NoSolics

Solid-phase extraction can rapidly separate lipid classes from acetone extracts of wood and pulp

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ABSTRACT: Solid-phase extraction was used to separate and quantify lipid classes in acetone extracts of wood and pulp. The method involves absorption of wood or pulp extracts onto an aminopropyl phase column, with each lipid class eluted by a sequence of solvents. Initially, the separation effectiveness and the required volume of solvents was monitored by thin-layer chromatography (TLC), and the purity of each class was confirmed by gas chromatography (GC). Once standard test conditions were established, TLC and GC were required infrequently, e.g., when sample sources were changed. The method is simple, rapid, quantitative, and uses small amounts of sample and solvents. This procedure could easily be implemented in research and in the pulp and paper industry.

KEYWORDS: Acetone, extraction, extractives, lipids, pitch, pulps, quantitative analysis, solid phases, wood.

Extractives in softwoods and hardwoods are liberated from wood chips at different stages of processing. These extractives can be deposited as pitch, either alone or in combination with fibers, fillers, defoaming agents, or coating binders *(1–3)*. They cause significant technical and economic problems for pulp and paper manufacturers, regardless of the pulping and bleaching procedures used, and have a detrimental impact on the environment *(1, 4–8)*.

Extractives are removed from both wood and pulp by organic solvents such as acetone, ethanol, ether, or dichloromethane. They are composed principally of lipids and related compounds, e.g., glycerides, steryl esters, fatty acids, resin acids, fatty alcohols, sterols, and alkanes *(9, 10)*. The concentration and composition of such lipid mixtures vary among wood species, within and among trees, with tree age, and with environmental conditions *(1)*.

Seasoned wood chips exhibit fewer pitch problems. During chip storage, extractives undergo volatilization, enzymatic hydrolysis, and air oxidation. Unfortunately, these reactions are slow, particularly in the colder months of the year. Further, long storage times inflate costs and promote microbial deterioration *(5, 11)*.

A biological pretreatment using a fungal inoculum [Cartapip™ (Sandoz)] has been developed to reduce extractives levels with a minimal amount of storage time *(12, 13)*. However, this fungus is poorly adapted to low temperatures and to some of the wood species used in the Canadian pulp industry. Efforts to identify microorganisms that are suited to Canadian conditions could be accelerated by developing a rapid, simple, quantitative method for separating and analyzing the complex lipid mixtures in wood chips. Such a method would be useful in understanding and optimizing reactions with wood extractives during biological pretreatments.

Traditional methods for fractionating and quantitatively analyzing extractives are time consuming. Such methods involve separating free resin

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and fatty acids by ion-exchange chromatography, saponifying the neutral fraction, and finally methylating the acid fractions prior to gas chromatography (GC). Faster methods for screening large numbers of samples for lipids have been reported in the literature *(14–17)*. Unfortunately, none allow the major fractions of wood extractives—fatty and resin acids, sterols, fatty alcohols, and mono-, di-, and triglycerides—to be separated, recovered, and quantified from a single sorbent matrix.

In this report, we describe a method for rapidly separating wood extractives into five different classes, then recovering and quantifying them. The method is a modified version of a solid-phase extraction (SPE) that was originally developed for pharmaceutical purposes *(18, 19)*. SPE has been used on pulp-mill process streams, but the application has been limited to concentration and isolation of a single lipid class—fatty and resin acids—prior to identification of individual compounds by GC *(20, 21)*.

In the work presented here, acetone extracts of wood or pulp were absorbed onto an aminopropyl phase column (Bond Elut). Five classes of wood extractives were eluted by a sequence of solvents. Each class could subsequently be recovered and quantified directly or analyzed further by GC and high-pressure liquid chromatography (HPLC). We present and discuss results for standard lipid mixtures and extracts of wood and pulp.

