride and n-hexane to decompose and remove the excess derivitization reagent and reaction by-products.

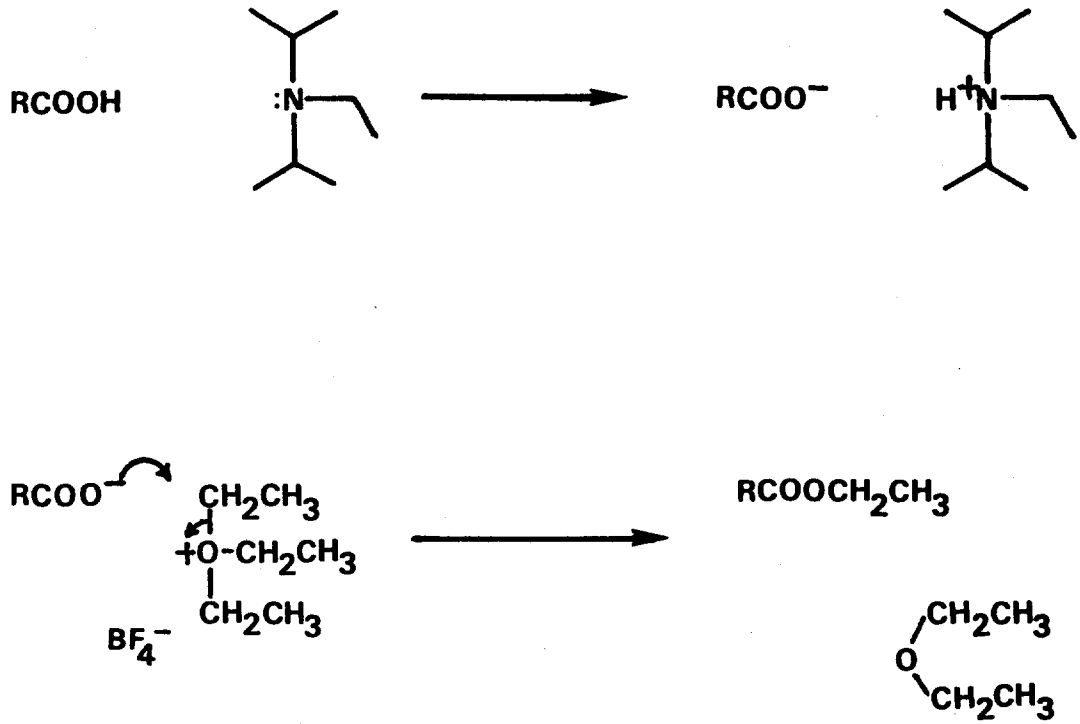



FIGURE 4

REACTION MECHANISM FOR THE ETHYLATION OF ORGANIC ACIDS WITH TRIETHYLOXONIUM TETRAFLUOROBORATE AND DIISOPROPYLETHYLAMINE

Triethyloxonium tetrafluoroborate (TEOTFB) is a slightly hydroscopic crystalline solid. Although the reaction is not sensitive to moisture and the commercially available TEOTFB can be used without further purification, old material which has been opened and stored for long periods of time accumulates water and it may be advisable to dry it. This can be easily accomplished by washing the solid with anhydrous diethyl ether since the TEOTFB is virtually insoluble. After washing, the excess ether can be removed with a stream of nitrogen. Although TEOTFB's stability and crystalline form makes it safer and more convenient to handle than diazomethane, it is still a potent alkylating agent and should be handled with care. Overall, the ethylation procedure is rapid, quantitative, does not degrade any of the analytes and produces no undesirable side products.

The extract obtained from this procedure was suitable for analysis by capillary GC/MS. The compound chosen for use as the internal standard for quantitation was n-propyl dehydroabietate. This ester has a retention time, structure and mass spectral fragmentation pattern which is very similar to the resin acids. The compound was prepared by reacting dehydroabietic acid with thionyl chloride in the presence of pyridine and then treating the resulting acid chloride with anhydrous n-propanol as described in Appendix B.

The analysis was performed by splitless injection on a 30 m DB-5 fused silica capillary. Relative retention time data and the relative areas of two or three characteristic ions per compound shown in Table 1 were used for qualitative identification. If there was any question about the identification, a background subtracted spectrum was obtained and compared against the corresponding spectrum from the daily calibration standard. Since most of the characteristic resin ester fragmentations involve loss of the carboxyl carbon, the spectra of the methyl and ethyl esters are virtually identical. The differences in the molecular ions affords the opportunity to analyze for naturally occurring resin acid methyl esters. Reference spectra of the principle resin acid ethyl esters are shown in Appendix C.

Extracted ion current areas of the ions listed in Table 1 were used for quantitation. Quantitation was accomplished using the internal standard technique using the base peak of n-propyl dehydroabietate as the reference. Concentrations were calculated for each ion and then the average of the two or three values was reported to help minimize errors which might occur if any of the individual ions had a minor interference.

If the final analysis procedure was to be capillary FID, it was found desirable to clean up the ethylated extracts. This was due to the need to remove certain interferences which were not problems when the extracts were analyzed by GC/MS. The silica-gel column chromatography procedure previously developed for methyl esters (32) was found to work equally well for the ethyl esters with no modifications or changes required. The slightly more polar character of ethyl O-methylpodocarpate (due to the methoxyl substituent on the C ring) causes it to be retained more strongly on the silica-gel column and if the fraction was cut too early before the Sudan I band, the recovery would be low. Thus, ethyl O-methylpodocarpate served as a useful quality control tool to insure that there were minimal losses of the resin acid esters in the silica-gel clean-up. The clean-up did not seem to be required for all samples. Generally, the lower the concentrations of the resin acids, hence the closer to the detection limit, the more important it was to include the clean-up but this had to be determined on a case by case basis by comparing GC/MS and GC/FID data. The clean-up does remove a substantial amount of colored non-volatile material from the extract and the useful life of injection port liners and columns seemed to be extended when the clean-up was applied routinely.

After the silica-gel clean-up, the extracts were spiked with n-propyl dehydroabietate as an internal standard and were analyzed by capillary GC/FID. Quantitation was accomplished using the internal standard technique relative to n-propyl dehydroabietate as the reference peak.

The column selected does not resolve oleic and linolenic acids or levopimaric and palustric acids. A glass SP-2340 capillary column and fused silica DB-1 and Carbowax 20M capillary

columns were also tested. Due to the complex nature of a biologically treated mill effluent extract, each of these columns had problems similar to the DB-5 column which was selected as the best compromise.

The reproducibility of the relative response factors was found to be quite high. Table 3 summarizes 132 determinations of the relative response factors covering a two year period. Over the entire period, the responses varied only 3 to 5.6 percent. Since the reproducibility of the response factors was so reliable, careful monitoring provided an effective quality control tool for the identification of chromatography problems. Generally, the neoabietic acid and palustric acid response factors were the earliest indicators of reactive sites in the injection port or on the column. Replacement of the injection port liner corrected the problems most of the time. Less frequently, the column had to be solvent washed with methanol, dichloromethane then pentane and in extreme cases, a section of the front of the column had to be removed.

The reproducibility of the response factors was also useful in monitoring the quality and stability of calibration standards. Comparison of the response factors for the non-chlorinated resin acids shows they vary by only 5 to 7 percent. This is in fair agreement with data reported for methyl esters (26). The differences in our data and that reported by Foster and Zinkel (26) were probably due to higher purity standards used in their work.

TABLE 3 FID RELATIVE RESPONSE FACTOR SUMMARY[a]

| Compounds | Response Factor | Relative Percent S.D. |
|---|---|---|
| Ethyl Oleate | 0.76 | 4.0 |
| Ethyl Linoleate | 0.86 | 5.3 |
| Ethyl Pimarate | 0.83 | 3.7 |
| Ethyl Sandracopimarate | 0.84 | 3.2 |
| Ethyl Isopimarate | 0.85 | 3.1 |
| Ethyl Palustrate | 0.88 | 3.8 |
| Ethyl Dehydroabietate | 0.89 | 3.1 |
| Ethyl Abietate | 0.88 | 4.1 |
| Ethyl Neoabietate | 0.94 | 4.1 |
| Ethyl 14-Chlorodehydroabietate | 1.14 | 4.1 |
| Ethyl 12-Chlorodehydroabietate | 1.11 | 4.2 |
| Ethyl Dichlorodehydroabietate | 1.22 | 5.6 |
| Ethyl n-Heptadecanoate (SS) | 0.87 | 5.6 |
| Ethyl O-Methylpodocarpate (SS) | 0.94 | 3.0 |

[a]Total number of determinations over a two year period = 132
(SS) Surrogate Spikes

The uniformity of the response factors can be used to good advantage in estimating the concentrations of resin acids for which reference standards are not readily available. For example, the peak eluting after ethyl isopimarate has been tentatively identified by its mass spectrum as ethyl 13-abieten-18-oate. This compound was first tentatively identified in pulp mill effluent by Keith (10) and we have frequently detected it in both treated and untreated pulp mill effluents. By estimating the response factor to be equal to that of ethyl isopimarate, the concentration of 13-abieten-18-oic acid can be estimated within 5 to 7 percent of the actual value.

## IV METHOD VALIDATION STUDIES

When the development and testing of the procedures described above had progressed satisfactorily, studies were undertaken to optimize various parameters and to validate and document the methods' performance. The studies included method comparisons with previously used GC/MS procedures, tests of instrument linearity, method precision, instrument precision, method detection limits, recovery studies and effluent and extract storage studies. The objective was to provide the analyst with all the data necessary to evaluate method performance and insure data quality. These studies are discussed below.

### A. Method Comparisons

One of the earliest validation studies involved comparing the results obtained using the capillary procedures described above with results obtained using a packed column GC/MS method which had been used in our laboratory previously. Thus, samples from two biologically treated bleached kraft mill effluents were split into appropriate proportions and analyzed by these methods. The results, summarized in Table 4 indicated that for both effluents, the capillary procedures gave lower results for the unsaturated fatty acids. In Mill A, there was a significant interference in the FID analysis for 14-chlorodehydroabietic acid and in Mill B, there was an interference for the 12-chlorodehydroabietic acid. Other studies which were being conducted at that time on the packed column GC/MS procedure gave indications that the unsaturated fatty acid data was suspect. For instance, the analysis of the same methylated extracts by capillary GC/MS gave substantially lower fatty acid concentrations suggesting there were co-eluting interferences in the packed column analyses. The difficulties with the analysis of the monochlorodehydroabietic acids indicated that the suitability of the procedure for these compounds would have to be tested and confirmed on a case by case basis.

Despite these apparent shortcomings, the agreement in the results and the advantages of the capillary procedures were judged sufficient to continue the development and testing studies.

TABLE 4 COMPARISON OF METHODS FOR THE ANALYSIS OF BIOLOGICALLY TREATED BLEACHED KRAFT MILL EFFLUENTS

| Compound | Mill A (µg/L) Methylated GC/MS | Mill A (µg/L) Ethylated GC/MS | Mill A (µg/L) Ethylated FID | Mill B (µg/L Methylated GC/MS | Mill B (µg/L Ethylated GC/MS | Mill B (µg/L Ethylated FID |
|---|---|---|---|---|---|---|
| Oleic Acid | 49 | 9 | 9 | 38 | 5 | 8 |
| Linoleic Acid | 8 | ND | 2 | 8 | 5 | ND |
| Pimaric Acid | 48 | 55 | 49 | 8 | 4 | 10 |
| Isopimaric Acid | 42 | 50 | 45 | 31 | 39 | 31 |
| Palustric Acid | 36 | 31 | 22 | 8 | 8 | 6 |
| Abietic Acid | 99 | 78 | 61 | 26 | 28 | 22 |
| Dehydroabietic Acid | 117 | 70 | 69 | 17 | 10 | 12 |
| Neoabietic Acid | 16 | 15 | 13 | 4 | 2 | 4 |
| 14-Chlorodehydroabietic Acid | 8 | 3 | Int[a] | NA[b] | NA | NA |
| 12-Chlorodehydroabietic Acid | 53 | 46 | 55 | 2 | 2 | 114 |
| Dichlorodehydroabietic Acid | 64 | 55 | 63 | 33 | 21 | 29 |
| Surrogate Spike Recovery | | | | | | |
| n-Heptadecanoic Acid | 93% | 95% | 80% | 113% | 77% | 88% |
| O-Methylpodocarpic Acid | 97% | 99% | 105% | 82% | 96% | 85% |

[a] Int = Interference
[b] NA = Not Analyzed

B. Response Factor Linearity

Another aspect of the method validation work involved testing the linearity of the FID analyses. The test was conducted by analyzing ethylated resin acid reference standards throughout the practical loading range of the column. The results are summarized in Table 5. As was shown in Table 3, the response factors are quite reproducible.

TABLE 5 FID LINEARITY SUMMARY

| Compounds | Relative Response Factors Mean | Rel.S.D. | Linear Range (ng injected) |
|---|---|---|---|
| Oleic Acid | 0.71 | 7 | 21.0 - 66.7 |
| Linoleic Acid | 0.84 | 12 | 2.3 - 73.1 |
| Pimaric Acid | 0.71 | 4 | 1.7 - 54.2 |
| Sandracopimaric Acid | 0.74 | 4 | 1.7 - 54.2 |
| Isopimaric Acid | 0.73 | 4 | 2.0 - 64.0 |
| Palustric Acid | 0.77 | 5 | 1.9 - 59.7 |
| Dehydroabietic Acid | 0.77 | 4 | 2.5 - 78.9 |
| Abietic Acid | 0.77 | 4 | 2.3 - 71.5 |
| Neoabietic Acid | 0.83 | 6 | 1.9 - 58.6 |
| 14-Chlorodehydroabietic Acid | 0.98 | 4 | 3.4 - 106 |
| 12-Chlorodehydroabietic Acid | 1.00 | 7 | 1.5 - 23.6 |
| Dichlorodehydroabietic Acid | 1.21 | 6 | 1.8 - 55.8 |
| n-Heptadecanoic Acid | 0.91 | 12 | 2.4 - 75.1 |
| O-Methylpodocarpic Acid | 0.90 | 4 | 2.3 - 72.9 |

The linearity of the GC/MS quantitation was also determined by the analysis of standards. The working range of concentrations where the relative standard deviation of the response factor was approximately equivalent to or less than the instrument precision (discussed below in Section IV-C) is summarized in Table 6. Generally speaking, this is a more restrictive range than specified in a similar EPA GC/MS procedure (43) in that the relative standard deviation used in our estimate is approximately half that recommended in EPA Method 625 (i.e., Rel. S.D. ≤35 percent).

