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Chemical Analysis and Biological Removal of Wood Lipids Forming Pitch Deposits in Paper Pulp Manufacturing

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1. Introduction

Wood extractives cause production and environmental problems in pulp and paper manufacturing. The lipophilic fraction is the most problematic, and it includes free fatty acids, resin acids, waxes, fatty alcohols, sterols, sterol esters, glycerides, and other oxidized compounds ***(1–5)***. During wood pulping and refining of paper pulp, the lipophilic extractives in the parenchyma cells and softwood resin canals are released, forming colloidal pitch ***(6)***. These colloidal particles can coalesce into larger droplets that deposit in pulp or equipment, forming the so-called pitch deposits, or remain suspended in the process waters. Pitch deposition results in low-quality pulp, and can cause the shutdown of mill operations ***(7)***. Economic losses associated with pitch problems in kraft mills often amount to 1% of sales. In addition, some wood extractives could have a detrimental environmental impact when released into wastewaters. This is especially important in modern environmentally-sound pulp manufacturing processes, where chlorine bleaching has been replaced by elemental chlorine-free (ECF) or totally chlorine-free (TCF) bleaching ***(8,9)***.

Traditionally, pitch deposition has been reduced by debarking and seasoning logs and wood chips, and by adding physicochemical control agents ***(10–13)***. However, the cost is high and often the results are far from satisfactory. As alternatives to the above, biological removal of wood extractives by treatment with enzymes ***(14–16)*** or microorganisms ***(17,18)*** has been suggested in recent years for pitch control. The screening for the most adequate organisms to

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degrade lipophilic extractives responsible for pitch deposition during pulping of different types of wood requires the use of simple and sensitive analytical methodologies. The broad range of molecular masses of lipophilic extractives and their structural diversity represent two important difficulties for their chemical analysis. In the present chapter, we describe an optimized methodology for the chromatographic separation and chemical identification of complex mixtures of wood lipids, without previous fractionation. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) are employed, using short- and medium-length high-temperature capillary columns (with thin films), respectively, that enable elution and separation of high-molecular-weight lipids ***(19–24)***. This methodology was successfully applied for the study of the biological removal of free and esterified wood sterols by selected fungal strains ***(25,26)***. The results show that some of the new fungi efficiently degrade steroids involved in pitch deposit formation in chlorine-free pulps, which are not removed by the other microbial preparations currently available. In this chapter, the GC-MS profiles of lipophilic extractives from eucalypt wood after solid-state fermentation (SSF) treatment with four selected fungi is used to illustrate this methodology.

For more detailed characterization, a simple solid-phase extraction (SPE) method using aminopropyl phase cartridges is described. This enables fractionation of the complex mixture of lipids isolated from woods and pitch deposits into major lipid classes, which can be subsequently characterized and quantified by GC and GC-MS. The advantages of SPE over traditional sample preparation methods include increased speed and simplicity, reduced solvent usage, and improved selectivity ***(27)***. The method outlined in this chapter is based on that previously developed by Kaluzny et al. ***(28)*** for separation of animal lipids. However, a different fractionation scheme with a reduced number of steps and cartridges, as well as a different solvent system, has been used for the fractionation of wood lipids.

2. Materials

1. GC equipment: Hewlett-Packard HP 5890 gas chromatograph equipped with a split-splitless injector and a flame ionization detection (FID) system (Hewlett-Packard, Hoofddorp, Netherlands).
2. GC-MS equipment: Varian Star 3400 gas chromatograph (Varian, Walnut Creek, CA) with an ion-trap detector (Varian Saturn 2000).
3. High-temperature capillary column (DB-5HT, 0.25 mm id, 0.1 μm film thickness) from J&W Scientific (Folsom, CA) especially processed for an extended temperature of 400°C (15 m for GC-MS analysis and 5 m for GC-FID analysis).
4. Accessories used for GC injector: Thermogreen LB-2 with extremely low bleeding (from 100°C to 350°C) and Therm-O-Ring™ seals processed for using at temperatures up to 375°C without being degraded (Supelco, Bellefonte, PA).