Experimental

Reagents

Trilinolein (C18:2,[cis, cis]-9,12) (99%), palmitic acid oleyl ester (99%), cholesteryl stearate (18:0) (99%), linoleic acid (C18:2) (99%), linoleic acid methyl ester (99%), arachidic acid (20:0) (99%), arachidic acid methyl ester (99%), 1-monopalmitoyl-*rac*-glycerol (99%), 1,3-dierucin (C22:1,[cis]-13) (99%), ß-sitosterol (>97%), ß-sitosteryl acetate (97%), and stearyl alcohol (99%) were obtained from Sigma Chemical Co. Ammonium molybdate (VI) tetrahydrate and sulfuric acid (95–98%) were obtained from Aldrich Chemical Co. Acetic anhydride AnalaR™ (98%) and pyridine AnalaR™ were obtained from BDH Inc. The solvents listed in **Table I** were all HPLC grade and were obtained from Aldrich.

I. Solvent systems and eluted lipid fractions

Fraction	Solvent	Solvent ratio, v/v [a]	Amount of solvent, mL	Lipid eluted [b]
Preliminary fractions				
A	$CHCl_3$:Hexane	(1:5)	7	TG, SE, W
B	Ether:Hexane	(8:1)	6	S, DG, MG, Fal
Final fractions				
C	Ether:AcOH	(98:2)	6	FA and/or RA
D	Hexane	...	12	SE, W
E	Ether	...	6	TG
F	Ether:Hexane	(2:8)	10	S, DG, Fal
G	Ether:MeOH	(2:1)	6	MG

[a]Volume ratio
[b]Acronyms: DG, diglycerides; FA, fatty acids; Fal, fatty alcohols; MG, monoglycerides; RA, resin acids; S, sterols; SE, steryl esters; TG, triglycerides; W, waxes.

Equipment

A Bond Elut/Vac Elut system and Bond Elut extraction cartridges (Analytichem International, Harbor City, CA) were used in this study. A Hewlett–Packard HP 5890 series II gas chromatograph equipped with HP 7673 auto injector and a flame-ionization detector was used for purity analysis. A Soxhlet extraction unit was obtained from Canadian Scientific Glassblowing Co. Thin-layer chromatograph (TLC) plates (250-µm layer, AL SIL G/UV) were from Whatman Ltd. The evaporator (TurboVap™) was from Zymark Ltd.

Preparation of standards

Different lipids and related compounds, representative of wood extractives, were selected as a standard solution to develop our analytical procedure. The standard solution included

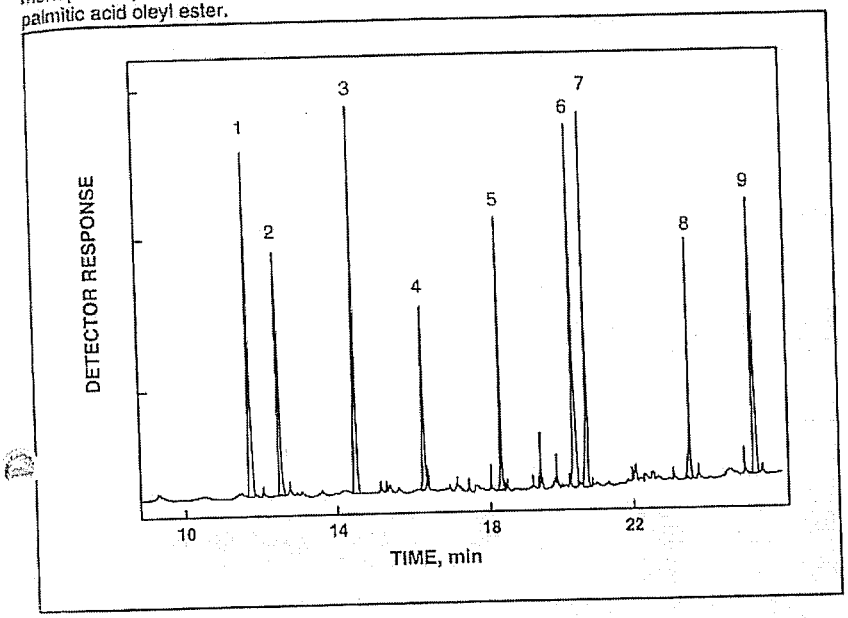
- Saturated and unsaturated fatty acids with linoleic acid (8 mg) and arachidic acid (2 mg)
- Tri-, di-, and monoglycerides with trilinolein (25 mg), 1,3-dierucin (5 mg), and 1-monopalmitoyl-*rac*-glycerol (5 mg)
- Sterol with ß-sitosterol (5 mg)
- Steryl ester with cholesteryl stearate (10 mg)
- Fatty alcohol with stearyl alcohol (5 mg)
- Fatty alcohol ester or wax with palmitic acid oleyl ester (5 mg).