C. Method Precision

Evaluation of the precision of various phases of the method was another aspect of the method validation work and the results obtained are summarized in Table 7. The inter-day precision of the FID response factors, as presented in Table 3, was quite good so the intra-day instrument precision shown in Table 7 was hardly surprising. The results show that the derivitization step was the major source of variability for linoleic acid and neoabietic acid, although the abietic acid and palustric acid results are

TABLE 6 GC/MS LINEARITY SUMMARY

| Compound | Ion | Mean | Rel S.D. | Linear Range (ng injected) |
|---|---|---|---|---|
| Oleic Acid | 55 | 2.50 | 12.68 | 4.9 - 228 |
| | 264 | 8.10 | 5.24 | 12.5 - 228 |
| | 310 | 24.72 | 13.45 | 12.5 - 228 |
| Linoleic Acid | 55 | 6.37 | 18.95 | 12.5 - 250 |
| | 67 | 5.11 | 0.82 | 12.5 - 250 |
| | 308 | 13.53 | 13.05 | 32.5 - 250 |
| Pimaric Acid | 121 | 1.87 | 5.47 | 3.9 - 182 |
| | 330 | 8.32 | 10.56 | 3.9 - 182 |
| Sandracopimaric Acid | 121 | 2.12 | 10.10 | 4.0 - 185 |
| | 330 | 7.34 | 11.96 | 4.0 - 185 |
| Isopimaric Acid | 241 | 3.60 | 5.89 | 4.7 - 219 |
| | 256 | 5.67 | 8.13 | 4.7 - 219 |
| | 330 | 11.70 | 11.08 | 4.7 - 219 |
| Palustric Acid | 241 | 2.62 | 7.44 | 3.4 - 204 |
| | 315 | 2.88 | 10.17 | 3.4 - 204 |
| | 330 | 3.20 | 10.01 | 3.4 - 204 |
| Abietic Acid | 241 | 5.14 | 8.20 | 5.8 - 270 |
| | 256 | 2.56 | 6.31 | 5.8 - 270 |
| | 330 | 4.47 | 8.25 | 5.8 - 270 |
| Dehydroabietic Acid | 239 | 0.89 | 12.99 | 5.2 - 244 |
| | 328 | 4.30 | 16.90 | 12.2 - 244 |
| Neoabietic Acid | 135 | 1.83 | 7.19 | 4.3 - 201 |
| | 330 | 2.71 | 7.67 | 4.3 - 201 |
| 7-Oxodehydroabietic Acid | 253 | 2.03 | 9.23 | 8.6 - 172 |
| | 342 | 4.11 | 15.15 | 22.1 - 172 |
| 14-Chlorodehydroabietic Acid | 273 | 1.29 | 7.42 | 6.6 - 310 |
| | 275 | 3.84 | 7.01 | 6.6 - 310 |
| 12-Chlorodehydroabietic Acid | 273 | 1.34 | 9.49 | 1.5 - 69 |
| | 275 | 4.12 | 8.52 | 3.5 - 69 |
| Dichlorodehydroabietic | 307 | 2.20 | 9.82 | 3.8 - 178 |
| | 309 | 3.24 | 11.40 | 3.8 - 178 |
| n-Heptadecanoic Acid | 88 | 1.73 | 9.27 | 12.5 - 250 |
| | 101 | 3.22 | 7.90 | 12.5 - 250 |
| O-Methylpodocarpic Acid | 227 | 1.58 | 11.13 | 4.7 - 248 |
| | 316 | 1.76 | 11.88 | 4.7 - 248 |

not much better. The silica-gel clean-up seems to contribute to the variability of the oleic acid data. By comparing the results of the instrument, derivatization and silica-gel steps with the total method precision, it can be concluded that the extraction procedure was the main source of variability for the majority of the compounds. It should be noted however, that the total method precision study was conducted on an effluent sample with concentrations of the analytes at very low levels in order to provide data for an estimate of the lower detection limits. Therefore, the results shown in Table 7 should be considered as a worse case representation of precision.

The instrument precision of the GC/MS analysis was estimated by measuring the variation in the response factors for the quantitation ions in 10 consecutive analyses of a single calibration standard and the results are summarized in Table 8. The total GC/MS method precision was determined by taking separate aliquots of an effluent sample through the entire procedure and these results are shown in Table 9. As one might anticipate, the GC/MS procedure was less reproducible than the FID procedure.

D. Estimated Method Detection Limits

Applying the method of Glaser et al.(44) to the FID method precision data affords an estimate of the lower detection limit. The results of this treatment of the data are shown in Table 10. The procedure is sensitive to how close the concentrations in the sample are to the actual detection limits. Although the precision data for abietic acid and dichlorodehydroabietic acid indicate higher than average variability, it is believed that the relatively high estimated detection limits are the result of

TABLE 7 FID METHOD PRECISION DATA

| | Relative S.D. | | | |
|---|---|---|---|---|
| Compound | Instrument | Derivatization | Silica-Gel Clean-Up | Total Method |
| Oleic Acid | 3.6 | 8.3 | 15.2 | 16.6 |
| Linoleic Acid | 3.2 | 21.0 | 22.3 | 14.8 |
| Pimaric Acid | 2.1 | 3.0 | 2.1 | 4.2 |
| Sandracopimaric Acid | 1.7 | 3.0 | 2.4 | 15.6 |
| Isopimaric Acid | 1.3 | 2.2 | 1.9 | 4.1 |
| Palustric Acid | 1.4 | 6.3 | 1.2 | 12.8 |
| Dehydroabietic Acid | 0.9 | 4.2 | 1.4 | 5.4 |
| Abietic Acid | 0.5 | 6.5 | 1.6 | 12.0 |
| Neoabietic Acid | 0.7 | 7.8 | 1.2 | 8.3 |
| 14-Chlorodehydroabietic Acid | 0.9 | 2.3 | 1.5 | 4.0 |
| Dichlorodehydroabietic Acid | 1.9 | 3.3 | 5.0 | 11.0 |

TABLE 8 GC/MS RESIN ACID INSTRUMENT PRECISION

| Compound | Response Factor[a] Ion | Mean | S.D. | Rel. S.D. |
|---|---|---|---|---|
| Oleic Acid | 55 | 3.62 | 3.1 | 14.2 |
| | 264 | 8.40 | 1.3 | 14.9 |
| | 310 | 21.68 | 3.1 | 14.2 |
| Linoleic Acid | 55 | 5.15 | 0.8 | 16.0 |
| | 67 | 4.53 | 0.4 | 9.9 |
| | 308 | 13.14 | 1.8 | 13.4 |
| Pimaric Acid | 121 | 2.15 | 0.3 | 11.7 |
| | 330 | 7.67 | 1.1 | 14.5 |
| Sandracopimaric Acid | 121 | 2.42 | 0.3 | 11.9 |
| | 330 | 6.81 | 1.0 | 14.8 |
| Isopimaric Acid | 241 | 4.39 | 0.7 | 15.5 |
| | 256 | 6.23 | 0.9 | 14.5 |
| | 330 | 6.81 | 1.0 | 14.8 |
| Palustric Acid | 241 | 3.30 | 0.5 | 14.0 |
| | 315 | 31.90 | 0.5 | 14.2 |
| | 330 | 3.08 | 0.5 | 15.8 |
| Abietic Acid | 241 | 5.42 | 0.9 | 16.2 |
| | 256 | 2.60 | 0.4 | 15.1 |
| | 330 | 3.94 | 0.6 | 15.1 |
| Dehydroabietic Acid | 135 | 1.96 | 0.3 | 14.2 |
| | 330 | 2.65 | 0.4 | 15.1 |
| Neoabietic Acid | 135 | 1.96 | 0.3 | 14.2 |
| | 330 | 2.65 | 0.4 | 15.1 |
| 7-Oxodehydroabietic Acid | 253 | 2.30 | 0.3 | 15.1 |
| | 342 | 3.46 | 0.6 | 18.7 |
| 14-Chlorodehydroabietic Acid | 273 | 1.52 | 0.2 | 14.1 |
| | 275 | 4.49 | 0.7 | 14.6 |
| 12-Chlorodehydroabietic Acid | 307 | 2.31 | 0.4 | 15.4 |
| | 309 | 3.35 | 0.5 | 15.0 |
| n-Heptadecanoic Acid | 88 | 1.78 | 0.2 | 12.3 |
| | 101 | 3.27 | 0.4 | 12.3 |
| O-Methylpodocarpic Acid | 227 | 1.97 | 0.3 | 13.5 |
| | 316 | 1.90 | 0.2 | 12.9 |

[a]Results summarize ten replicate analyses of a single calibration standard

TABLE 9 GC/MS METHOD PRECISION DATA AND DETECTION LIMIT ESTIMATE

| Compound | Rel. S.D. | MDL[a](ppb) |
|---|---|---|
| Oleic Acid | 30.0 | 4.2 |
| Linoleic Acid | 23.0 | 0.8 |
| Pimaric Acid | 16.0 | 3.3 |
| Sandracopimaric Acid | 25.0 | 0.9 |
| Isopimaric Acid | 13.0 | 3.0 |
| Palustric Acid | 23.0 | 2.6 |
| Dehydroabietic Acid | 17.0 | 8.5 |
| Abietic Acid | 26.0 | 9.6 |
| Neoabietic Acid | 24.0 | 0.7 |
| 14-Chlorodehydroabietic Acid | 16.0 | 10.2 |
| 12-Chlorodehydroabietic Acid | 21.0 | 1.0 |
| Dichlorodehydroabietic Acid | 14.0 | 20.9 |

[a]MDL=Method Detection Limit

TABLE 10 ESTIMATED FID METHOD DETECTION LIMIT

| Compound | MDL[a](ppb) |
|---|---|
| Oleic Acid | 2.2 |
| Linoleic Acid | 0.8 |
| Pimaric Acid | 0.8 |
| Sandracopimaric Acid | 1.3 |
| Isopimaric Acid | 0.8 |
| Palustric Acid | 1.1 |
| Dehydroabietic Acid | 1.7 |
| Abietic Acid | 4.7 |
| Neoabietic Acid | 2.3 |
| 14-Chlorodehydroabietic Acid | 2.0 |
| Dichlorodehydroabietic Acid | 5.4 |

[a]MDL = Method Detection Limit

the high analyte concentrations in the sample; and that these estimates would probably be adjusted downward as a result of an iterative application of the procedure. Table 9 also includes estimated detection limits derived from a similar treatment of the GC/MS precision data.

The estimation procedure of Glaser et al.(44) depends totally on the standard deviation of the results near the

detection limit. Thus, any improvements in the reproducibility translate into a reduction in the detection limits. It follows then, that the poorer reproducibility of the GC/MS method would translate into generally higher detection limits as can be seen in Table 9. From the earlier observations concerning the major source of variability in the FID method, it can readily be seen that improvement of the extraction procedure would result in an improvement in detection limits. In reality, a detection limit is limited not only by reproducibility, but by other factors such as the level of interferences. Thus, there is a practical limit to the improvements that can be obtained through increasing the reproducibility of the isolation procedure. The GC/MS procedure is capable of confirming the identification of compounds below the estimated lower detection limit; lower levels just cannot be reliably quantitated.

## E. Recovery Studies

The recovery of analytes spiked into samples provides some information on the accuracy of the method and can serve as a useful element in a quality assurance program. Thus, recovery spikes have been conducted on a regular basis and the results of 25 such determinations are summarized in Table 11. In general, the recoveries were high and reproducible for all the compounds except palustric and neoabietic acids. These problems were particularly noticeable at low concentration levels.

The acid catalyzed isomerization of neoabietic, palustric, and levopimaric acids to give an equilibrium mixture which is predominantly (95 percent) abietic acid is well documented in the literature (35-42). In an effort to minimize the potential for this isomerization to occur during the extraction process as a result of acidifying the effluent, recoveries at extraction pHs of 2, 3, 4, and 6 were tested and the results are summarized

TABLE 11 FID METHOD RECOVERY DATA

| Compound | Percent Recovery |
|---|---|
| Oleic Acid | 84 |
| Linoleic Acid | 90 |
| Pimaric Acid | 91 |
| Sandracopimaric Acid | 93 |
| Isopimaric Acid | 93 |
| Palustric Acid | 64 |
| Dehydroabietic Acid | 101 |
| Abietic Acid | 88 |
| Neoabietic Acid | 48 |
| 14-Chlorodehydroabietic Acid | 90 |
| Dichlorodehydroabietic Acid | 95 |

in Table 12. There seemed to be an improvement in the recovery of neoabietic and palustric acids as the extraction pH was increased but the recoveries of most of the other analytes decreased.

TABLE 12 RECOVERIES OF RESIN AND FATTY ACIDS AT VARIOUS pHs

| Compound | 4 x pH 3 | 4 x pH 3 | 4 x pH 4 | 3 x pH 6 |
|---|---|---|---|---|
| Oleic Acid | 98 | 102 | 97 | - |
| Linoleic Acid | 80 | 89 | 87 | 75 |
| Pimaric Acid | 89 | 83 | 81 | 71 |
| Sandracopimaric Acid | 111 | 108 | 106 | 94 |
| Isopimaric Acid | 102 | 98 | 88 | 76 |
| Palustric Acid | 48 | 61 | 67 | 69 |
| Dehydroabietic Acid | 101 | 96 | 85 | 77 |
| Abietic Acid | 66 | 95 | 84 | 78 |
| Neoabietic Acid | 26 | 57 | 77 | 82 |
| 14-Chlorodehydroabietic Acid | 84 | 86 | 71 | 91 |
| Dichlorodehydroabietic Acid | 96 | 86 | 76 | 64 |

Since most of the analytes seemed to require extraction at low pH to get quantitative recoveries but the acid sensitive compounds benefited by extraction at higher pH, a series of experiments were conducted to determine if a two step (i.e. extraction at high pH followed by extraction at low pH) procedure would resolve the difficulty. Extraction at an initial pH of either 3 or 4 followed by extraction at pH 2 did not give acceptable recoveries of neoabietic acid or palustric acid as would be expected from the above data.