5. Standard compounds (palmitic acid, dehydroabietic acid, sitosterol, cholesteryl oleate, and triheptadecanoin).
6. Aminopropyl phase cartridges (500 mg) from Waters (Division of Millipore, Milford, MA).
7. Solvents: acetone, hexane, chloroform, diethyl ether, and acetic acid.
8. Wood chips from *Eucalyptus globulus* (e.g., 2–4 × 20–40 mm).
9. Fungal strains: *Bjerkandera adusta* CBS 230.93, *Phlebia radiata* CBS 184.83, *Pleurotus pulmonarius* CBS 507.85, and *Ceriporiopsis subvermispora* CBS 347.63 conserved at the Centraalbureau voor Schimmelcultures fungal culture collection (Utrecht, The Netherlands).
10. SSF equipment: Horizontal rotary fermentor including six 2-L bottles with a capacity for 350 g of wood chips (28 cm rotating diameter), which were individually sterilized at 120°C for 30 min and inoculated under sterile conditions (as described below) before being assembled into the fermentor, where they were flushed with sterilized wet air (165 mL/min), rotated 1 h/d at 1 rpm, and maintained at 28°C ***(29)***.

3. Methods

The methods described below outline (1) the GC and GC-MS conditions to analyze lipophilic compounds within a wide molecular-mass range in the same chromatographic analysis in a short period of time, (2) the fractionation of the wood extracts using a SPE procedure, and (3) the fungal treatment of wood chips to remove lipophilic extractives, the efficiency of which is analyzed by the above chromatographic methods.

3.1. Chromatographic Analyses

High-temperature, short- and medium-length capillary columns with thin films have been used for the rapid identification and quantification of lipophilic wood extractives with no prior derivatization nor fractionation (*see* **Note 1**). This analytical method is being used routinely in our laboratory for the evaluation of biological removal of extractives from eucalypt and pine woods, which implies the analysis of a great number of samples.

3.1.1. GC

The capillary column used for GC analyses was of 5-m length because it enables simultaneous analysis and quantification of the main classes of lipophilic extractives (fatty acids, steroid hydrocarbons, sterols, sterol esters, and triglycerides) in the same chromatographic run (in a short period of time) as shown in **Fig. 1** (*see* **Note 1**). The injector and the detector temperatures were set at 300°C and 350°C, respectively. Sample volumes of 1 µL were injected in the splitless mode. Helium was used as the carrier gas. The oven was temperature-programmed from 100°C (1 min) to 350°C (3 min) at a rate of 15°C/min.

