# Eucalypt Wood and Pulp Localization of Sterols Involved in Pitch Deposition Using Filipin Fluorescent Staining

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*Sitosterol is one of the main compounds found in pitch deposits during totally chlorine free (TCF) bleaching of* Eucalyptus globulus *kraft pulp. Filipin staining is used for the first time as a selective method to localize sitosterol in eucalypt pulp and wood. This polyene antibiotic reacts specifically with several 3β-hydroxysterols, such as sitosterol, forming fluorescent complexes. These hydroxysterols represent ~75% of the total free sterols in* E. globulus *wood. Filipin staining showed sitosterol in the walls and lumen of ray parenchyma cells. Distinct fluorescence was observed also in the bordered pits of fibres, as well as in tylose-occluding vessels. In general, various wood elements in pulp reflected the filipin reaction pattern observed in wood, but fluorescent aggregates were found also on the surface of unbleached fibres. Parenchyma cells in pulps showed fluorescent pits, especially at their stubby ends. A significant removal of sitosterol from various pulp elements was observed after TCF bleaching.*

*Le sitostérol est l'une des principales composantes des dépôts de poix lors du blanchiment sans aucun chlore de pâte kraft d'*Eucalyptus globulus. *La coloration avec de la filipine est employée pour la première fois à titre de méthode sélective permettant de localiser le sitostérol dans la pâte et le bois d'eucalyptus. Cet antibiotique polyénique réagit spécifiquement avec plusieurs 3β-hydroxystérols (comme le sitostérol représentant quelque 75 % des stérols libres totaux du bois de* E. globulus*) formant des complexes fluorescents. La coloration avec de la filipine a permis de déceler du sitostérol dans les parois et les canaux médullaires des cellules du parenchyme. Une fluorescence évidente a aussi été observée dans les ponctuations aérolées des fibres, ainsi que dans les tyloses bouchant les vaisseaux. En général, les divers éléments de bois dans la pâte ont conservé la structure de réaction à la filipine observée dans le bois, mais des agrégats fluorescents ont été trouvés à la surface des fibres non blanchies. Les cellules du parenchyme dans les pâtes présentaient des ponctuations fluorescentes, et principalement aux extrémités aplaties. Une grande partie du sitostérol a été éliminée des divers éléments de la pâte après le blanchiment sans aucun chlore.*

## INTRODUCTION

Eucalypt wood is an important raw material for paper pulp manufacturing in Spain, Portugal, Brazil, Australia and other countries. Eucalypt pulps represent 10% of the world production of chemical pulp [1]. Moreover, high-quality totally chlorine free (TCF) kraft pulps from *Eucalyptus globulus* wood are being produced in some of these countries. The introduction of some environmentally sound TCF bleaching sequences has increased pitch troubles in eucalypt pulp manufacturing because of deposition of wood extractives that were destroyed by chlorine-containing bleaching agents. These pitch deposits have strong economic impact since they lower pulp quality and can cause shutdown of mill operations.

The presence of phenolic extractives is a characteristic of most eucalypt species and an extensive literature about their chemical nature and behaviour is available [1–3]. However, little information has been reported on eucalypt lipophilic extractives (resin) [4,5]. Recently, more attention has been focussed on this wood fraction, since lipophilic compounds have been identified as responsible for pitch deposition during manufacturing of kraft pulps from fast-growing *E. globulus* [6–10]. Moreover, a promising biotechnological approach for pitch control during eucalypt pulp manufacturing has been reported, based on the capacity of some fungi to degrade wood steroids [11–14].

The anatomical distribution of non-structural wood constituents has been investigated more extensively in softwoods than in hardwoods. The extractives nature and cellular location are known to be important for wood physical and chemical properties, and protection against fungal decay [15]. In hardwoods, most histochemical studies focussed on the polar fraction of extractives, and the location of lipophilic extractives has received scarce attention [16–18]. Various staining procedures have been reported to localize lipids in vegetal tissues, often associated with wound responses.

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However, most of them are specific for suberin or cutin, or based on general lipid stains such Red Oil or Sudan [19,20]. In the present paper, we present a specific and rapid microscopy method to localize the lipophilic extractives responsible for pitch deposits during manufacturing of high-quality TCF-bleached kraft pulp from *E. globulus* wood using filipin, a polyene antibiotic forming fluorescent complexes with sterols [21,22].