Therefore, efforts were focused on extraction schemes where the initial extractions were conducted at either pH 5 or 6 and the results are summarized in Table 13. These studies showed that, in general, the recoveries of most of the analytes were higher when the initial extraction was followed by a pH 2 rather than a pH 3 extraction. When the number of extractions at the high pH was reduced, the recovery of palustric acid and in particular neoabietic acid dropped. There seemed to be no significant improvement in using pH 6 in the initial extraction and the emulsion problems were slightly greater at the higher pH. Considering all of the above, the optimum extraction scheme consisted of three extractions at pH 5 followed by two extractions at pH 2. Table 14 summarizes the routine quality assurance recovery data collected using this extraction protocol and there has been definite improvement in the recoveries of neoabietic and palustric acids.

TABLE 13 RECOVERIES USING SEQUENTIAL EXTRACTIONS AT TWO pHs

| Compound | Percent Recovery | | | | | | |
|---|---|---|---|---|---|---|---|
| | Mill A | | | Mill B | | Mill C | |
| | 3x pH5 2x pH3 | 2x pH5 2x pH3 | 2x pH5 3x pH2 | 3x pH6 2x pH2 | 3x pH6 3x pH3 | 3x pH6 2x pH2 | 3x pH6 3x pH3 |
| Oleic Acid | 112 | 107 | 76 | 87 | 80 | 90 | 78 |
| Linoleic Acid | 101 | 97 | 81 | 87 | 80 | 91 | 77 |
| Pimaric Acid | 84 | 83 | 91 | 92 | 87 | 89 | 79 |
| Sandracopimaric Acid | 110 | 104 | 120 | 88 | 83 | 86 | 79 |
| Isopimaric Acid | 89 | 85 | 106 | 92 | 88 | 89 | 84 |
| Palustric Acid | 80 | 71 | 78 | 85 | 73 | 83 | 80 |
| Dehydroabietic Acid | 94 | 92 | 99 | 92 | 91 | 85 | 84 |
| Abietic Acid | 89 | 83 | 100 | 88 | 94 | 83 | 83 |
| Neoabietic Acid | 61 | 39 | 93 | 76 | 70 | 78 | 79 |
| 14-Chlorodehydroabietic Acid | 82 | 85 | 114 | 98 | 91 | 82 | 85 |
| Dichlorodehydroabietic Acid | 78 | 79 | 119 | 80 | 89 | 80 | 83 |
| n-Heptadecanoic Acid | 102 | 100 | 78 | 86 | 79 | 99 | 90 |
| O-Methylpodocarpic Acid | 94 | 89 | 95 | 84 | 89 | 84 | 83 |

TABLE 14 pH 5 - pH 2 RESIN ACID QA RECOVERY SUMMARY

| Compound | Percent Recovery[a] Mean | S.D. | Rel.S.D. |
|---|---|---|---|
| Oleic Acid | 81 | 12.1 | 14.9 |
| Linoleic Acid | 86 | 9.2 | 10.7 |
| Pimaric Acid | 91 | 14.6 | 16.1 |
| Sandracopimaric Acid | 88 | 5.1 | 5.8 |
| Isopimaric Acid | 96 | 10.8 | 11.3 |
| Palustric Acid | 81 | 8.6 | 10.6 |
| Dehydroabietic Acid | 91 | 6.0 | 6.6 |
| Abietic Acid | 89 | 5.4 | 6.1 |
| Neoabietic Acid | 79 | 7.2 | 9.2 |
| 14-Chlorodehydroabietic Acid | 87 | 6.3 | 7.2 |
| Dichlorodehydroabietic Acid | 88 | 8.3 | 9.5 |

[a]Summary of 26 recovery determinations

Recently, Voss and Rapsomatiotis (5) reported a different approach to resolving the problem of acid catalyzed isomerization of the resin acids. They studied the recoveries of oleic, linoleic, isopimaric and dehydroabietic acids using a volume of methyl t-butylether equal to the volume of the sample and an extraction pH range of 2 to 12. Their findings indicated pH 9 was the optimum for these compounds using this approach. The high proportion of solvent eliminated the emulsion problems that are typical of high pH extractions of pulp mill effluents.

The difference in the findings of Voss and Rapsomatiotis (5) and those shown in Table 12 may be due to the large proportion of extraction solvent used by those workers (twice the sample volume vs. 90 percent of the sample volume). Although it has been our experience and the experience of others (45) that methyl t-butylether gives equivalent results to diethylether, it may be that the high water solubility of ether (10 percent) creates a water solvent mix which stabilized the solubility of the resin acid salts making them more difficult to extract. Finally, the difference may be due to the use of Voss and Rapsomatiotis (5) of an effluent which had previously been passed through XAD resin. This may have removed other surface active components which, in whole effluent as used in our studies, may contribute to micelle formation or otherwise stabilize the water solubility of the resin acid salts, thereby making them more difficult to recover.

## F. Effluent Preservation and Storage Stability

The validation studies described above provided the necessary method performance data so other aspects of sample handling could be addressed. In particular, the optimum preservation techniques and maximum storage times for effluent samples were investigated.

The studies investigated refrigerated storage of samples preserved at pH 2 and pH 10. As can be seen in Figure 5, the pH 2 preserved samples showed a rapid loss of both neoabietic acid and palustric acid with a concurrent increase in the concentration of abietic acid. This was undoubtedly caused by the acid catalyzed isomerization reactions discussed above. Although the other analyte concentrations remained constant during this period of time, the difficulties with the acid labile resin acids indicated that acid preservation would be unsatisfactory for even as few as seven days.

Similar effluent samples which were refrigerated at pH 10 showed no significant changes in the concentrations of any of the analytes during the test period of 29 days. Since this was the last day tested, it was not determined just how long samples could be stored before degradation would occur. Of the preservation procedures tested, refrigeration at pH 10 was chosen as the optimum.

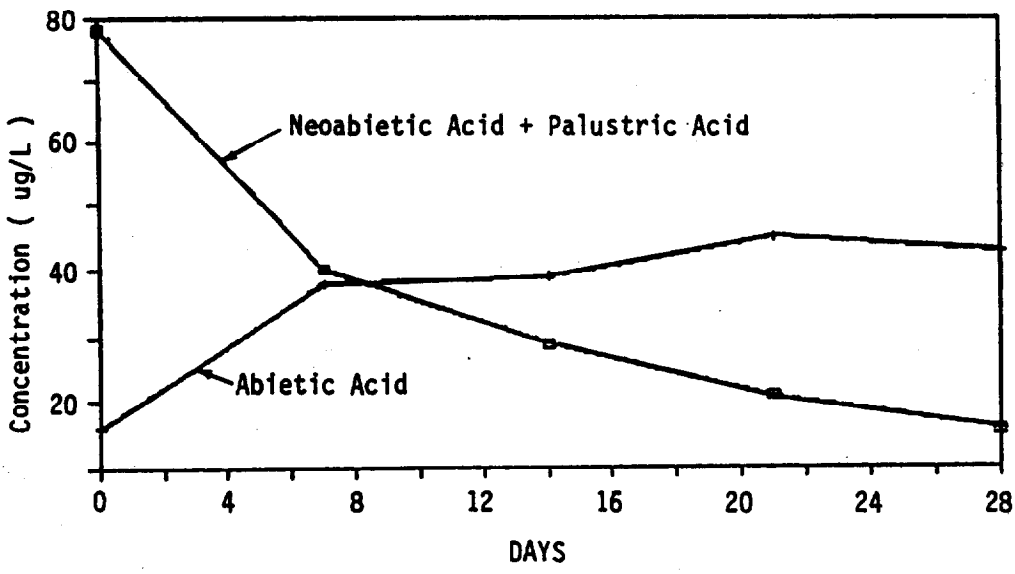



FIGURE 5 STABILITY OF NEOABIETIC, PALUSTRIC AND ABIETIC ACIDS IN pH 2 PRESERVED EFFLUENT

G. Extract Preservation and Storage

Resin esters are also known to be unstable in hydrocarbon solvents (46,47), especially in the presence of light (48-50). They are also unstable in halogenated solvents (27,51). In the case of the hydrocarbon solvents, the problem is thought to be disproportionation or auto-oxidation reactions; possibly due to the presence of free radicals. Traces of HCl are thought to be responsible for the isomerization reactions caused in halogenated solvents. Since using the procedure described above, the extracts ultimately end up in hexane, studies were undertaken to determine the optimum storage conditions for the effluent extracts.

Methanol stock solutions of the underivatized resin acids were found to be stable for months when refrigerated. Refrigeration was also all that was required to stabilize underivatized effluent extracts (which were still in ether) and derivatized extracts (which were in hexane) for up to 35 days (the longest time period tested). However, once the extracts were run through the silica-gel column, neoabietic, palustric, and abietic acids were found to degrade significantly within a week.

To resolve this difficulty, the use of antioxidants which are free radical scavengers was investigated. It was found that addition of methylenebis(di-t-butylphenol) to a final concentration of 0.01 percent in conjunction with refrigeration was effective in the stabilization of calibration standards and silica-gel cleaned up extracts for up to 90 days. It was found that 1,3,5-trimethyl-2,4,6-tris(3,5-di-t-butyl-4-hydroxybenzyl)-benzene was also effective in stabilizing the extracts and standards but was found to accumulate in the injection port due to its low volatility. This accumulation eventually led to unacceptable response factors and irreproducibility and the compound was judged unsuitable for use. The methylenebis(di-t-butylphenol) chromatographed nicely, giving a peak which did not interfere with any of the analytes. It was observed that when extracts were stored at room temperature overnight, significant losses of neoabietic and palustric acids were observed even in preserved samples.

In retrospect, it was concluded that the stability of the extracts prior to the silica-gel clean-up was probably due to natural free radical scavengers in the matrix which were co-extracted with the resin acids. The brown colored lignin material may be responsible for this stabilization. The silica-gel clean-up removes this material, thereby possibly contributing to the instability of the extracts.

Since some laboratories prefer to use dichloromethane as an extraction solvent, the effectiveness of methylenebis(di-t-butylphenol) for dichloromethane extract stabilization was investigated. When no preservative was used, neoabietic acid and palustric acid degraded rapidly (within 24 hours) even with refrigeration. Both derivatized and underivatized extracts were similarly unstable. When the preservative was tested, it was found to be unable to resolve the stability problems even when added at substantially higher concentrations. Apparently, the antioxidant was incapable of stabilizing the degradation of the solvent which generates traces of HCl which presumably caused the acid mediated isomerization and degradation of the acid labile components.

## V SUMMARY

(1) A method for the analysis of resin and fatty acids in pulp mill effluents based on fused silica capillary column technology has been described. The method utilizes triethyloxonium tetra-

fluoroborate to form ethyl esters and quantitation by either GC/MS or FID. Evaluations of the precision of all phases of the procedure, estimates of detection limits and spike recovery data have been presented and they indicate that the greatest improvement in the method could be achieved by optimizing the extraction step. Refrigeration of pH 10 preserved effluent samples and methylenebis(di-t-butylphenol) preserved extracts has been shown to be the best means of storage.

(2) The method is fairly simple, uses a commercially available column and provides an alternative to the use of diazomethane for derivatization. While accomplishing these goals the method remains reproducible and sensitive, and therefore will be a useful tool in the evaluation of the environmental significance of resin and fatty acids.

(3) The procedure for the analysis of resin and fatty acids in pulp mill effluents is presented in standard methodology format in Appendix D. The only difference between the procedure used to develop the information described in this report and that described in Appendix D is that the quantitation procedures have been changed to be more consistent with techniques used in EPA methods.

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# APPENDIX A

# PREPARATION OF O-METHYLPODOCARPIC ACID

## PREPARATION OF O-METHYLPODOCARPIC ACID

A 100 mL, three neck round bottom flask was fitted with a septum, thermometer and condenser with a nitrogen inlet. Diethylene glycol (20 mL), 0.52g of NaOH and a Teflon-coated stirring bar were added to the flask and the apparatus was purged with dry nitrogen to remove air. The contents were heated and stirred to approximately 80°C until all the sodium hydroxide had dissolved.

Two grams of methyl-O-methylpodocarpate (Aldrich, recrystallized from methanol) was added all at once. The resulting mixture was heated and stirred at a gentle reflux. After several hours, 160 µL of water was added via syringe through the septum over a period of 20 minutes (i.e. 8 µL every minute). The reaction was allowed to continue to reflux overnight.

An aliquot was removed via syringe the following morning and ethylated with triethyoxonium tetrafluoroborate. The ethylated aliquot was analyzed by FID to determine the completeness of the reaction.

When it was determined the reaction was complete, the mixture was allowed to cool and was slowly poured into 60 mL of cold water in a separatory funnel. Two 20 mL portions of water were used to wash out the reaction flask and all aqueous phases were combined.

The aqueous phases were then extracted with two 40 mL portions of diethyl ether to remove unreacted starting material (note: be sure the reaction mixture is at or very near room temperature or excessive pressure will be generated during the extraction due to the boiling of the diethyl ether).

The aqueous phase was then acidified with 6N HCl and was extracted with four 40 mL portions of diethyl ether. The combined ether phases were back-washed with water until the water washes were neutral. The diethyl ether was then removed by roto-evaporation.

The resulting crude product was chromatographed on activated silica-gel eluting first with n-hexane and gradually working up to 90 percent hexane-10 percent diethyl ether. The fraction containing the O-methylpodocarpic acid was concentrated by roto-evaporation and the resulting solid was recrystallized from methanol.

# APPENDIX B

## PREPARATION OF n-PROPYL DEHYDROABIETATE

## PREPARATION OF n-PROPYL DEHYDROABIETATE

Five grams of dehydroabietic acid were dissolved in 165 mL of anhydrous ether along with five drops of dry pyridine (redistilled and stored over KOH pellets for 24 hours) in a round bottom flask under a dry nitrogen atmosphere. The solution was then cooled to near 0°C using an ice slush bath. A total of 9.7 mL of freshly distilled thionyl chloride was slowly added via syringe over approximately 5 minutes. A white precipitate began forming immediately. The slush bath was removed and the mixture was allowed to come to room temperature and stirring continued for 5 hours.