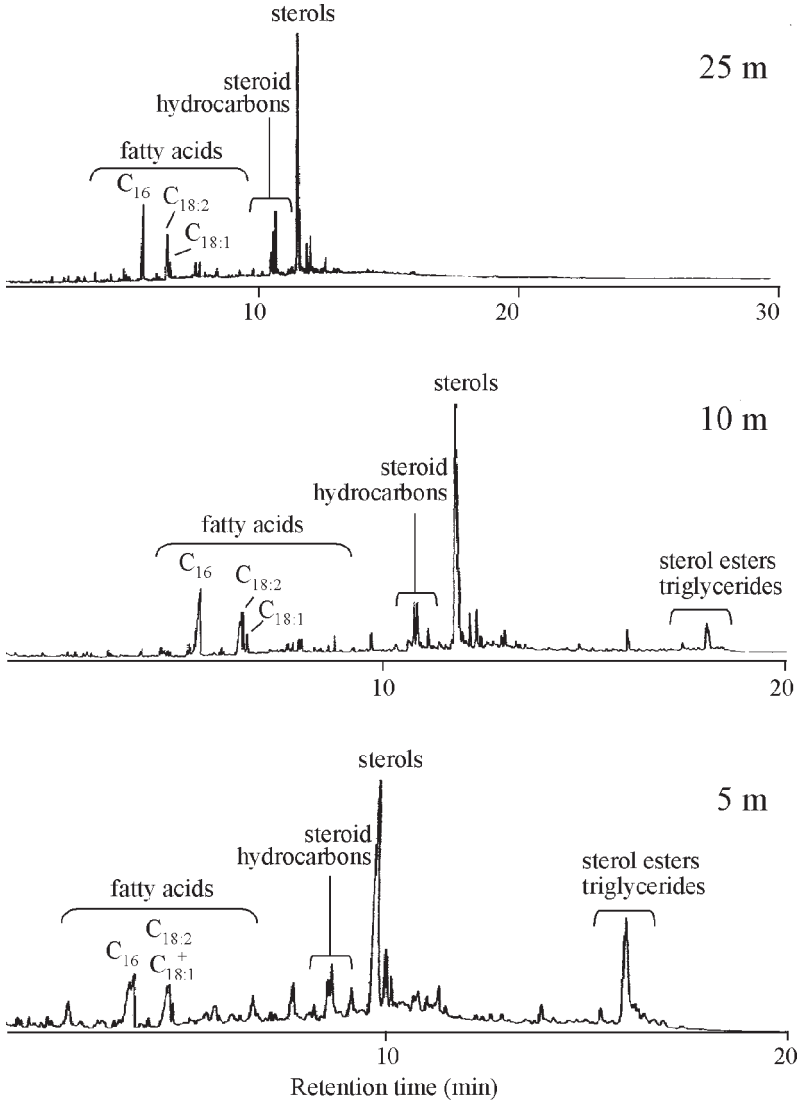

Fig. 1. GC-FID chromatograms of the lipid extract of *E. globulus* wood obtained with high-temperature capillary columns of different lengths. The identity of major compounds is shown in the chromatograms.

A mixture of standard compounds (*see* **Subheading 2.**) was used to elaborate a calibration curve for the quantification of wood extractives. All peaks were quantified by area.

3.1.2. GC-MS

The capillary column used for GC-MS analyses was of 15-m length (*see* **Note 1**). Sample volumes of 1 µL were injected with an autoinjector using a septum-equipped programmable injector (SPI) system. The temperature of the injector during the injection was 120°C, and 0.1 min after the injection was programmed to 380°C at a rate of 200°C/min and hold 10 min. Helium was used as the carrier gas. The oven was temperature programmed from 120°C (1 min) to 380°C (5 min) at 10°C/min. The temperatures of the ion trap and the transfer line were set at 200°C and 300°C, respectively. Compounds were identified by computer comparison of the mass spectra with those in the Wiley and NIST libraries, by mass fragmentography, and when possible by comparison with standard compounds.

3.2. SPE Fractionation

The SPE fractionation scheme described here has been used when a more detailed characterization of some compounds that eluted close together or were present in very minor amounts was required.

The SPE implies a physical extraction process involving a liquid and a solid phase. Process parameters include the stationary phase of the SPE cartridge, the conditioning of the phase, the volume and quantity of the sample, the choice of solvents, the volumes used, and the flow rates, as well as the suppliers of the columns. Different stationary phases are available from several suppliers ***(30)***. In the SPE method described here, the lipophilic compounds of interest are retained in the column and elute from the column in order of increasing polarity. Aminopropyl phase cartridges (500 mg) were used. The column separations described here can be performed with different quantities of lipid extract (5–20 mg). The cartridge was loaded and eluted by gravity (*see* **Note 2**). It is necessary to avoid allowing the columns to become completely dry between the different elution steps. The procedure for the fractionation of eucalypt wood lipids follows **(Fig. 2)**: The aminopropyl column was conditioned with hexane (4 mL). Next, the dried chloroform extract (containing free fatty acids, squalene, steroid hydrocarbons, waxes, free sterols, sterol esters, and triglycerides) was taken up in a minimal volume (0.5 mL) of hexane:chloroform (4:1) (*see* **Note 2**), and loaded into the cartridge column, leaving the entire lipid mixture in the column. The column was first eluted with 8 mL hexane. The fraction A (containing sterol esters, waxes, and hydrocarbons) was saved, and a new tube was placed below the column. Next, the column was loaded with 6 mL hexane:chloroform (5:1) and the fraction B was eluted (containing triglycerides) and saved. The column was subsequently loaded with 10 mL chloroform and the fraction C was eluted (containing free sterols) and saved. Finally,