## MATERIALS AND METHODS

### Samples

*Eucalyptus globulus* wood, pulps, process water and pitch deposits were supplied by the ENCE mill in Pontevedra, Spain. Brown kraft pulp and process water samples were collected after the last washing step before oxygen prebleaching. Bleached kraft pulp was obtained at the end of a TCF sequence including oxygen prebleaching, a chelating stage and two hydrogen peroxide stages. Wood samples (industrial chips) of freshly cut 12–14 year old trees (debarked trunk), and never-dried brown and TCF bleached pulps were stored at –20°C.

### Chemical Analyses

Acetone extracts were obtained by Soxhlet extraction (8 h) of milled wood and dried pulp samples, as well as from pitch specks (small black deposits) picked out from TCF pulp sheets. The process water samples were extracted using a method optimized for sterol analysis, which included three treatments in a separatory funnel with a mixture of hexane:acetone (2:1) at pH 12 [23]. After extraction (triplicate), the solvents were evaporated to dryness under vacuum and the samples redissolved in chloroform. The lipophilic fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian Saturn 2000 chromatograph equipped with a 15 m x 0.25 mm DB-5HT (0.1 μm film) capillary column, coupled to an ion trap detector (ITD). Samples were injected in the splitless mode, and helium was used as the carrier gas. The temperature of the injector was 120°C; 0.1 min after injection it was programmed to 380°C at 200°C/min and to hold 10 min. The oven was programmed from 120°C (1 min) to 380°C (5 min) at 10°C/min. The temperatures of the ITD and transfer line were 200 and 300°C, respectively. Compounds were identified using the Wiley and Nist libraries, and standard compounds. Peaks were quantified by area, and a mixture of standard compounds (palmitic acid, stigmasta-3,5-diene, sitosterol, cholesterol oleate and triheptadecanoin) was used to obtain calibration curves.

### Microscopy Studies

Wood chips were soaked in water for 15 min under vacuum, and cross and radial sections (7–10 μm thickness) were obtained at –20°C using a cryomicrotome. Brown and TCF pulps were suspended in phosphate buffer saline (PBS), and samples were taken for filipin staining. Extractives-free controls were prepared by extracting wood sections or pulp samples with acetone in a Soxhlet apparatus for 3–8 h, followed by water and PBS washing. Positive controls were prepared by adding sterol solutions (1 mg/mL) in acetone to extractive-free samples. The filipin solution was prepared by dissolving 2.5 mg of filipin (Sigma, St. Louis, MO, USA) in 1 mL of dimethylformamide (Merck, Darmstadt, Germany), and mixing with 50 mL of PBS [24]. The solution was applied to eucalypt wood sections and pulp suspensions mounted in glass slides (as well as the controls described above), and incubated in the dark for 20 min at room temperature. After filipin staining, the preparations were rinsed with PBS and mounted in glycerol containing 1% Mowiol 40-88 (Aldrich, Steinheim, Germany) as an antifade agent [25]. Wood and pulp samples were immediately examined with a fluorescence microscope (Zeiss Axioplan) using epifluorescence. Filipin was excited with ultraviolet light using a 360/40D filter, the fluorescence emitted was analyzed through a 460 nm barrier filter, and the image was captured with a CCD device (Photometrics CH250/A). Similar exposure times were used in all cases to facilitate sitosterol distribution analysis. The specimens were examined simultaneously by light microscopy using phase contrast.