Two hundred grams of n-propanol (freshly distilled from sodium metal) were added to the mixture and the reaction was heated to reflux for 6 hours. Reaction progress was monitored by withdrawing aliquots, methylating with diazomethane and analyzing by GC-FID.

When the reaction was complete, the solvent and excess reagents were removed by distillation. The resulting oil was taken up in 20 mL fresh diethyl ether and transferred to a separatory funnel. Two additional 25 mL portions were used to wash the reaction vessel. The combined organic phases were then washed with three 100 mL portions of saturated potassium carbonate.

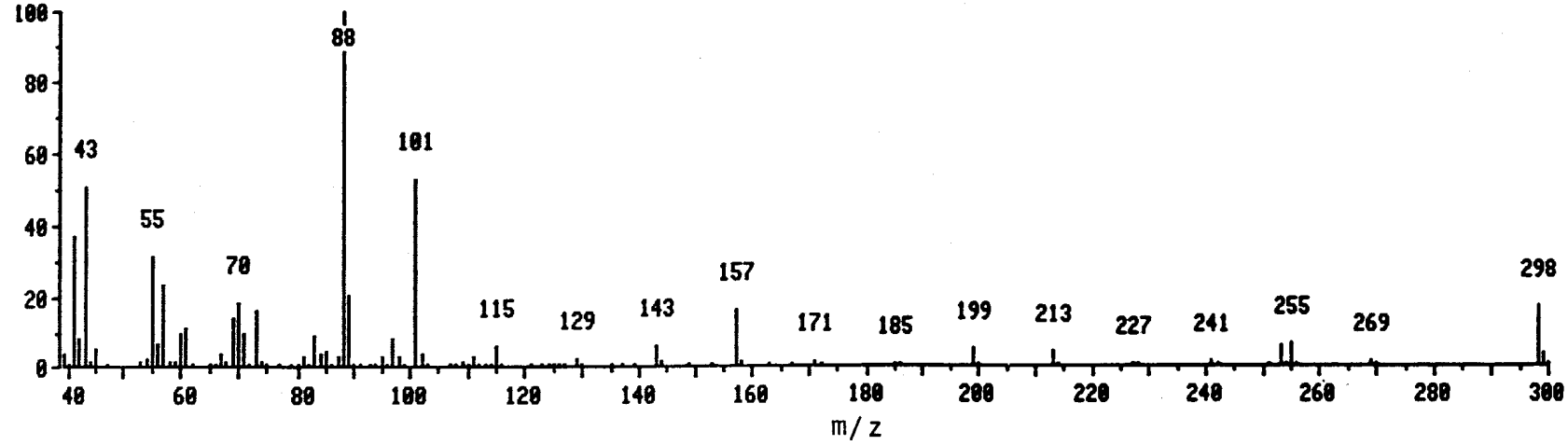
The base washed diethyl ether phase was dried over potassium carbonate and concentrated by roto-evaporation. The resulting viscous oil was taken up in pentane and chromatographed on neutral alumina using pentane as the eluant.

Concentration of the n-propyl dehydroabietate fractions by roto-evaporation afforded a clear viscous oil which solidified upon refrigeration and eventually became a low melting waxy solid.

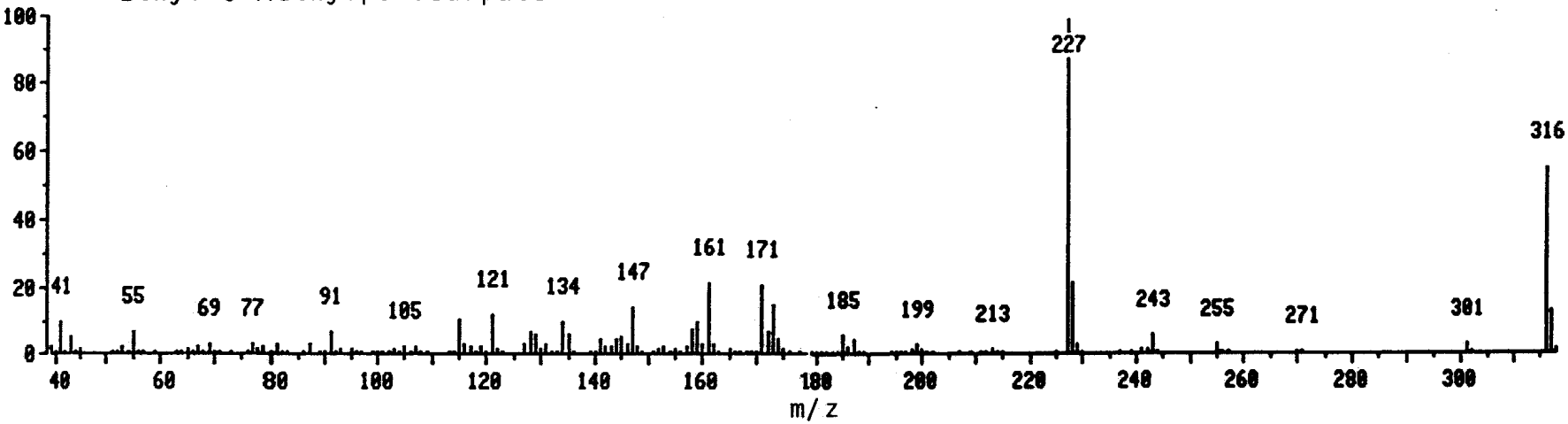
# APPENDIX C

## REFERENCE MASS SPECTRA OF n-PROPYL DEHYDROABIETATE AND ETHYL ESTERS OF RESIN ACIDS, FATTY ACIDS AND RECOVERY SURROGATES