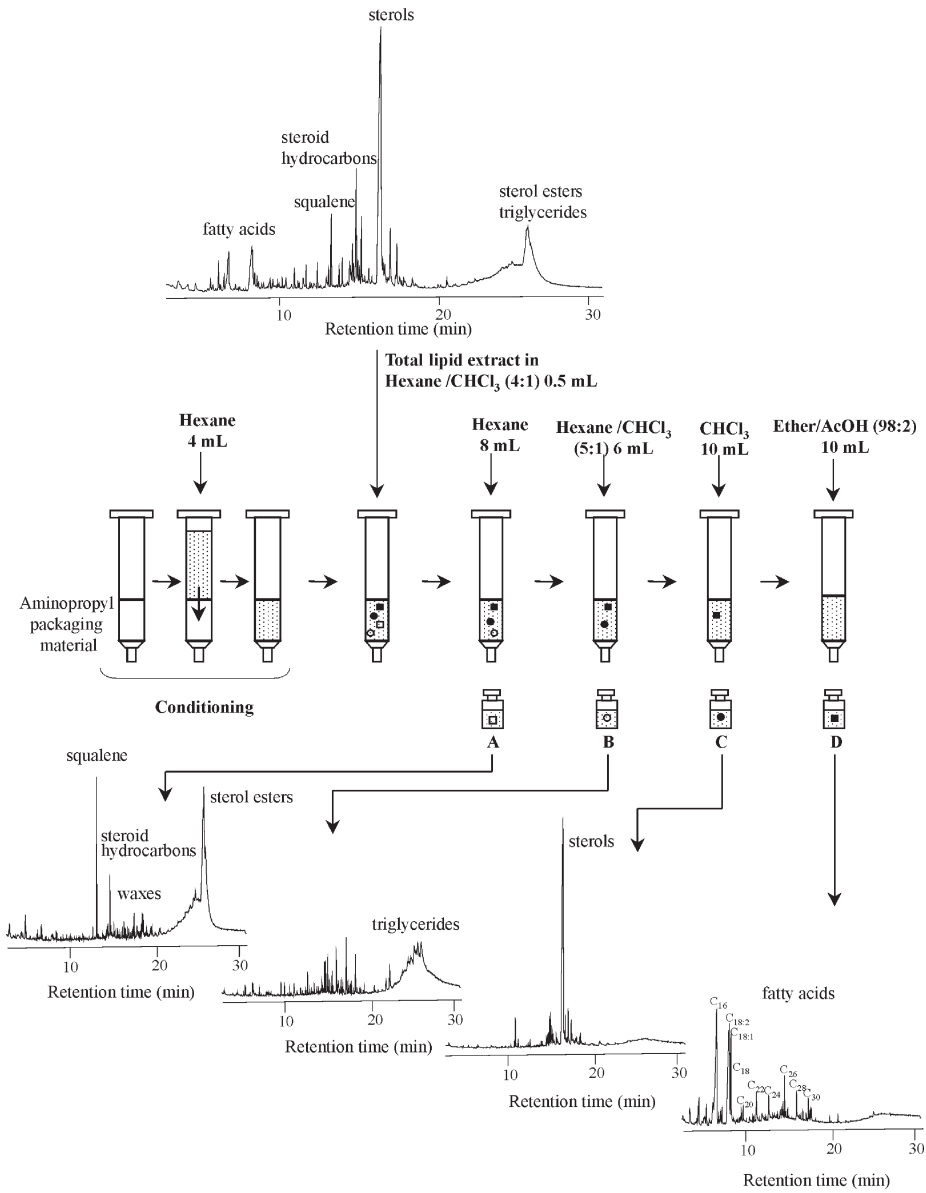

Fig. 2. Elution scheme used for the separation and isolation of lipophilic extractives from *E. globulus* wood in SPE aminopropyl columns and GC-MS (15-m column) chromatograms of the total lipid extract and the four fractions obtained (A–D).

the column was loaded with 10 mL diethyl ether:acetic acid (98:2), and the fraction D was eluted (containing free fatty acids) and saved. Each isolated fraction was dried under nitrogen and weighed. GC and GC-MS were used to monitor the purity and to determine the amount of solvent needed to elute each fraction (**Fig. 2**).

3.3. Biotechnological Control of Pitch Deposits

After a large screening of fungal strains from different origins (*see* **Note 3**), the four fungi listed above were selected for the removal of lipophilic extractives involved in pitch deposit formation from eucalypt and other woods used as raw material for chlorine-free paper pulp manufacturing. Inocula were grown in liquid media, and fungal pellets were used to inoculate wood chips. In order to remove the most recalcitrant lipophilic extractives (such as free and esterified sterols in the case of eucalypt), the inoculated wood was incubated for several weeks under SSF conditions. These are similar to those in chip piles at the pulp mill during natural seasoning of wood, which is traditionally used for decreasing pitch troubles. Therefore, the treatment described could be extrapolated to the mill scale, and can be considered as a controlled seasoning of wood using selected fungal inocula (instead of depending on the action of the wood-inhabiting microorganisms as in the case of natural seasoning).