The standard chemicals were dissolved in chloroform (3 mL) and stored under nitrogen at –4°C for no longer than a week.

Extracts from wood and pulp

A fresh aspen log (Northwood Pulp and Timber Ltd.) was removed from an area near Eagle Lake, some 30 km

1. Separation of standard lipids mixture on fused-silica capillary column. Legend: 1, linoleic acid methyl ester; 2, acetic acid stearyl ester; 3, arachidic acid methyl ester; 4, trilinolein; 5, monopalmitoyl-*rac*-glycerol; 6, dierucin; 7, cholesteryl stearate; 8, ß-sitosteryl acetate; 9, palmitic acid oleyl ester.



east of Prince George, BC. A lodgepole pine tree was removed from the Alex Fraser University of British Columbia Research Forest Centre. The logs were hand-debarked and chipped, and the wood chips were screened, mixed, and stored at –20°C. The frozen chips were milled in a blender and screened through 1.0-mm mesh. One gram of sawdust was placed in an extraction thimble and extracted with 120 mL of acetone for 6 h using a Soxhlet extraction apparatus (8 cycles/h). The extraction solution was evaporated to dryness using a TurboVap.

One gram of aspen thermomechanical pulp (TMP), produced from fresh, sound aspen chips in the Vancouver laboratory of Paprican, was extracted in the same manner as the sawdust. The TMP was prepared as follows: Chips were presteamed for 15 min, heated for 5 min at 125°C at a pressure of 110 kPa, and then defibrated through a pressurized laboratory refiner (Sunds Defibrator). The final consistency was about 20%.

Purity analysis

TLC was used to monitor the separation of the lipid fractions from the sorbent matrix and to track the amount of solvent required to remove each fraction. Samples were spotted on a TLC plate using a capillary glass tube. The plate was developed in hexane–diethyl ether–acetic acid (70:30:1, v/v/v) and air-dried for a few minutes. The separated spots were visualized by spraying on a molybdate oxidizing solution consisting of ammonium molybdate (VI) tetrahydrate (5 g) in sulfuric acid (10 mL) and ethanol (90 mL), and then heating the plate at 150°C.

The purity of each fraction isolated from the standard mixture was further analyzed by GC. Wood extractives were not analyzed for purity because the sample mixture was too complex and contained unknowns. However, GC analysis of resin and fatty acids gave values approximately equal to those obtained by gravimetric analysis.

Derivatization and GC analysis of fatty and resin acids. Two mL of diazomethane ether solution and one drop of absolute methanol were added to the dried fraction containing the fatty and resin acids. After 10 min, as the color of the reaction was still yellow, the ether was evaporated under nitrogen and the fatty and resin acids methyl esters were redissolved in 1.0 mL of ethyl acetate. The purity was then analyzed by GC using a DB-5 fused-silica capillary column (ID, 0.25 mm; length, 30 m) and the following temperature program: 160°C for 2 min, raising the temperature 7°C/min to 310°C and holding for 3.5 min. A typical chromatograph for a standard mixture is shown in Fig. 1.