Ethyl n-Heptadecanoate

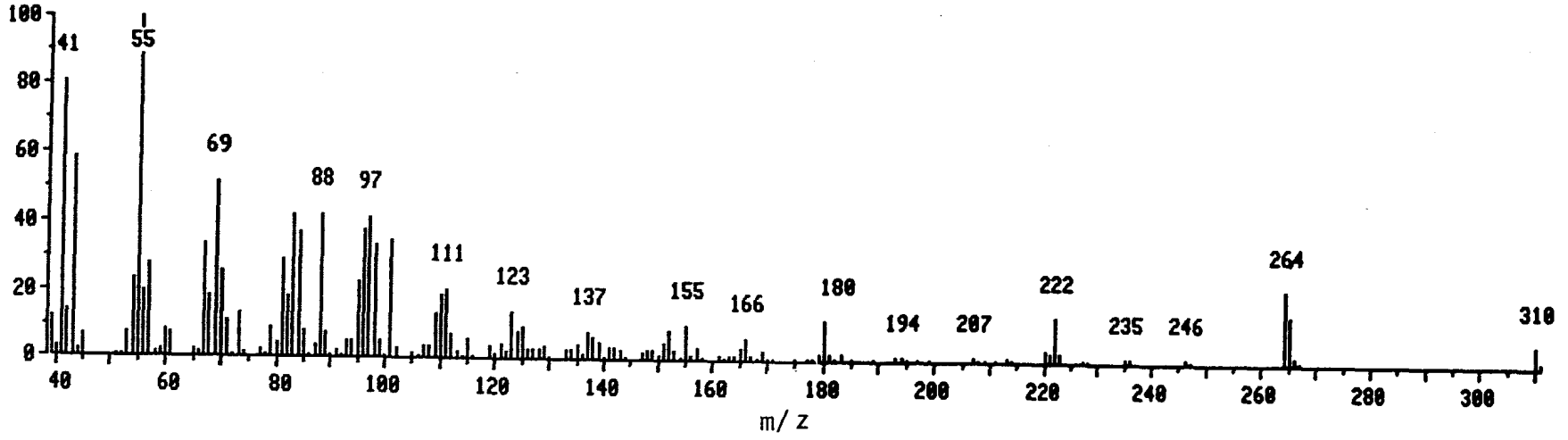



Ethyl O-Methylpodocarpate

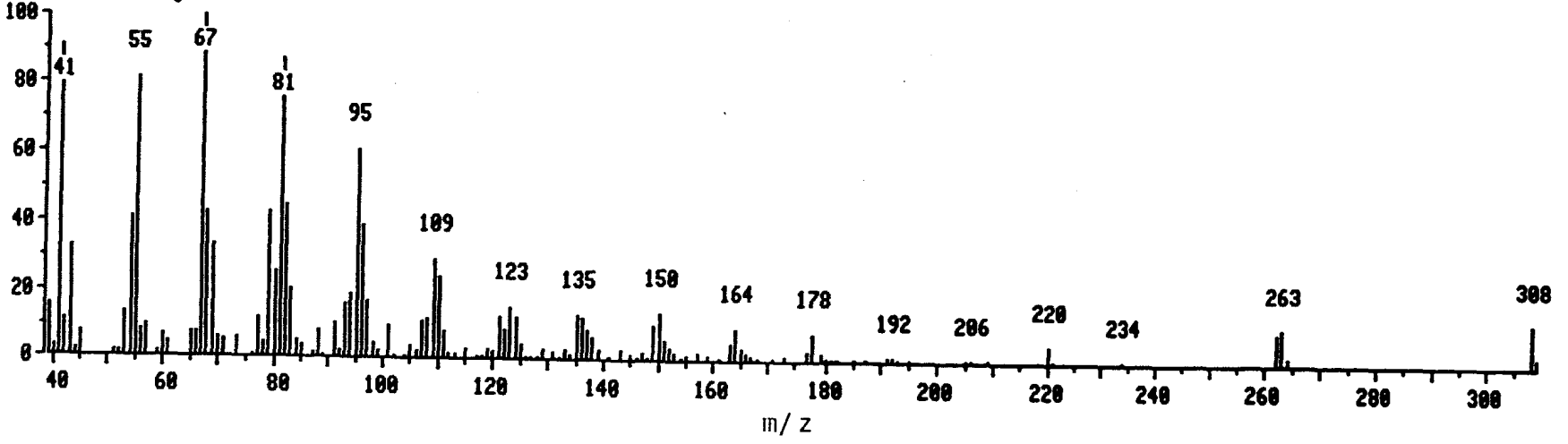



C-1

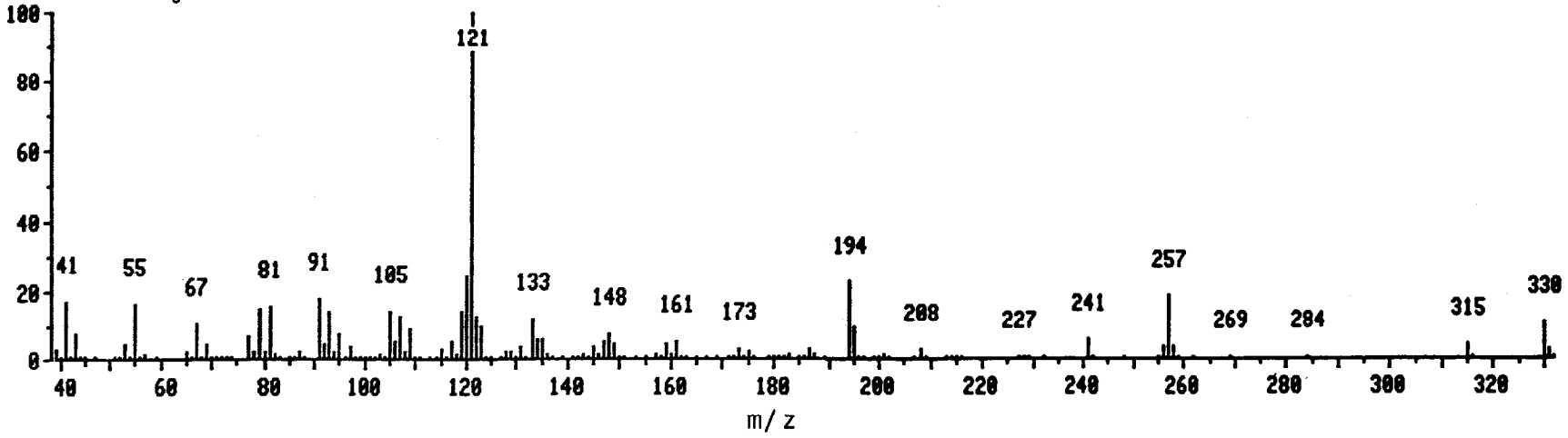
Ethyl Oleate



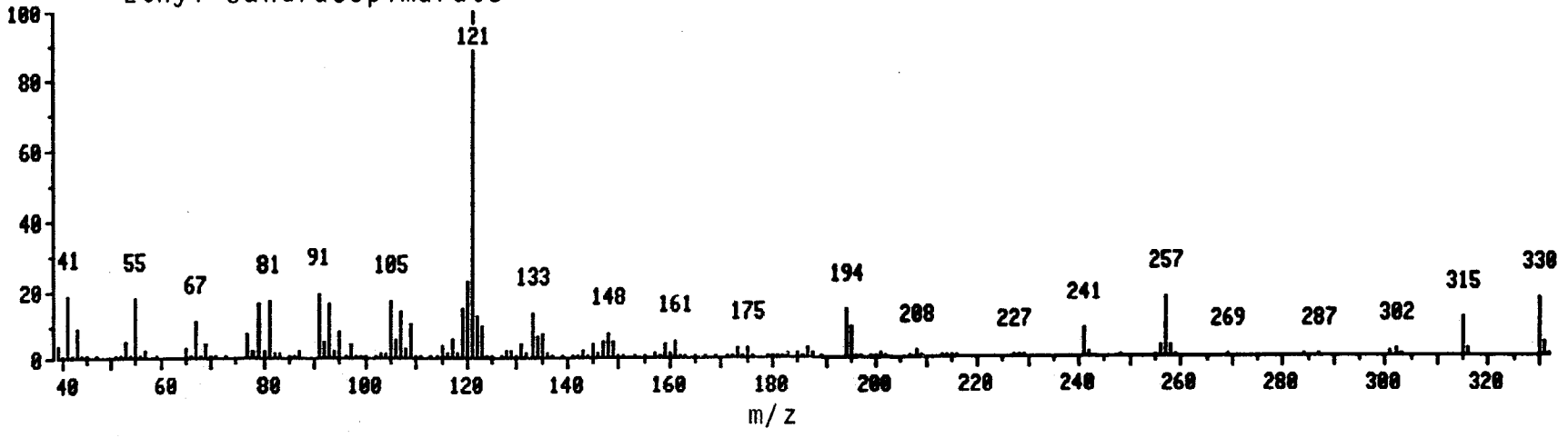

Ethyl Linoleate



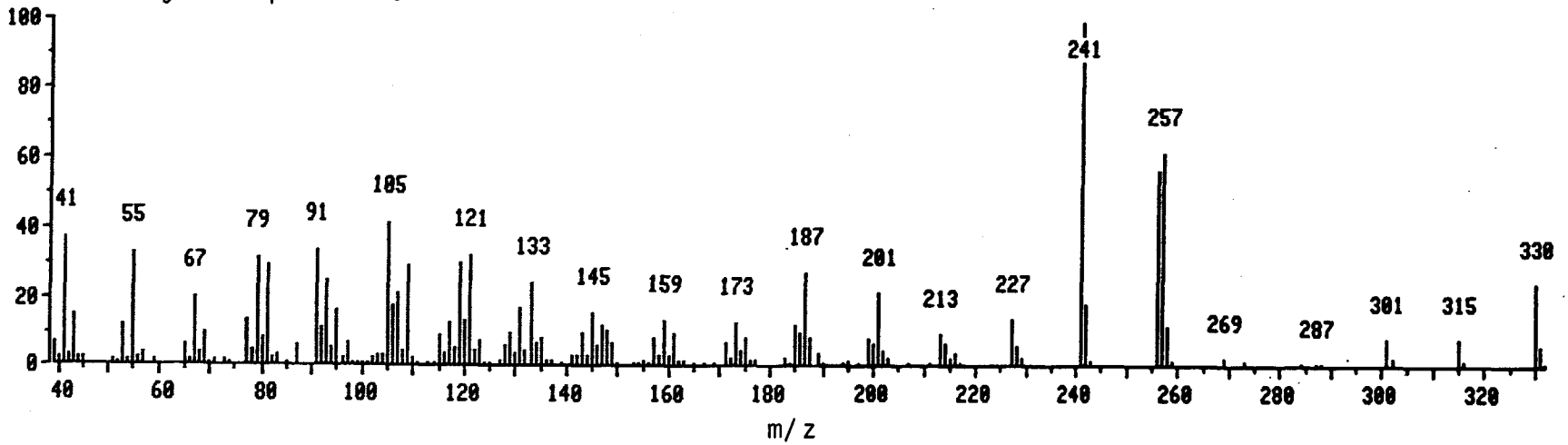
Ethyl Pimarate



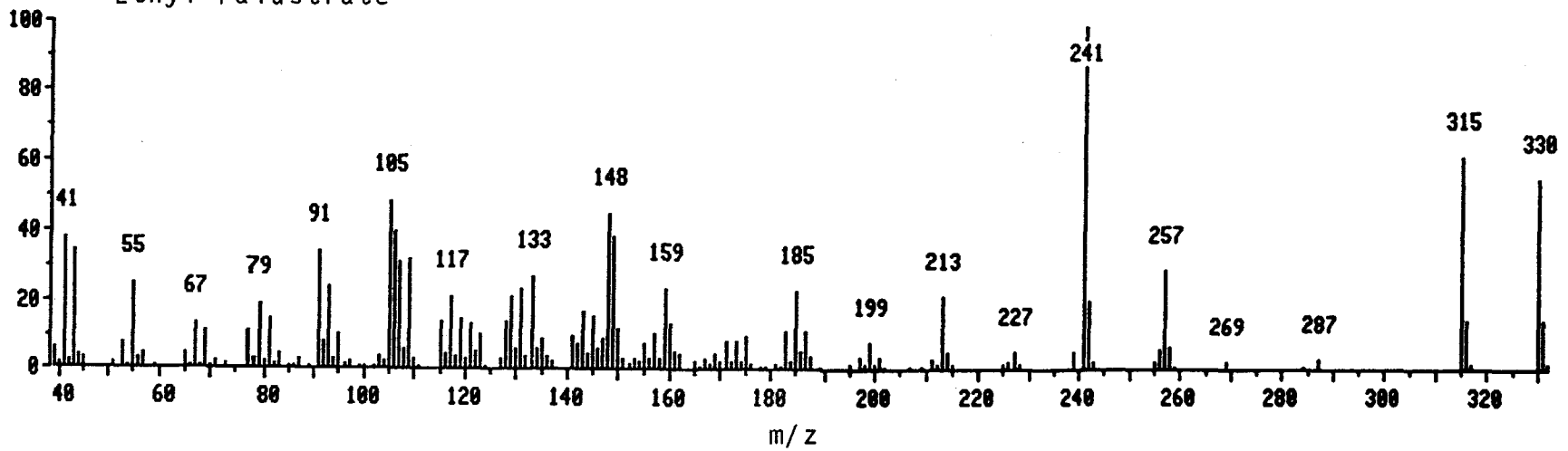

Ethyl Sandracopimarate



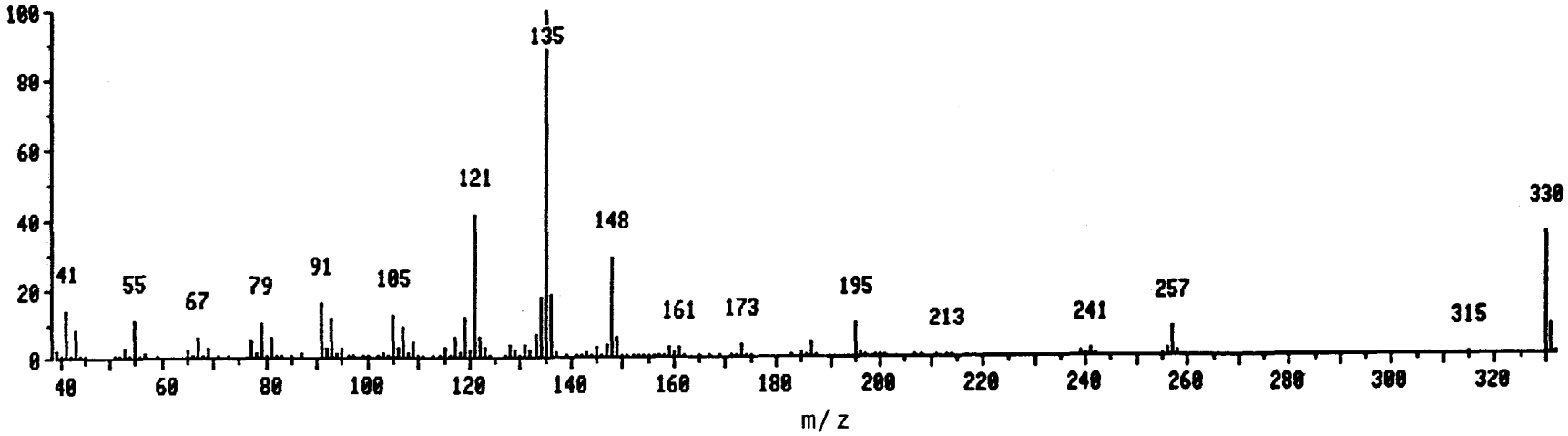
Ethyl Isopimarate




Ethyl Palustrate

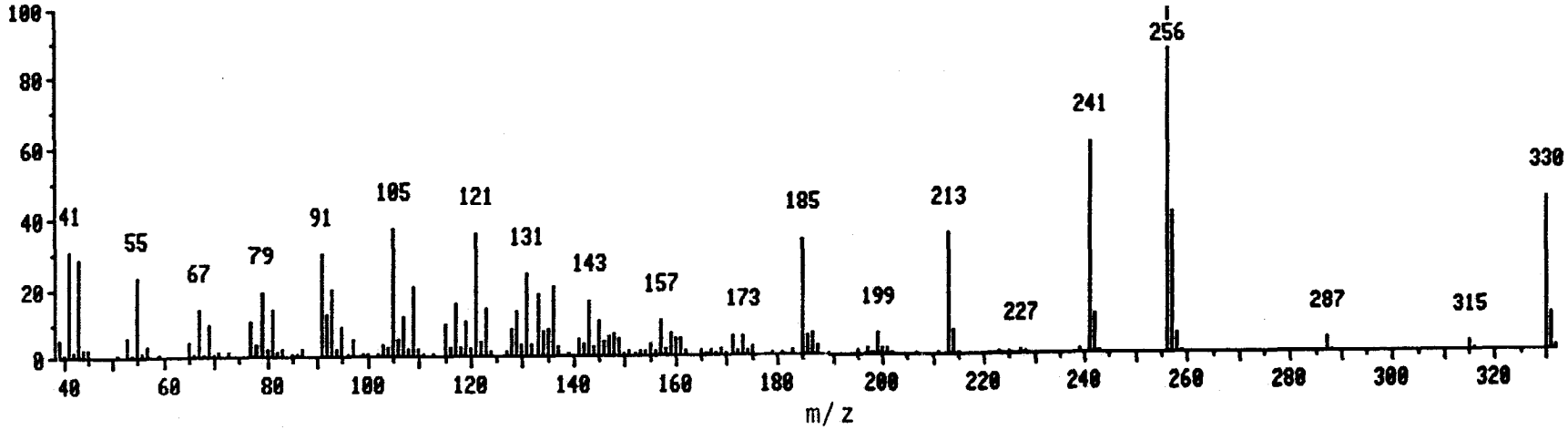


Ethyl Neoabietate

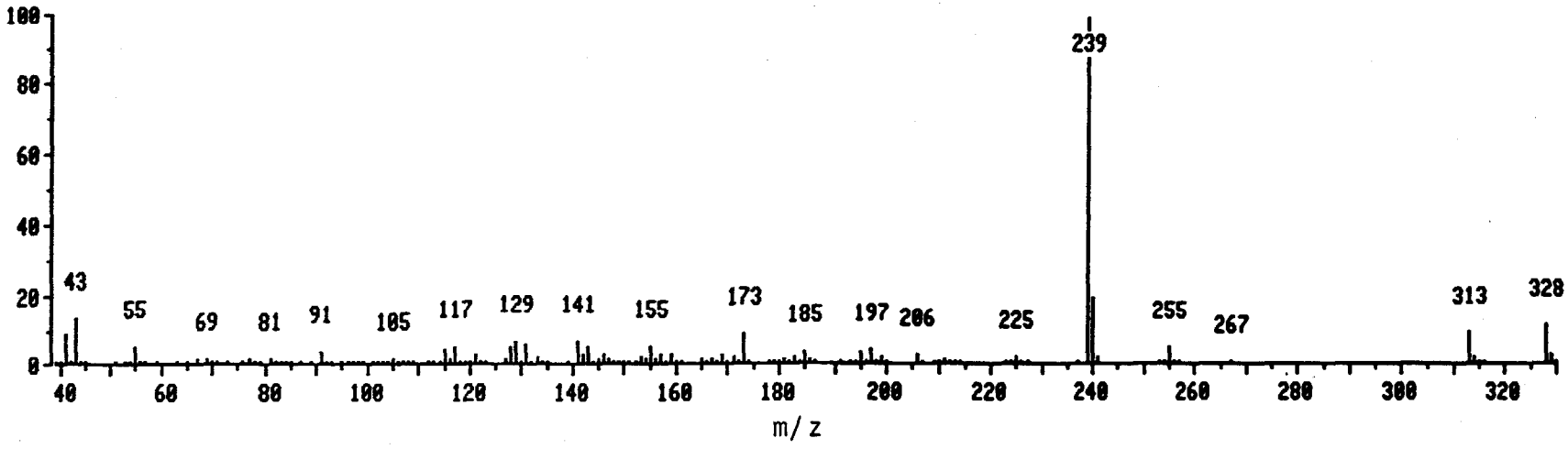




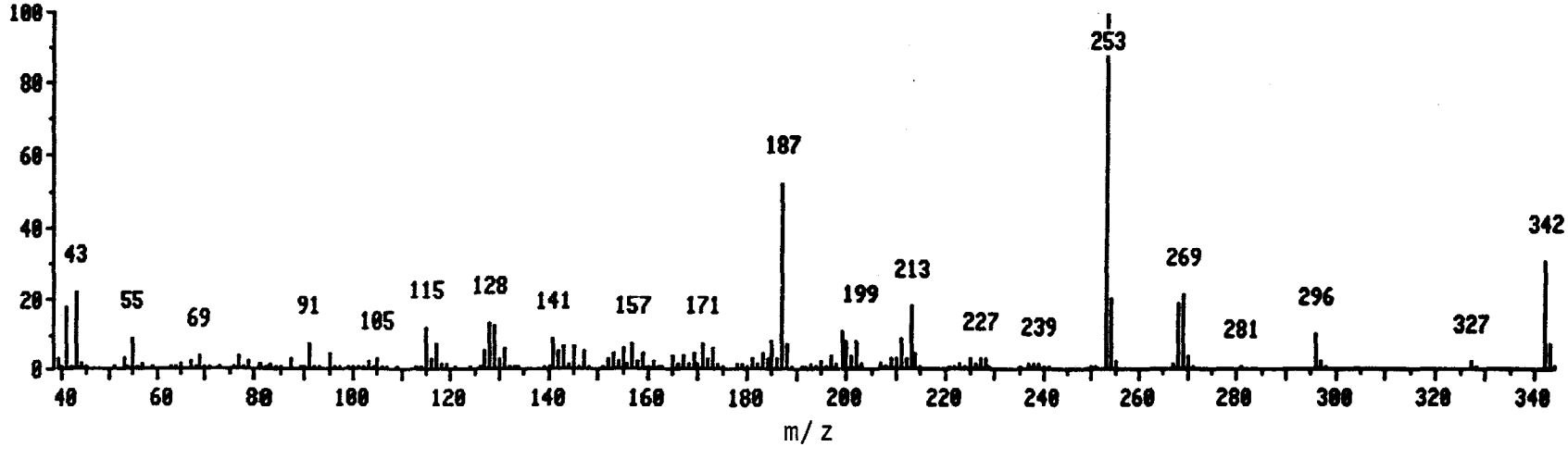

Ethyl Abietate



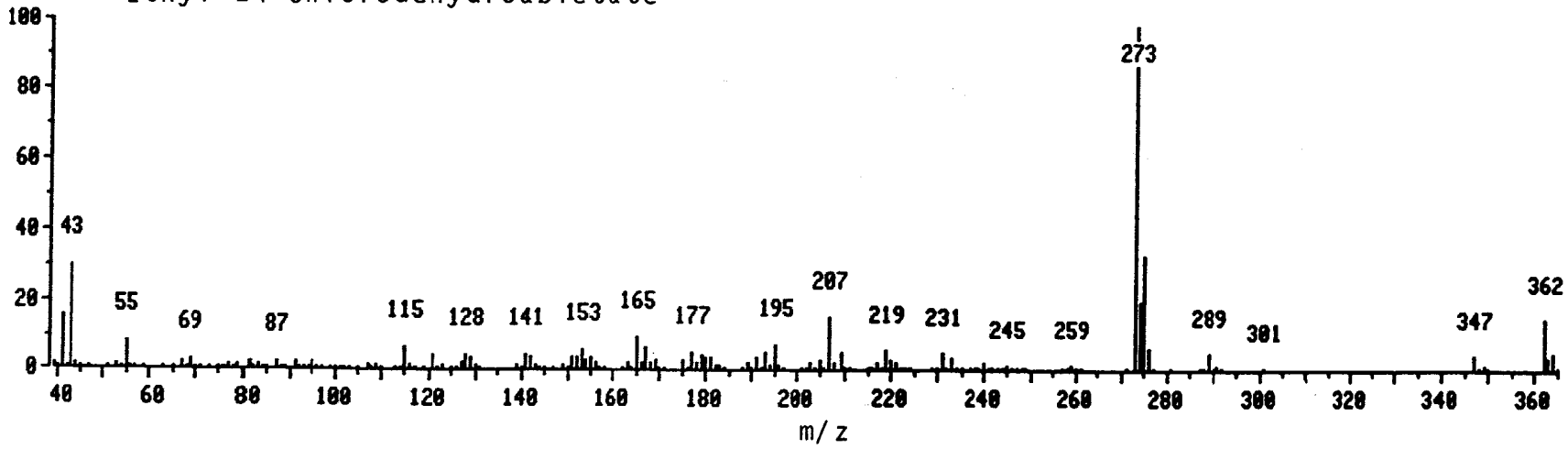
Ethyl Dehydroabietate



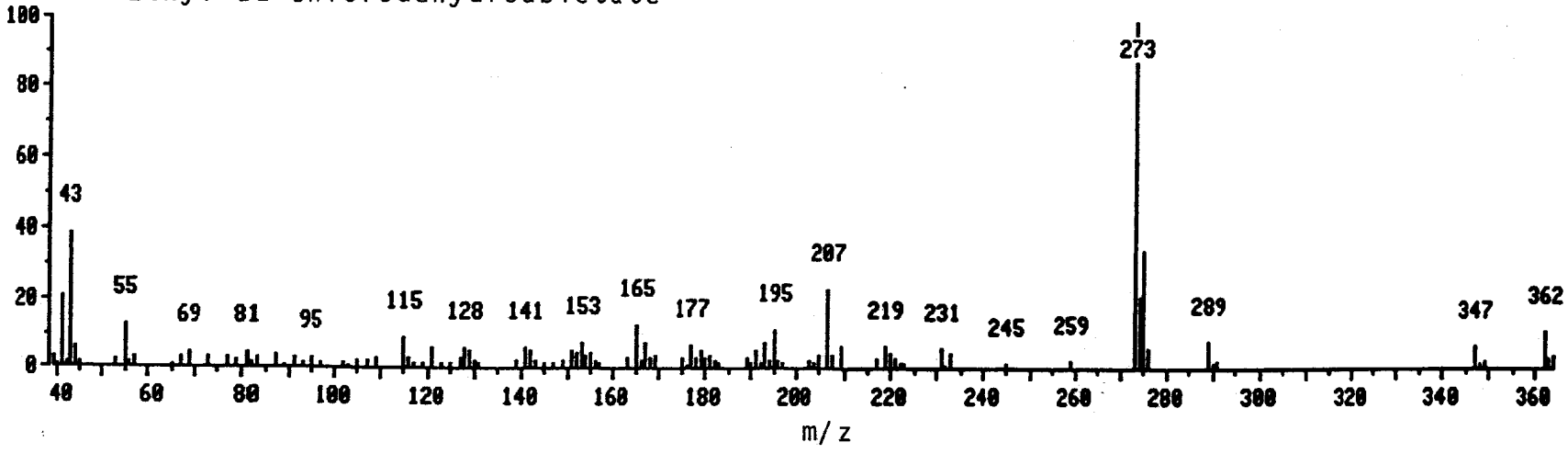

Ethyl 7-Oxodehydroabietate



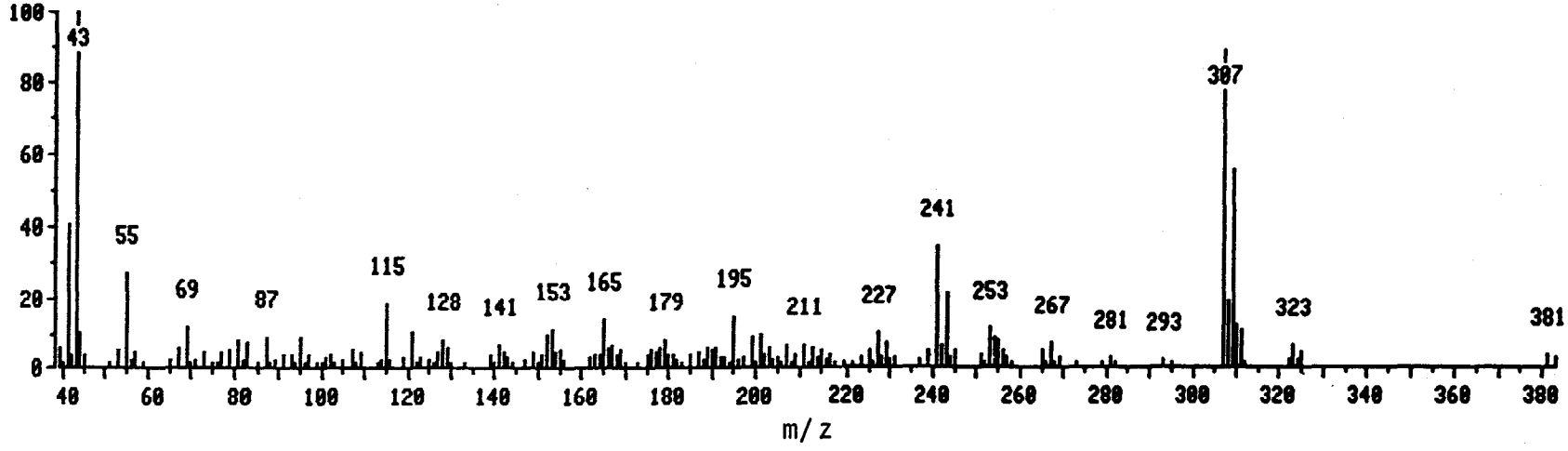
Ethyl 14-Chlorodehydroabietate



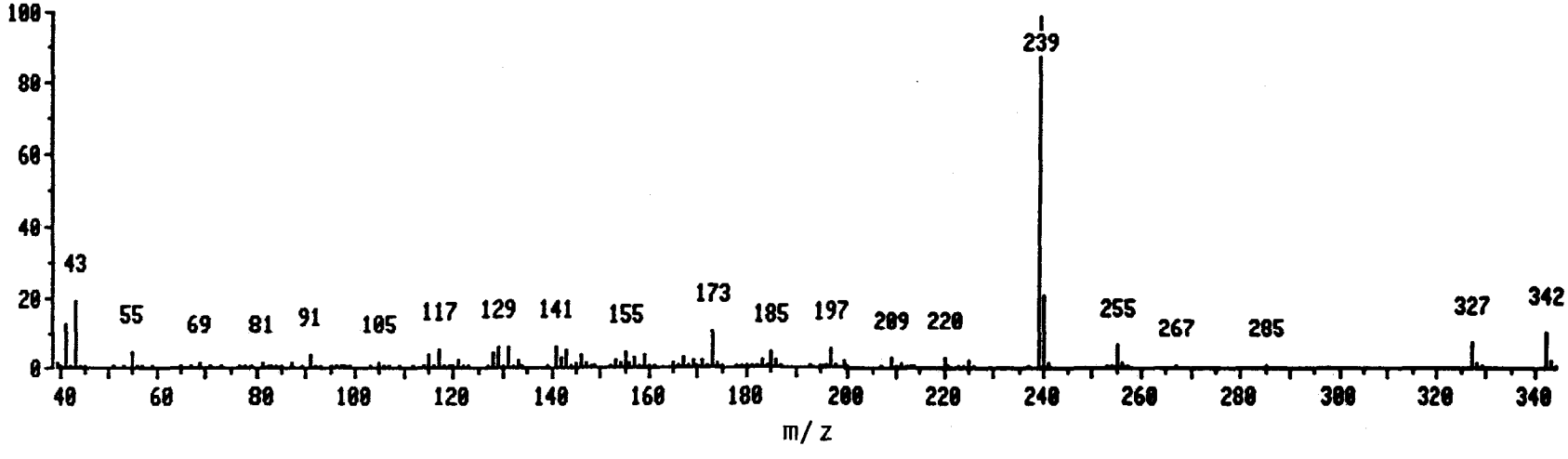

Ethyl 12-Chlorodehydroabietate



Ethyl 12,14-Dichlorodehydroabietate




n-Propyl Dehydroabietate



# APPENDIX D

# NCASI METHOD RA/FA-85.01

NCASI METHOD RA/FA - 85.01

# RESIN AND FATTY ACIDS BY EXTRACTION/ETHYLATION GC/FID AND GC/MS DETERMINATION

## 1.0 Scope and Application

1.1 The method is used to determine the concentration of resin and fatty acids in water samples. This method has been applied to the following compounds:

| Resin Acids | Fatty Acids |
|---|---|
| Pimaric Acid | Oleic Acid |
| Sandracopimaric Acid | Linoleic Acid |
| Isopimaric Acid | |
| Palustric Acid | |
| Abietic Acid | |
| Dehydroabietic Acid | |
| Neoabietic Acid | |
| 14-Chlorodehydroabietic Acid | |
| 12-Chlorodehydroabietic Acid | |
| Dichlorodehydroabietic Acid | |

1.2 This method has been used to analyze untreated and biologically treated pulp mill effluents, and receiving waters.

## 2.0 Summary of Method

2.1 Method RA/FA-85.01 provides extraction, derivatization, clean-up and gas chromatographic conditions for detecting ppb levels of resin and fatty acids. Prior to analysis, samples are extracted under acidic conditions with diethyl ether as a solvent using a separatory funnel. After concentration, the acids are converted to their ethyl esters using triethloxonium tetrafluoroborate as the derivatizing agent. After clean-up using an activated silica-gel column, the esters are determined by capillary gas chromatography using a flame ionization detector or mass spectrometer.

2.2 The sensitivity of the method usually depends on the level of interferences rather than on instrumental limitations. Table 1 lists the limits of detection that can be generally obtained in biologically treated effluent. Actual detection limits would have to be determined on each type of matrix.

TABLE 1 ESTIMATED FID METHOD DETECTION LIMIT

| Compound | MDL[a] (ppb) |
|---|---|
| Oleic Acid | 2.2 |
| Linoleic Acid | 0.8 |
| Pimaric Acid | 0.8 |
| Sandracopimaric Acid | 1.3 |
| Isopimaric Acid | 0.8 |
| Palustric Acid | 1.1 |
| Dehydroabietic Acid | 1.7 |
| Abietic Acid | 4.7 |
| Neoabietic Acid | 2.3 |
| 14-Chlorodehydroabietic Acid | 2.0 |
| Dichlorodehydroabietic Acid | 5.4 |

[a]MDL = Method Detection Limit