3.3.1. Inoculum Preparation

Flasks (1 L) with 100 mL of a medium containing (per L) 10 g glucose, 2 g ammonium tartrate, 1 g KH_2PO_4, 1 g yeast extract, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g KCl, and 1 mL of a mineral solution (containing 10 mg $B_4O_7Na_2 \cdot 10\ H_2O$, 7 mg $ZnSO_4 \cdot 7\ H_2O$, 5 mg $FeSO_4 \cdot 7\ H_2O$, 1 mg $CuSO_4 \cdot 5\ H_2O$, 1 mg $MnSO_4 \cdot 4\ H_2O$, 1 mg $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, and 100 mL water) were sterilized by autoclaving at 120°C for 20 min ***(31)***. Each flask was inoculated with two plugs from a fungal colony grown on 2% malt-extract agar slants, and was maintained under stationary incubation at 28°C for 15 d. After this time period the mycelia were washed and homogenized under sterile conditions. The washed and homogenized mycelia (preinoculum) were grown in 1-L flasks with 200 mL of the above glucose-ammonium-yeast extract medium for 5 d, and the pellets produced were washed and used to inoculate the eucalypt chips.

3.3.2. Fungal Treatment of Wood

The sterilized wood chips inoculated with fungal pellets (0.5 mg/kg wood) were incubated under SSF conditions (corresponding to the water-holding capacity) in the rotary fermentor described previously. After an adequate incubation period (typically 21 d), the treated chips were dried in an aerated oven at 60°C, ground to sawdust using a knife mill, extracted with acetone in a Soxhlet