Derivatization and GC analysis of sterols, fatty alcohols, and di- or monoglycerides. To each dried fraction of sterols, fatty alcohols, and di- or monoglycerides, 2 mL of acetic anhydride/pyridine (5:1, v/v) was added. The solutions were heated in a water bath at 90°C for 2 h. The solvent was partially evaporated under a nitrogen stream to a final volume of 1.0 mL. If the final volume was less than 1.0 mL, ethyl acetate was added, and fractions of 1 µL or more were analyzed by GC (using the same column and conditions as described for the fatty and resin acids) to check their purity.

GC analysis of steryl esters, fatty alcohol esters, and triglycerides. The dried fractions of steryl esters, fatty alcohol esters (waxes), and triglycerides were redissolved in 1.0 mL of hexane for GC analysis using the same column and conditions as described previously.

Results and discussion

Development of separation technique using model compounds

Before applying solid-phase extraction (SPE) to wood or pulp extracts, we modified and optimized the lipid separation method of Kaluzny *(18)* for a standard lipid mixture representative of the major components present in wood extractives. However, the major wood steryl ester, ß-sitosteryl ester, was not available commercially and was replaced by a closely related compound, cholesteryl ester.

The mixture was fractionated as follows. The Bond Elut cartridge column, conditioned with hexane (2 x 2 mL), was loaded with 0.5 mL of the standard lipid mixture in chloroform, and a vacuum of 5 kPa was applied. The column was sequentially eluted as outlined in Fig. 2 using the solvents listed in Table I. The steryl esters, fatty alcohol esters, and triglycerides were recovered in the first fraction and were saved as Fraction A for the next separation step. The mixture of sterol, fatty alcohol, diglyceride, and monoglyceride was eluted with a mixture of ether and hexane and saved as Fraction B. Finally, the remaining fatty acid and resin acid was removed from the column with Solvent C and saved as Fraction C.

Fraction A was dried under nitrogen, redissolved in 1 mL of hexane, and bonded to a new column that had been conditioned as described. Solvent D removed from this column the steryl ester and the fatty alcohol ester, which were saved as Fraction D. Solvent E eluted the triglyceride, which was saved as Fraction E.

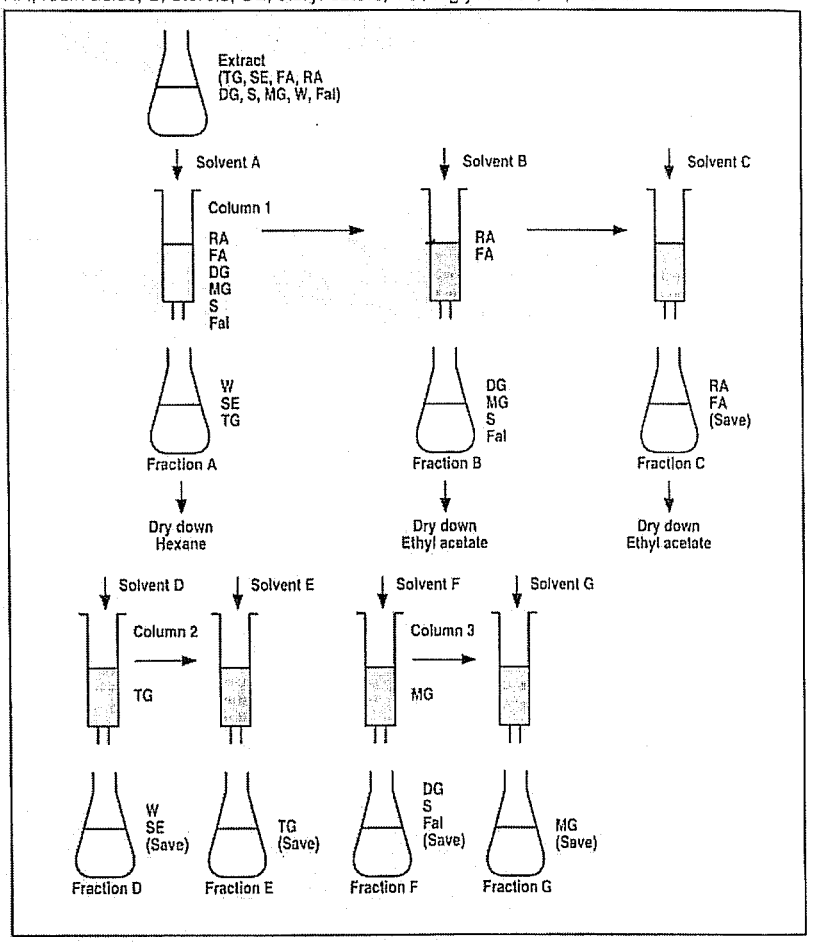
Fraction B was dried under nitrogen and reconstituted in 0.5 mL ethyl acetate before being loaded into a newly conditioned column. Solvent F was passed through the column, and the eluate containing sterol, fatty alcohol, and diglyceride was saved as Fraction F. Finally, the monoglyceride was desorbed and removed from the column using Solvent G, and the eluate was saved as Fraction G.