2.3 Where doubt exists over the identification of a peak on the gas chromatogram or when interfering peaks are found, confirmation techniques such as GC/MS should be used. The GC/MS method is summarized in Section 10.5.

## 3.0 Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware and other sample processing hardware. All these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory blanks as outlined in Section 9.1.

3.2 Glassware must be scrupulously cleaned. Clean all glassware by detergent washing with hot water and rinses with tap water. The glassware should then be drained dry and heated in a suitable oven at 400°C for at least 6 hours. After drying and cooling, the glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants.

3.3 Matrix interferences may be caused by other compounds that are co-extracted from the sample. The extent of these interferences, which have usually proved to be fatty acids, ($C_{20}$ and higher) will vary considerably from sample to sample, depending upon the matrix and diversity of the waste being sampled. In cases where the quantitation of the interfered compounds is critical, the GC/MS analytical procedure as outlined in Section 10.5 is employed.

## 4.0 Safety

4.1 The toxicity or carcinogenicity of chemicals used in this method has not been precisely defined; each chemical should

be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining awareness of OSHA regulations regarding safe handling of chemicals used in this method.

5.0 Apparatus and Equipment

5.1 Glassware

5.1.1 Separatory funnel: 500 mL, with Teflon stopper

5.1.2 Beaker: 400 mL

5.1.3 Funnel: 80 mm, glass powder

5.1.4 Erlenmeyer Flask: 250 mL

5.1.5 Graduated cylinders: 250 mL, 100 mL

5.1.6 Centrifuge tubes: 15 mL and 50 mL with Teflon lined screw cap

5.1.7 Centrifuge tube: 15 mL graduated conical with ground glass stopper

5.1.8 Concentrator tube, Kuderna-Danish: 15 mL

5.1.9 Evaporative flask, Kuderna-Danish: 250 mL attach to concentrator tube with springs

5.1.10 Snyder column, Kuderna-Danish: three-ball macro

5.1.11 Chromatographic column: Constructed by joining a 10 cm x 8.0 mm o.d., 6.0 mm i.d. glass tube to the bottom of a 10 cm test tube. The end of the tubing is tapered to give a small orifice

5.1.12 Vials: 2 dram with Teflon-lined screw caps

5.2 Boiling chips: Approximately 10/40 mesh carborundum

5.3 Analytical filter pulp: Used to plug chromatographic column (Schleicher & Schuell No. 289 or equivalent)

5.4 Pipets: Pasteur, glass, disposable with 1 mL latex bulb

5.5 Pipets: 1.0 mL volumetric or equivalent

5.6 Syringes: 100 µl, 250 µl, and 10 µl

5.7 Centrifuge: Bench top model

5.8 Water bath: Constant temperature capable of temperature control (± 2°C). The bath should be used in a hood

5.9 pH meter: Calibrated using two-point procedure

5.10 Drying oven: Set at 130 ±5°C.

5.11 Balance: Analytical, capable of weighing to the nearest 0.0001 g

5.12 Evaporation/concentration assembly: Pierce 19797 Uni-Vap Evaporator or equivalent

5.13 Gas chromatograph/detector system

5.13.1 Gas chromatograph: analytical system complete with a temperature-programmable gas chromatograph suitable for splitless injection and all required accessories including syringes, analytical columns, and gases.