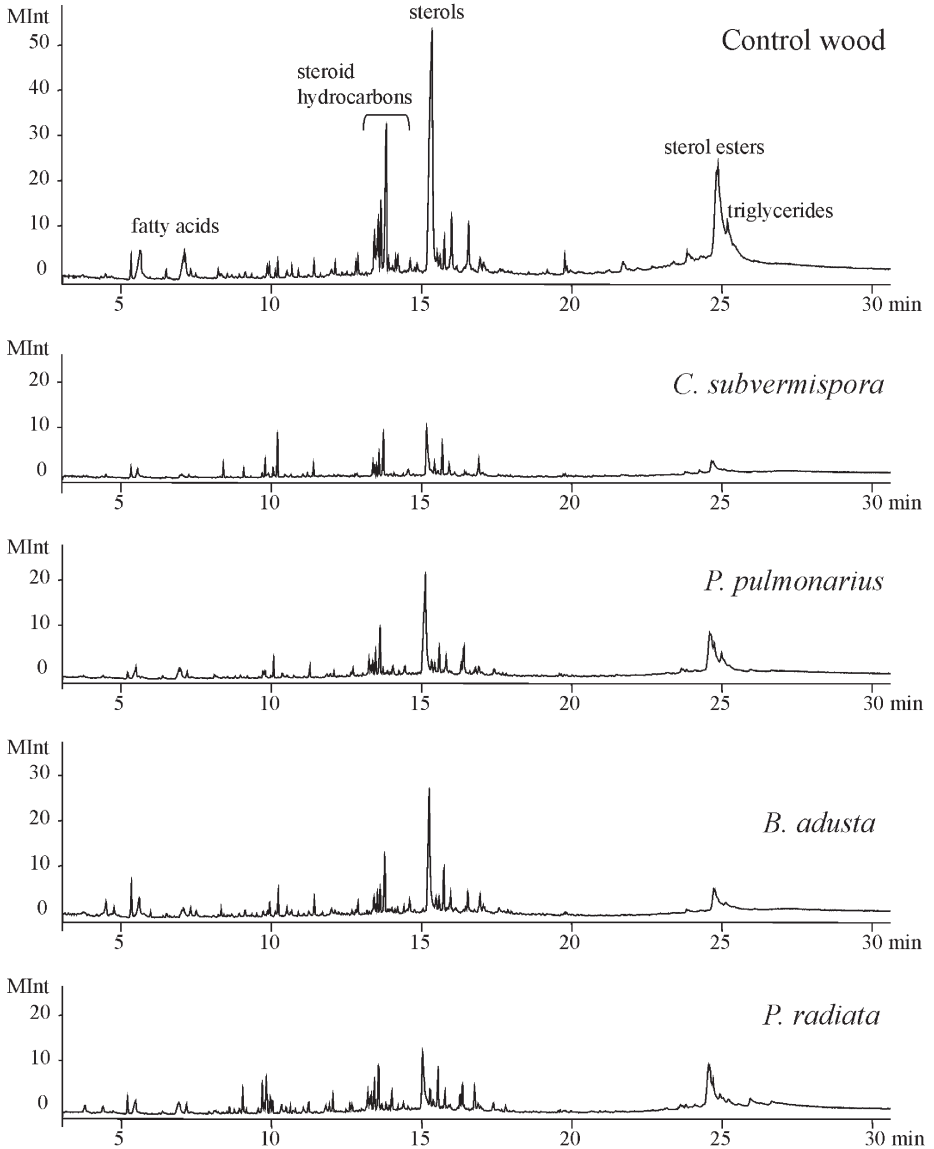

Fig. 3. Total-ion GC-MS (15-m column) chromatograms of the lipophilic fraction of the acetone extracts from the *E. globulus* wood treated with the selected fungal strains (21 d).

apparatus for 8 h, and the lipophilic compounds dissolved in chloroform and analyzed by GC-MS as previously described. After pretreating wood with these fungi, the abundance of most pitch-forming compounds, such as free and

esterified sterols, was strongly decreased, as illustrated in **Fig. 3**, which shows the GC-MS profiles of lipophilic extractives from eucalypt wood after SSF treatment with the four selected fungi and the noninoculated control. These results show that the four fungi selected are promising organisms for the biological control of pitch in paper-pulp manufacturing, when applied as a SSF pretreatment of the raw material (*see* **Note 3**).