Each isolated fraction was dried under nitrogen and weighed to calculate its recovery. The results are presented in **Table II**. During the development of the separation, the composition of each eluted fraction was monitored by TLC, as seen in Fig. 3. The volume of solvent required was carefully recorded. The purity of each isolated lipid class was confirmed by GC.

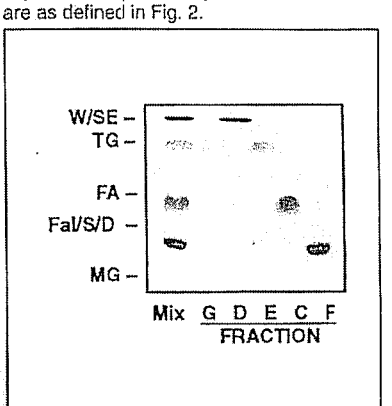
Using our solvent system, we were unable to separate the sterol from the diglyceride and the fatty alcohol (Fig. 3, Fraction F). As reported by Kaluzny *(18)*, it is very difficult to separate the diglycerides from the sterols because of the similar free hydroxyl group present in both compounds that bonds strongly to the column. However, most of the individual compounds present in this fraction have been successfully separated and quantified by GC.

GC analysis and the derivatization step it requires were needed while the method was being developed. However, when the order and volume of solvents had been established, only infrequent verifications were necessary, e.g., when the wood species was changed.

2. Elution sequence for separating and isolating lipid classes from acetone extracts of wood and pulp. Legend: DG, diglycerides; FA, fatty acids; Fal, fatty alcohols; MG, monoglycerides; RA, resin acids; S, sterols; SE, steryl esters; TG, triglycerides; W, waxes.



3. Thin-layer chromatogram of lipid mixture before separation and lipid classes after separation. Lipid acronyms on the left axis are as defined in Fig. 2.



II. Recovery and purity of standard solution separated by solid-phase extraction[a]

Fraction	Lipid class[b]	Recovery, %	Contamination, %
C	FA and/or RA	100±3.7	0.3±0.1
D	SE/W	99±2.5	0.4±0.1
E	TG	98±4.2	1.6±0.5
F	S/DG/Fal	96±5.8	1.8±0.4
G	MG	98±3.4	0.3±0.2

[a]Results are the mean of at least three experiments. Purity was analyzed by GC. Contamination is carried over from other lipid classes.
[b]Acronyms are as defined in Table I.