5.13.2 Column: 30 m x 0.25 mm bonded-phase DB-5 fused silica capillary column (J&W Scientific or equivalent).

5.13.3 GC Detector: Flame ionization with appropriate data system.

5.13.4 Mass Spectrometer: Capable of scanning from 35 to 450 amu every 1 sec or less, utilizing 70 volts (nominal) electron energy in the electron impact ionization mode. A computer system must be interfaced to the mass spectrometer. The system must allow the continuous acquisition and storage on machine-readable media of all mass spectra obtained throughout the duration of the chromatographic program. The computer must have software that can search any GC/MS data file for ions of a specific mass and that can plot such ion abundances versus time or scan number. This type of plot is defined as an Extracted Ion Current Profile (EICP). Software must also be available that allows integrating the abundance in any EICP between specified time or scan number limits.

6.0 Reagents

6.1 Non-spectrograde, hexane, cyclohexane, methylene chloride distilled in glass (Burdick and Jackson)

6.2 Diethyl ether: Redistilled USP grade

6.3 Methanol: Redistilled reagent grade

6.4 Reagent water: Organic free such as produced by a Barnstead Model D2798 NANO-pure-A water purification system

6.5 Sodium hydroxide (5 percent): Dissolve 25 g NaOH in reagent water and dilute to 500 mL

6.6 Sulfuric acid (1:4): Slowly add 1 part concentrated $H_2SO_4$ to 4 parts reagent water

6.7 Indicator solution: Dissolve 10 mg of Sudan I and 150 mg of azulene in 20 mL hexane, store in Teflon-lined screw cap scintillation vial

6.8 Preservative solution: Dissolve 15 mg of Ethanox 702$^{tm}$ [methylene bis(di-t-butylphenol)] in 20 mL of hexane, store in Teflon-lined screw cap scintillation vial

6.9 Sodium sulfate: Anhydrous powder, store at 130°C

6.10 Silica gel: 70 to 150 mesh, activated, store at 130°C

6.11 Diisopropylethylamine (1M): Dissolve 3.88 g of diisopropylethylamine (Aldrich) in 30 mL of dichloromethane (Burdick & Jackson). The solution is stored in a Teflon-lined screw capped bottle in a freezer.

6.12 Triethyloxomium tetrafluoroborate (1M): Dissolve 6.09 g of triethyloxonium tetrafluoroborate (Fluka) in 32 mL of dichloromethane and store in a freezer. Caution: weighing should be conducted in a hood and gloves should be worn. The crystalline triethyloxonium tetrafluoroborate should be stored in a freezer. If the reagent becomes saturated with water, it can be washed with anhydrous ether since it is virtually insoluble.

6.13 Potassium Chloride (Saturated): Excess KCl is added to reagent water and is allowed to stand overnight. Solid KCl should remain present to insure the solution is saturated. If blank problems are traced to this reagent, the KCl can be baked at 400°C for several hours prior to preparing the solution.

6.14 Surrogate spiking solution: Prepare the stock solution by weighing (to the nearest 0.1 mg) 50 $\pm$5 mg of C17:0 and 50 $\pm$5 mg of 0-methylpodocarpic acid and dissolve to volume with methanol in a 50 mL ground-glass stoppered volumetric flask. Transfer the stock solution to an amber bottle with Teflon-lined screw cap and refrigerate at 4°C. Prepare the spiking solution by pipetting 5.0 mL of the stock solution into a 50 mL ground-glass stoppered volumetric flask and dilute to volume with methanol. Transfer the spiking solution into five 25 mL scintillation vials with Teflon-lined screw caps and refrigerate at 4°C.

6.15 Internal standard spiking solution: Prepare the

spiking solution by weighing (to the nearest 0.1 mg) 75 ±15 mg of n-propyl dehydroabietate and dissolve to volume with hexane in a 100 mL ground-glass stoppered volumetric flask. Transfer the spiking solution to five 25 mL scintillation vials with Teflon-lined screw caps and refrigerate at 4°C.

6.16 Calibration standard stock solution: Prepare stock solutions of individual compounds by weighing (to the nearest 0.1 mg) 25 ±1 mg of each compound of a known purity. Dissolve the material in methanol and bring to volume in a 25 mL ground-glass stoppered volumetric flask. Transfer the individual stock solutions to 25 mL scintillation vials with Teflon-lined screw caps and refrigerate at 4°C. Prepare the calibration stock solution by pipetting volumetrically the appropriate volumes of each stock solution into a single container, so that the final concentration of each compound is approximately 1 mg/mL. Mix the solution thoroughly and transfer it to four 25 mL scintillation vials with Teflon-lined screw caps and refrigerate at 4°C.

## 7.0 Sample Collection, Preservation and Storage

7.1 Collection: Grab samples must be collected in glass containers having a Teflon-lined screw cap. Automatic sampling equipment which comes in contact with the sample should be constructed of glass, Teflon, or stainless steel. Composite samples should be refrigerated during the sampling period.

7.2 Preservation: All samples must be preserved by adjusting to pH 10, with NaOH, and refrigerating. This should be done as soon as possible after sample collection. Samples must be shipped in iced containers as quickly as possible.

7.3 Storage: Samples may be stored in the refrigerator (4°C) for up to 30 days.

## 8.0 Calibration

8.1 Calibration Standard: Prepare a calibration standard by adding 1.0 mL of the surrogate spiking solution (Section 6.14) and 1.0 mL of the calibration standard stock solution (Section 6.16) to a 15 mL Teflon-lined screw cap centrifuge tube. Proceed with the derivatization as outlined in Section 10.2. After the standard has been derivatized, add 100 µL of the internal standard spiking solution (Section 6.15) and 200 µL of the preservative solution (Section 6.8). Adjust the volume of the derivatized extract to 1.0 mL and transfer it to a labeled two dram vial with Teflon-lined screw cap.

8.2 Daily Calibration: Prior to the analysis of samples, analyze 1.0 µL of the calibration standard. Tabulate peak areas for each compound and calculate relative response factors (RRF) as follows:

$$RRF = \left(\frac{A_{is}}{C_{is}}\right) \quad X \quad \left(\frac{C_s}{A_s}\right)$$

where: $A_s$ = Response of the compound of interest
$A_{is}$ = Response of the internal standard
$C_{is}$ = Concentration of the internal standard
$C_s$ = Concentration of the compound of interest

If the RRF of any of the compounds falls outside the acceptable ranges, as shown in Table 2 the calibration standard must be re-analyzed. If the standard fails to pass the established criteria a second time, analysis must stop until the problem has been solved.

TABLE 2 RESIN ACID RESPONSE FACTOR ACCEPTABLE RANGES GC/FID PROCEDURE

| Compound | Lower Limit | Upper Limit | Mean |
|---|---|---|---|
| Oleic Acid | 0.70 | 0.82 | 0.76 |
| Linoleic Acid | 0.77 | 0.95 | 0.86 |
| Pimaric Acid | 0.77 | 0.89 | 0.83 |
| Sandracopimaric Acid | 0.79 | 0.89 | 0.84 |
| Isopimaric Acid | 0.79 | 0.90 | 0.85 |
| Palustric Acid | 0.81 | 0.95 | 0.88 |
| Dehydroabietic Acid | 0.84 | 0.94 | 0.89 |
| Abietic Acid | 0.80 | 0.95 | 0.88 |
| Neoabietic Acid | 0.86 | 1.02 | 0.94 |
| 14-Chlorodehydroabietic Acid | 1.05 | 1.24 | 1.14 |
| 12-Chlorodehydroabietic Acid | 1.01 | 1.20 | 1.11 |
| Dichlorodehydroabietic Acid | 1.08 | 1.36 | 1.22 |
| n-Heptadecanoic Acid | 0.77 | 0.97 | 0.87 |
| O-Methylpodocarpic Acid | 0.88 | 1.00 | 0.94 |

## 9.0 Quality Control

9.1 Blanks: Before processing any samples or whenever a new reagent is prepared, the analyst should demonstrate through the analysis of a blank that utilizes all glassware and reagents required for sample analyses that all materials are interference free. The blank samples should be carried through all stages of the sample preparation and measurement.

9.2 Frequency: A minimum of ten percent of routine samples should be allocated for quality control. In addition to

this, representative samples from each new or untested source or sample matrix should be treated as a quality control sample. Laboratory replicates and fortification should be conducted on each quality control sample to document method performance as indicated by precision and recovery.

9.3 Replicates: Replicates consist of running two or more separate aliquots of the sample through the entire analytical procedure. The concentration determined for each analyte, the range of concentrations determined by the replicates and the mean should be tabulated in a method precision log.

9.4 Recovery: Using the mean concentration determined by the replicate analyses, determine the spiking level which will give a maximum of three times the background. Spike the sample with the determined amount of the calibration standard working solution and proceed to analyze the sample in the normal manner. Using the results of that analysis, calculate the percent recovery as follows:

$$\text{Percent Recovery} = \frac{\text{Level Measured - Background}}{\text{Level Spiked}} \times 100$$

where the background is the mean of the replicate determinations described above.

9.5 Surrogate spike recovery: The laboratory is required to spike all samples with the surrogate standard (Section 6.14) to monitor spike recoveries. The spiking level used should be two to three times the level of analytes expected to be found in the sample. The acceptable range of recoveries for the surrogate spikes is 65 to 120 percent. If the recovery for any surrogate does not fall within these limits, steps must be taken to isolate the problem before proceeding any further. The calculations for recovery are:

$$\text{Percent Recovery} = \frac{\text{level of surrogate measured}}{\text{level of surrogate added}} \times 100$$

10.0 Procedures

10.1 Extraction: Remove the sample and surrogate spiking solution from the refrigerator and allow them to come to room temperature. Shake the sample to insure homogeneity and transfer a 250 mL aliquot to a 400 mL beaker. Add the appropriate amount of surrogate spike solution to the sample. Adjust the sample to pH 5.0 $\pm$ 0.2, using the 5 percent NaOH and 1:4 $H_2SO_4$. Transfer the sample to a 500 mL separatory funnel. Add 75 mL of diethyl ether to the beaker, swirl and add to the separatory funnel. Shake the sample vigorously for 1 minute with frequent venting. After the phases have separated drain off the aqueous layer

into the beaker and transfer the ether emulsion into two 50 mL centrifuge tubes. Cap the centrifuge tubes and centrifuge at high speed for 2 to 3 minutes. Using a disposable Pasteur pipette with a 1 mL latex bulb, transfer the ether layer to a 250 mL Erlenmeyer flask. Transfer the remaining aqueous layer to the 400 mL beaker. Use reagent water (sparingly) to wash out the centrifuge tubes, adding the wash to the beaker. Return the sample to the separatory funneland repeat the extraction two more times using 50 mL of diethyl ether each time. The total volume of diethyl ether used to extract the sample at pH 5.0 $\pm$ 0.2 should be 175 mL (75mL, 50mL, 50mL).

After the third extraction at pH 5.0, re-adjust the pH of the sample to 2.0 $\pm$ 0.2 with 1:4 $H_2SO_4$. Transfer the sample to the separatory funnel and extract two times using 50 mL of diethyl ether each time combining all the ether extracts. Transfer the ether extract to the Kuderna-Danish assembly, rinsing the Erlenmeyer with 20 to 30 mL of diethyl ether. Combine the diethyl ether washes with the sample in the Kuderna-Danish. Add 1 to 2 carborundum boiling chips and secure the assembly in the water bath. Watch the sample carefully and remove from the water bath when the Kuderna-Danish receiving tube is about $\frac{1}{4}$ full. Do not allow the sample to go to dryness. Transfer the concentrated extract to a 15 mL centrifuge tube using three 1 mL ether rinses. Cap the tube, label, and store in the refrigerator. The extract can be stored in this manner for 35 days.

10.2 Derivatization: Concentrate the extract to approximately 0.2 to 0.3 mL using the evaporation/concentrator assembly with a stream of dry nitrogen. If the extract or standard contains methanol, add about 0.5 mL of cyclohexane and re-concentrate. Repeat this process until the solvent is made up solely of cyclohexane. Add 1.0 mL of the diisopropylethylamine (Section 6.11) followed by 1.0 mL of the triethyloxonium tetrafluoroborate (Section 6.12). Cap the tube and shake for 30 seconds. Add 1.0 mL of the saturated KCl solution (Section 6.13), cap the tube and shake. Add 1.0 mL of hexane and shake for one minute. Centrifuge the sample if an emulsion forms. Withdraw the organic phase using a Pasteur pipette and transfer to a 15 mL graduated conical centrifuge tube. Repeat the hexane extraction with two additional 1 mL portions of hexane. Combine the organic phases and concentrate using the evaporator/concentrator assembly to approximately 0.4 mL.

10.3 Column clean-up: Prepare the chromatographic columns by gently pushing a plug of filter pulp to the bottom, taking care not to pack it too tightly. Dry pack the column with 4.0 cm of activated silica gel, gently tapping the column sides while packing. Add 1.0 cm of powdered anyhydrous sodium sulfate to the top of the silica gel. Store the packed columns in the drying oven at 130°C until needed. Remove the column from the drying

oven and allow it to cool to room temperature. Add 20 µL of the indicator solution (Section 6.7) to the concentrated hexane extract of the derivatized sample. Place a 15 mL screw-cap centrifuge tube as a receiver and pre-elute the column with 1 mL of hexane. Just prior to exposure of the sodium sulfate layer to the air, transfer the sample to the column, using two hexane washes of 0.2 to 0.3 mL each. Rinse the sample tube with 0.2 to 0.3 mL of 95 percent hexane/5 percent diethyl ether (v/v), adding it to the column prior to the exposure of the sodium sulfate layer. When this wash drops down to the sodium sulfate fill the column reservoir with 95 percent hexane/5 percent diethyl ether. Maintain the reservoir full with this solvent mixture throughout the remainder of the clean-up procedure.