4. Notes

1. Several authors have used short GC capillary columns, which allow the elution and separation of high-molecular-mass lipids, for the analysis of wood lipophilic extractives, although they do not allow the best resolution ***(20,21)***. In the present study, the range of temperature of the analysis has been extended by using high-temperature capillary columns. On the other hand, capillary columns with thin films, which are necessary for an optimal analysis of high-molecular-mass lipids such as waxes, sterol esters, and triglycerides ***(32)*** are preferred for this study. Therefore, the column finally selected for the chromatographic analyses was a DB5-HT capillary column of 0.25 mm id with a film thickness of 0.1 μm. Previous procedures for the analysis of wood extractives used conventional temperature columns and different film thickness or internal diameters ***(20,21)***. Different column lengths (from 25 to 2 m) and different temperature programs were investigated for the analysis of the lipid extract from eucalypt wood by GC-FID. As the main objective was to obtain short elution times, high-temperature programming rates, which enhance the speed of the analysis, were preferred. **Figure 1** shows the chromatograms of eucalypt wood lipophilic extractives obtained with different column lengths and program temperature rates. It can be observed that the high-molecular-mass lipids (sterol esters and triglycerides) start to elute as the column length decreases. Shorter lengths (2 m) were also attempted, but the resolution was not good enough for quantitative purposes. The length of the column used should be a compromise between the optimum, in terms of resolution, and the need to limit the exposure time of the sample to high temperatures to the minimum ***(32)***. After this comparative study, a 5-m capillary column was selected for the rapid analysis of wood extractives, since it enables elution and separation of compounds with a wide range of molecular masses (from fatty and resin acids to sterol esters and triglycerides) in the same chromatographic analysis, in a short period of time (20 min), and with enough resolution for quantification of the different component groups. The chromatograms obtained by GC-FID using the 5-m capillary column had to be reproducible in GC-MS in order to identify the different compounds. However, GC-MS systems cannot support very short columns. The minimum column length of a capillary column of 0.25 mm id suggested by the manufacturers of the Varian Saturn 2000 GC-MS system was limited to 15 m, although shorter columns (10–12 m) have also been successfully used. The utilization of a high-temperature capillary column made it possible to increase the final temperature up to the 380°C necessary for the detection of

sterol esters and triglycerides in a 15-m capillary column in a short period of time (30 min). The good reproducibility of chromatograms of the eucalypt wood extractives obtained by GC-FID with a 5-m column and by GC-MS with a 15-m column, can be observed by comparing the chromatograms of **Figs. 1** and **2**. Although some triglycerides and sterol esters from eucalypt wood may elute closely, their differentiation is possible in the 15-m column by their mass spectra. On the other hand, this has not been a problem for the quantification in GC-FID when the purpose was categorizing the extractives into chemical classes as in the case of the quantification of wood extractives degradation after fungal treatment. However, when a more accurate characterization of some compounds was required, the extract was fractionated by the simple SPE procedure described in this chapter.

2. The separation and isolation of neutral and polar lipid classes for subsequent use or analysis has been the subject of many reports in the literature. The advantages of SPE include smaller sample and solvent requirements and ease of use compared to conventional solvent extraction techniques. The principles of SPE and the methods of isolation and fractionation of lipids in biological and food matrices have been extensively reviewed ***(27,30,33)***. A basic method for the separation of individual neutral and polar lipid classes using aminopropyl bonded SPE cartridges was developed by Kaluzny et al. ***(28)*** for the separation of lipids from bovine adipose tissue. Chen et al. ***(34)*** used a SPE method for the fractionation of wood extractives and described a procedure that involved the use of three different SPE cartridges in a multi-step fractionation that resulted in seven fractions. However, both the GC elution order described and the purity of some of the SPE fractions obtained is questionable. Using the SPE method outlined in **Fig. 2,** lipophilic wood extractives are also fractionated in aminopropyl-bonded SPE cartridges; however, only one cartridge is used per sample and some elution steps have been eliminated, resulting in only four final fractions. The present method has been optimized for eucalypt wood extractives ***(35)***. The column was loaded and eluted by gravity. Gravity flow is essential when the full resolving power of the sorbent is required ***(30)***, and it demands little additional equipment. However, when a major flow-rate is needed, positive displacement or vacuum can be applied. The recovery rate was approx 95%, and the purity of each isolated fraction was confirmed by GC and GC-MS using the chromatographic methods described above. In the SPE procedure outlined here, lipids elute from the aminopropyl column in order of increasing polarity. Polar lipids such as fatty acids or those having a polar group such as sterols are likely to interact more strongly with the aminopropyl group on the columns through hydrogen bonding to the primary amine group ***(27)***. On the other hand, it must be reemphasized that the relative compositions of the solvent mixtures (shown in the methods section) should not be changed, since the physical environment of the columns may be altered. This may result in less than optimal separation, recovery, and/or purity of the fractions obtained ***(28)***. For example, we found that when the sample is taken up in pure chloroform and the column subsequently eluted with