III. Analysis of acetone extracts from a softwood, a hardwood, and an aspen mechanical pulp[a]

Lipid class	Hardwood (aspen), mg	Softwood (lodgepole pine), mg	Hardwood pulp (aspen), mg
Triglycerides	12.1±0.2	19.1±0.5	1.0±0.1
Steryl esters/Waxes	8.9±0.5	2.1±0.1	5.8±0.3
Fatty acids/Resin acids[b]	6.8±0.2	8.5±0.6	4.5±0.2
Sterols/Diglycerides/ Fatty alcohols	4.8±0.4	1.4±0.2	1.3±0.1
Monoglycerides	3.4±0.3	2.2±0.2	1.4±0.1
Total lipids	36.0±0.5	33.3±0.6	14.0±0.3

[a]Weight of components in 1.0 g of o.d. wood or pulp. For wood, the data are the means of five samples, for pulp, the data are the means of three samples.
[b]Resin acids are included in this class for softwoods

Quantitative analysis of extractives from wood and pulp samples

In testing our method with wood and pulp samples, we first determined the minimum amount of wood chips that could be ground and extracted with reproducible results. We found that a constant amount of extractives (≈ 40 mg/g dry wood) was obtained with dry wood weights varying from 0.5 g to 5 g. For convenience, we chose to extract 1 g of ground wood chips. The dried acetone extracts from both wood and pulp were redissolved into 2 mL of diethyl ether, filtered through a short anhydrous sodium sulfate column, washed twice with 4 mL of the same solvent, evaporated under nitrogen to dryness, and weighed to determine the total lipid content.

The dried eluate from each extraction was redissolved in 1.0 mL of chloroform, and 0.5 mL of the chloroform solution was loaded into the conditioned Bond Elut cartridge and eluted as described previously. **Table III** summarizes the results of the extracts from both aspen and lodgepole pine wood and from aspen TMP. The woods and pulp were randomly sampled and extracted several times, and the separation of the different lipid classes was highly reproducible. Separation times were typically 1–2 h per sample, with a recovery rate of 95–99%. The method was applicable to both hardwood and softwood species. In softwoods, both fatty and resin acids were recovered in Fraction C.

The high degree of recovery and purity of the different fractions separated by the rapid method—and the possibility of further characterizing each component within a fraction by analytical methods such as GC or HPLC—make this technique attractive. The method is an alternative to the lengthy, costly, and complex traditional analysis used in wood and paper industries, in which acidic compounds are separated from neutrals by ion exchange, neutral lipids are hydrolyzed, and both fractions are methylated and finally analyzed by GC *(22, 23)*.

Several alternatives to the traditional methods have been proposed recently. Sitholé and co-workers *(24)* showed that they could process both high-boiling-point lipid fractions and fatty acid methyl esters by GC in 30 min by using a short column. However, as noted by the authors, the separation of the eluting fractions needs to be developed further. Similarly, while gel-permeation chromatography has been used to fractionate the resin from *radiata* pine into triglycerides, waxes, fatty acids and sterols, and resin acids, the authors recognized that the accuracy is poorer than traditional methods, particularly for the fraction containing the fatty acids and the sterols *(25)*.

Nuclear magnetic resonance spectroscopy can also determine the amounts of fatty acids, resin acids, triglycerides, and fatty acid esters in wood extractives. In this case, the wide application of this method is limited by the large amount of lipid samples necessary to obtain an acceptable spectrum, the time required to run each sample, and the expensive instrumentation to analyze the sample *(26)*.

Conclusion

The developmental work described here has demonstrated that a solid-phase-extraction technique can be used to separate and quantify acetone

extracts from different wood species and pulps. For screening large numbers of samples, SPE is fast, cheap, versatile, and accurate, and the technique does not require highly trained personnel. Without automation, an operator can process up to five extracts within an hour. The Bond Elut/Vac Elut system is much cheaper than GC or HPLC, and Bond Elut extraction cartridges are commercially available. The column material is also available commercially, and columns can be packed with smaller or larger amounts of matrix to fit the needs of the user. Most of the solvents are nontoxic and used in small amounts, making this method relatively safe for users and environmentally acceptable. ◫

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