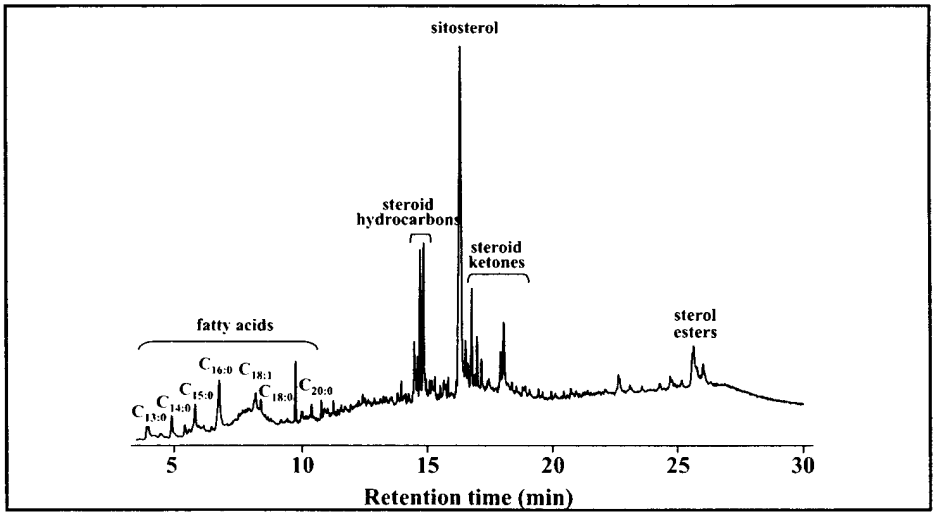


**Fig. 1. Major lipophilic extractives in pitch deposits collected during TCF bleaching of *E. globulus* kraft pulp analyzed by GC-MS using a short (15 m) capillary column. $C_{13:0}$ = tridecanoic acid, $C_{14:0}$ = myristic acid, $C_{15:0}$ = pentadecanoic acid, $C_{16:0}$ = palmitic acid, $C_{18:1}$ = oleic acid, $C_{18:0}$ = stearic acid, $C_{20:0}$ = arachidic acid.**

## RESULTS

### Chemical Identification of Compounds Responsible for Pitch Deposits

Over 70% of the material in the pitch specks from *E. globulus* TCF pulp was soluble in acetone, and nearly all the extractable material was soluble in chloroform, revealing a lipophilic nature. The GC-MS analysis (Fig. 1) showed sitosterol as the main constituent of the pitch deposits, together with sterol esters, steroid ketones and hydrocarbons, and various free fatty acids. Similar chromatographic profiles were obtained when analyzing the lipophilic compounds from eucalypt wood, brown and TCF pulps, and process liquids. However, several differences were observed, including a lower percentage of sterol esters in the process water sample, and higher percentages of triglycerides in wood, and fatty acids in deposits and wood. Moreover, a strong decrease of total lipophilic compounds was observed during pulp manufacturing (from wood

**TABLE I**
**MAIN LIPOPHILIC COMPOUNDS DURING MANUFACTURING OF TCF-BLEACHED KRAFT PULP FROM *Eucalyptus globulus* WOOD***

| | Wood (mg/kg) | Brown pulp (mg/kg) | TCF pulp (mg/kg) | Process water (mg/10 L)** | Pitch specks (%) |
|---|---|---|---|---|---|
| Fatty acids | 277 | 6 | 1 | 0 | 26 |
| Squalene | 38 | 5 | 1 | 3 | 0 |
| Steroid hydrocarbons | 109 | 49 | 25 | 8 | 16 |
| Sitosterol | 494 | 234 | 90 | 221 | 27 |
| Other sterols | 151 | 92 | 39 | 68 | 9 |
| Steroid ketones | 217 | 34 | 13 | 5 | 11 |
| Sterol esters | 517 | 106 | 95 | 80 | 21 |
| Triglycerides | 132 | 0 | 0 | 0 | 0 |

* In all cases, the standard deviation for replicates was less than 5% of the mean values presented.
** Analyzed using a method optimized for sterol analysis in process liquids [23].

to TCF pulp). The quantitative results from GC-MS analyses of lipophilic compounds during the manufacture of TCF kraft pulp from *E. globulus* wood are summarized in Table I.

### Microscopy Localization of Sitosterol

Since sitosterol was involved in pitch deposit formation during the manufacture of eucalypt TCF pulps, a method was developed for its rapid localization using filipin. Distinct signals corresponding to sitosterol–filipin complexes were observed in both *E. globulus* wood and pulps using epifluorescence microscopy. In all cases, the same preparations were examined by phase-contrast microscopy for identification of wood and pulp elements. The autofluorescent background from other wood components (lignin, cellulose and phenolic extractives) was minimized by the excitation and barrier filters used. In this way, no distinct fluorescent signals were observed in wood sections and pulp samples without filipin (images not shown).

Figures 2B and 3A show the highest concentration of fluorescent signals in the parenchyma rays, although scattered fluorescence was observed also in fibres. Many of the fluorescent signals in the parenchyma cells were found at the vicinity of pits connecting cells, those in transverse walls often showing stronger fluorescence than lateral pits (Fig. 2D). The presence of sterols was also shown in spherical deposits characteristic of ray cells (Figs. 2A,B, 3A). Some fluorescence was visible in the tyloses found inside heartwood vessels (Fig. 3C), whereas empty vessels corresponding to sapwood did not show fluorescent signals. A distinct fluorescence was also found in the bordered pits of wood fibres (Fig. 3B). The fluorescence observed in parenchyma rays and fibre pits disappeared completely after 8 h acetone extraction of sections before filipin staining (extractive-free controls) (Fig. 3D), but strongly fluorescent crystals were observed when sitosterol in acetone was added (images not shown).