chloroform:hexane (1:5), as proposed by Chen et al. ***(34)***, triglycerides and sterols may coelute in fraction A. This does not occur if the sample is taken up in pure hexane and subsequently eluted with hexane. However, since the eucalypt wood extractives were not totally soluble in pure hexane, it was necessary to use the mixture hexane:chloroform (4:1) described here, in which these samples were completely soluble. The proportion of chloroform should not be increased, since higher amounts of the more polar solvent may result in some triglyceride elution in fraction A. The SPE fractionation method described here has also been successfully applied to the fractionation of the extracts of pitch deposits occurring during the kraft pulping of eucalypt wood.

3. The results obtained after GC and GC-MS analysis of wood chips treated with a variety of fungi from several taxonomic groups showed different patterns of degradation of compounds responsible for pitch deposit formation ***(25,26)***. Commercial preparations for pitch biocontrol during manufacturing of mechanical pulps from pine and other softwood are currently available ***(1)***. These are based on the use of the so-called sap-stain fungi (from the group of ascomycetes) being able to hydrolyze triglycerides and degrade the fatty acids. The four fungi described here are among the most promising strains for pitch biocontrol in those cases where more recalcitrant wood extractives (including free and esterified sterols) are at the origin of deposits, as shown in **Fig. 3**. The experiments described here were at the laboratory scale, and the wood samples (chips of different sizes) were inoculated with plugs from fungal cultures (fungal screening experiments in flasks) or pellets from liquid cultures (SSF bioreactor experiments). Scaling-up the fungal treatment implies optimization of inocula for large-scale use (considering supports, stabilizers, and enhancers of fungal growth). After pretreating wood with these fungi, which belong to the group of basidiomycetes, the abundance of free and esterified sterols strongly decreased. Laboratory pulping of pretreated wood has shown that the biological removal of sterols from the raw material results in pulps with reduced amounts of these compounds ***(36)***, as well as in a decrease in the potential toxicity of the process effluents. This is because natural extractives (which were degraded by chlorine-containing bleaching reagents but survive peroxide bleaching of pulp) are among the most toxic compounds in effluents from manufacturing chlorine-free paper pulps ***(8,9)***. The pulp yield after biological pretreatment (47–50%) could be further optimized by modifying the duration of the biological pretreatment (which always results in some loss of wood weight) or by improving the fungal strains used to treat wood (with the purpose of increasing lipid degradation and decreasing carbohydrate hydrolysis). The final selection of the most promising fungal treatment for industrial control of pitch deposition should be a compromise between the optimum in terms of extractive removal, the lowest decrease of pulp yield, and the conservation of those pulp properties of interest for the different types of paper to be produced ***(36,37)***. The ultimate goal is a sustainable production of paper pulp—i.e., a pulp mill in ecological balance with nature by the use of biotechnological tools enabling a higher closure of circuits, and a better performance of environmentally sound TCF bleaching processes.

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