Just prior to the azulene (present as a narrow purple band) reaching the bottom of the silica gel packing, replace the screw cap centrifuge tube receiver with the 15 mL graduated centrifuge tube. Continue to collect eluant in this receiver until the Sudan I (present as a broad orange band) begins to elute from the column. At this time replace the receiver with the original screw cap centrifuge tube and continue to collect another 7 to 10 mL of eluant. The azulene should begin to elute after passing 3 to 5 mL of the solvent mixture. The Sudan I should begin to elute within 5 to 8 mL after the azulene.

Add the appropriate amount of internal standard spiking solution (Section 6.15) and 200 µL of the Ethanox 702 preservative solution (Section 6.8) to the 15 mL graduated centrifuge tube which contains the azulene and the compounds of interest. Concentrate this fraction using a stream of dry nitrogen to 0.2 to 0.3 mL. Cap both fractions, label appropriately and store in the refrigerator.

10.4 GC/FID Procedure

10.4.1 Gas Chromatography Conditions: The recommended gas chromatographic operating conditions for the instrument are: Hydrogen carrier gas ($\mu$ = 42 cm/sec at 200°C), Nitrogen detector make-up gas 30 mL/min. The injection port temperature is 280°C and the oven is programmed from an initial temperature of 130°C after a 1 minute hold at 6°C/min for 6 minutes and then 2°C/min to 280°C holding for 10 minutes. The detector temperature is 320°C.

10.4.2 Analysis: Inject 1 to 2 µL of the sample extract. Tabulate peak areas for each compound of interest. If the peak area of any of the compounds exceeds the linear range of the instrument the extract must be diluted and re-analyzed. Using the internal standard technique (Section 11.0) calculate the concentration for each compound. An example of a GC/FID chromatogram for resin and fatty acids is shown in Figure 1.

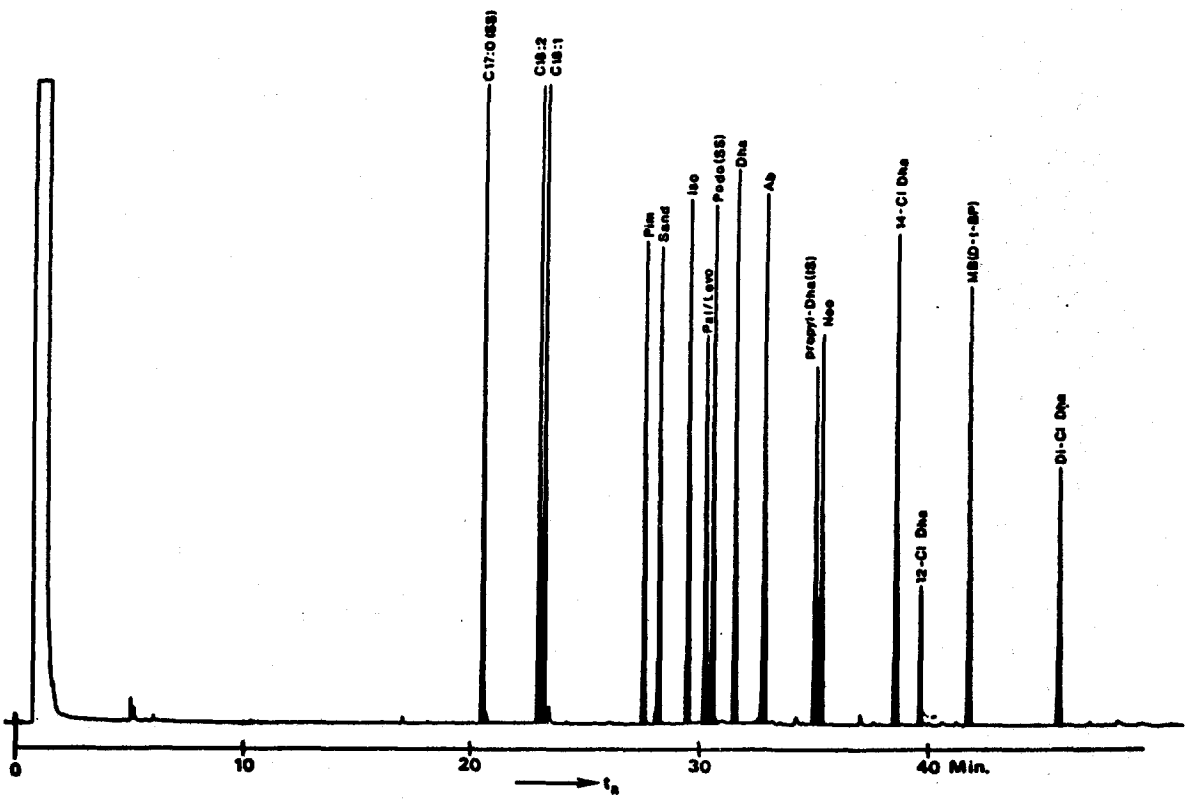



FIGURE 1

FID CHROMATOGRAM OF RESIN AND FATTY ACID ETHYL ESTERS
TEMP. PROGRAM - 130°C (1 min) $\xrightarrow{6°C/min\ (6\ min)}$ $\xrightarrow{2°C/min}$ 280°C
INJ. PORT TEMP. - 280°C DETECTOR TEMP. - 320°C

10.5 GC/MS Procedure

10.5.1 Extraction and derivatization: The extraction and derivatization steps are the same as outlined in Sections 10.1 and 10.2. The silica-gel clean-up step (Section 10.3) may be omitted entirely if the sample is designated for GC/MS analysis only. The internal standard is added after derivatization and the extract is concentrated to a suitable volume.

10.5.2 Calibration: Prior to any analysis of standards or samples, the mass spectrometer must be tuned in such a manner that a mass spectrum of DFTPP, meeting all criteria in Table 3, can be obtained.

Preparation of the calibration standard is identical to the GC/FID procedure outlined in Section 8.1. The relative response factors are calculated using the integrated areas of extracted ion current profiles (EICP) for the characteristic ions shown in Table 4.

TABLE 3 DETPP KEY MASSES AND ABUNDANCE CRITERIA[ab]

| Mass | Ion Abundance Criteria |
|---|---|
| 51 | 30 to 60% of mass 198 |
| 68 | Less than 2% of mass 69 |
| 70 | Less than 2% of mass 69 |
| 127 | 40 to 60% of mass 198 |
| 197 | Less than 1% of mass 198 |
| 198 | Base peak, 100% relative abundance |
| 199 | 5 to 9% of mass 198 |
| 275 | 10 to 30% of mass 198 |
| 365 | Greater than 1% of mass 198 |
| 441 | Present but less than mass 443 |
| 442 | Greater than 40% of mass 198 |
| 443 | 17 to 23% of mass 442 |

[a] J.W. Eichelberger, L.E. Harris, and W.L. Budde, "Reference Compound To Calibrate Ion Abundance Measurement In Gas Chromatography-Mass Spectrometry." Analytical Chemistry 47,995 (1975).

[b] 50 ng DFTPP injected using a Grob type splitless injection and the following gas chromatographic conditions: Injection port temperature 280°C, oven programmed from 160°C after a 1 minute hold at 6°C/minute to 210°C. Mass spectrometer conditions are set to scan from 45 to 445 amu at 216.7 amu/sec.

10.5.3 GC/MS operating conditions: The recommended operating conditions for the instrument are: Helium carrier gas (36 cm/sec at 200°C), injection port temperature is 280°C and the oven is programmed from an initial temperature of 140°C after a 1 minute hold at 4°C/min to 280°C holding for 20 minutes. The mass scan range is m/z 50 to 400 and the scan speed is 162.5 amu/sec. An example of a GC/MS chromatogram is shown in Figure 2.

TABLE 4 GC/MS QUANTITATION IONS

| Compound | Quantitation Ions | |
|---|---|---|
| | Primary | Secondary |
| Ethyl Oleate | 55 | 264, 310 |
| Ethyl Linoleate | 55 | 67, 308 |
| Ethyl Pimarate | 121 | 330 |
| Ethyl Sandracopimarate | 121 | 330 |
| Ethyl Isopimarate | 241 | 256, 330 |
| Ethyl Palustrate | 241 | 315, 330 |
| Ethyl Dehydroabietate | 239 | 328 |
| Ethyl Abietate | 241 | 256, 330 |
| Ethyl Neoabietate | 135 | 330 |
| Ethyl 14-Chlorodehydroabietate | 273 | 275 |
| Ethyl 12-Chlorodehydroabietate | 273 | 275 |
| Ethyl Dichlorodehydroabietate | 307 | 309 |
| Ethyl Heptadecanoate (SS) | 88 | 101 |
| Ethyl O-Methylpodocarpate (SS) | 227 | 316 |
| Propyl Dehydroabietate (IS) | 239 | |

10.5.4 Analysis: The extract is analyzed by splitless injection on a 30 m DB-5 column using helium as the carrier gas.

10.5.5 Qualitative identification: Obtain EICPs for the primary ions and the secondary ions listed in Table 4. The following criteria must be met in order to make a qualitative identification. The characteristic masses of each compound of interest must maximize in the same or within two scans of each other. The retention time must fall within ± 30 seconds of the retention time of the authentic compound. The relative peak areas of the characteristic masses in the EICPs must fall within ± 20 percent of the relative intensities of these masses in a reference compound obtained from the previously analyzed calibration standard.

10.5.6 Quantitation: Using the internal standard technique as outlined in Section 11.0, calculate the concentration, using the peak areas of each primary ion. If the sample produces an interference for the primary ion, use a secondary characteristic ion for quantitation.

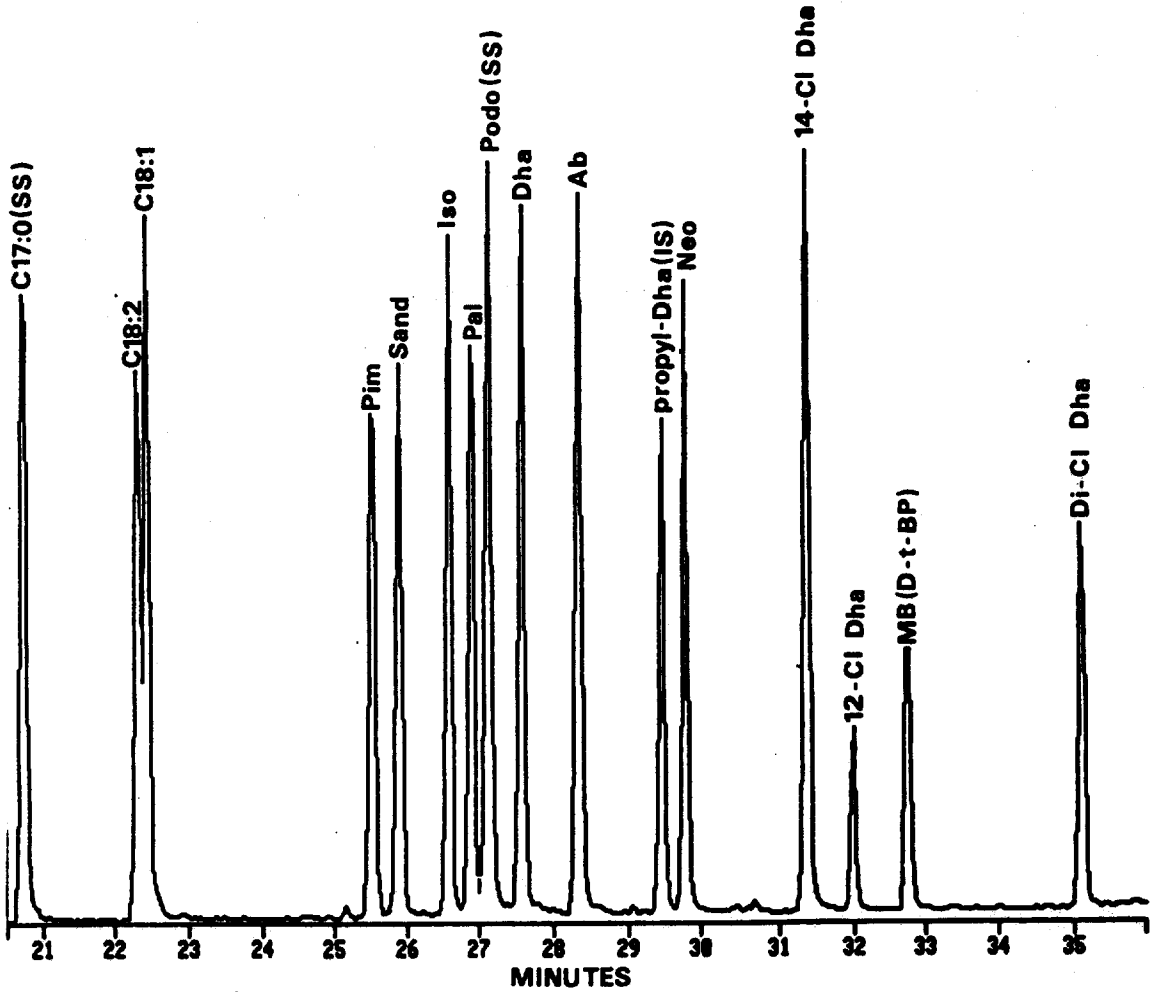



FIGURE 2

GC/MS TOTAL ION CHROMATOGRAM OF RESIN AND FATTY ACID ETHYL ESTERS. CONDITIONS: 140°C (1 MIN) $\xrightarrow{4°/MIN}$ 280°C INJECTION PORT TEMPERATURE - 280°C, MASS SCAN RANGE m/z = 50 to 400, SCAN SPEED 162.5 AMU/Sec

## 11.0 Calculations

11.1 Compound Concentration: Calculate the concentrations for each compound as follows:

$$\text{Conc. } (\mu g/L) = (A_s) \times (RRF) \times (C_{is}/A_{is})$$

Where:
$A_s$ = Response of the compound of interest
RRF = Relative response factor of compound of interest
$C_{is}$ = Concentration of the internal standard (µg/L)
$A_{is}$ = Response of the internal standard