Fluorescent sitosterol–filipin complexes were more easily detectable in pulps, because of lower lignin content and better sitosterol accessibility in pulp elements than in whole tissues. Filipin staining revealed differences in sitosterol concentration in various cell types identified in the brown kraft pulp from *E. globulus* wood (Fig. 4). Strongly fluorescent signals were found in parenchyma cells, some still associated with fibres. The average size of parenchyma pits increased during cooking. The altered pits lost the fluorescence found in wood sections, but strong signals were still visible in the stubby ends of the parenchyma cells (Fig. 4A,B). Significant differences in fluorescence pattern and intensity were observed between fibres, revealing differences in sitosterol distribution (Fig. 4D). Fibres showed strong fluorescence at the cell wall surface, and weaker signals in pits. In some cases, large fluorescent aggregates were found associated with fibres. Despite being difficult to identify in wood sections, some vascular tracheids were found in pulp, being characterized by their larger diameter, spindle-like shape and abundant pitting with discrete filipin fluorescence (Fig. 4C,D). The TCF-bleached pulp from *E. globulus* exhibited lower fluorescence than the brown pulp, in agreement with chemical analyses showing a strong decrease of sitosterol and other lipophilic extractives content during bleaching (see Table I). Some parenchyma cells in bleached pulp still showed intense sitosterol–filipin fluorescence, especially at their stubby ends (Fig. 5B). However, very

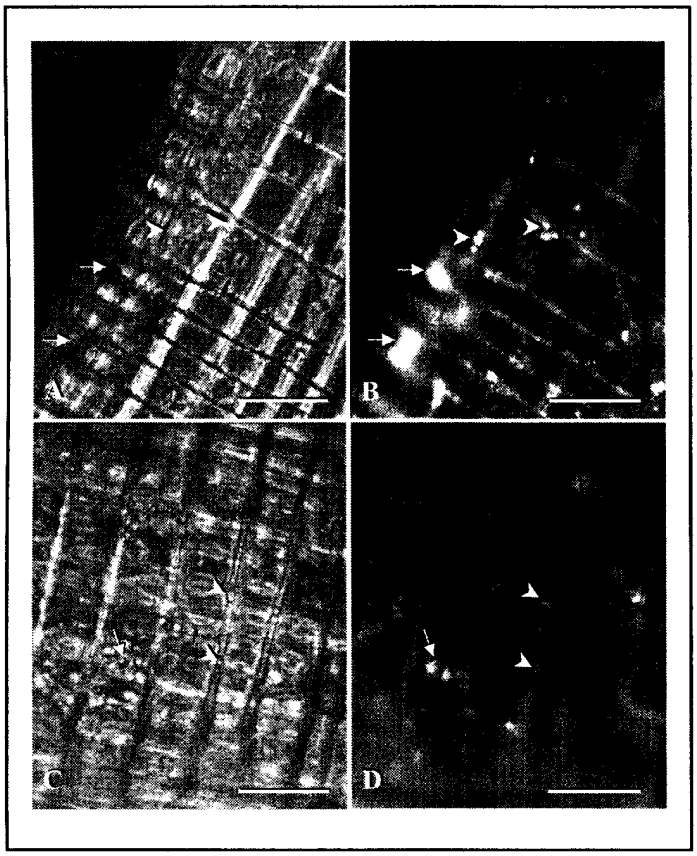


**Fig. 2. Radial sections of *E. globulus* wood under phase-contrast (A,C) and ultraviolet microscopy (B,D) showing fluorescent sterol-filipin complexes in ray parenchyma cells. A,B) Sterol localization in cell walls (arrowheads) and lumen deposits (arrows). C,D) sterol localization around pits in transversal walls (arrow) and in small dots scattered throughout the cell wall (arrowheads). The scale bars represent 20 μm.**

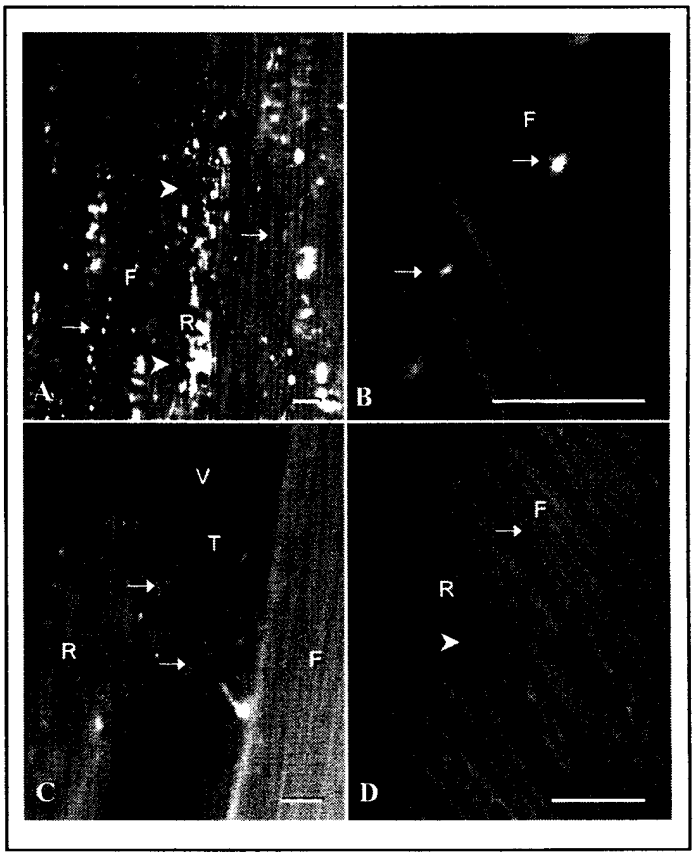


**Fig. 3. Radial (A–C) and tangential (D) sections of *E. globulus* wood under ultraviolet microscopy showing fluorescent sterol-filipin complexes in different tissues. A) Sterol localization in both fibres (arrows) and ray parenchyma cells with larger fluorescent deposits (arrowheads). B) fluorescent fibre pits (arrows). C) fluorescent signals (arrows) in tyloses occluding heartwood vessels. D) non-fluorescent fibres (arrow) and uniseriate rays (arrowhead) in extractive-free control obtained after 8 h extraction (alteration of tissue morphology is observed). The scale bars represent 20 μm. F = fibre, R = parenchyma ray cell, V = vessel, T = tylose.**

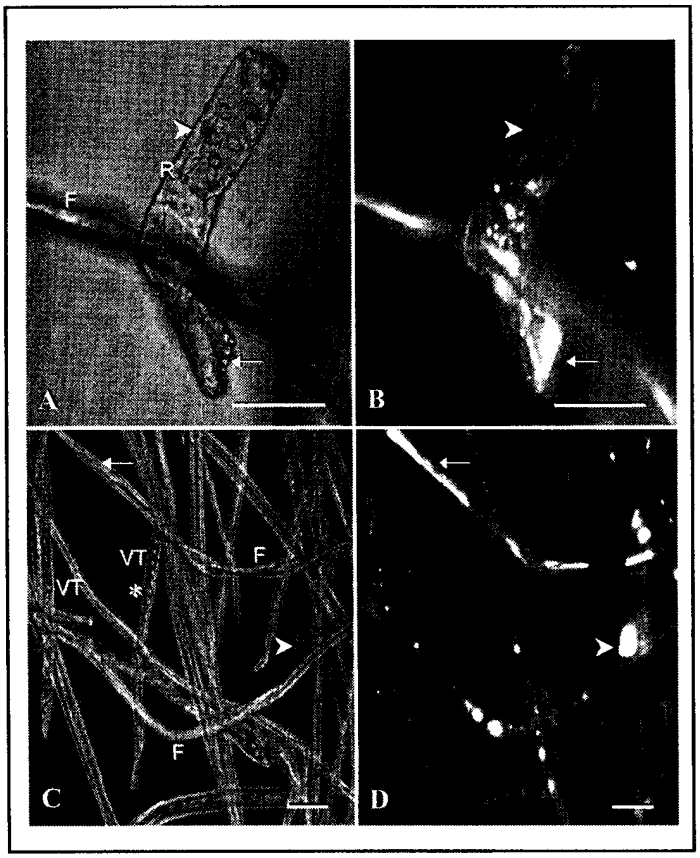


**Fig. 4. Brown kraft pulp from *E. globulus* wood under phase-contrast (A,C) and ultraviolet microscopy (B,D) showing fluorescent sterol-filipin complexes. A,B) Ray parenchyma cell with strongly fluorescent stubby end (arrow) and enlarged pits lacking fluorescence (arrowhead). C,D) pulp fibres showing an irregular distribution of fluorescent deposits on the cell wall surface (arrow), large fluorescent aggregates (arrowhead), and vascular tracheids with strongly pitted walls (asterisk). The scale bars represent 20 μm. F = fibre, R = parenchyma ray cell, VT = vascular tracheid.**

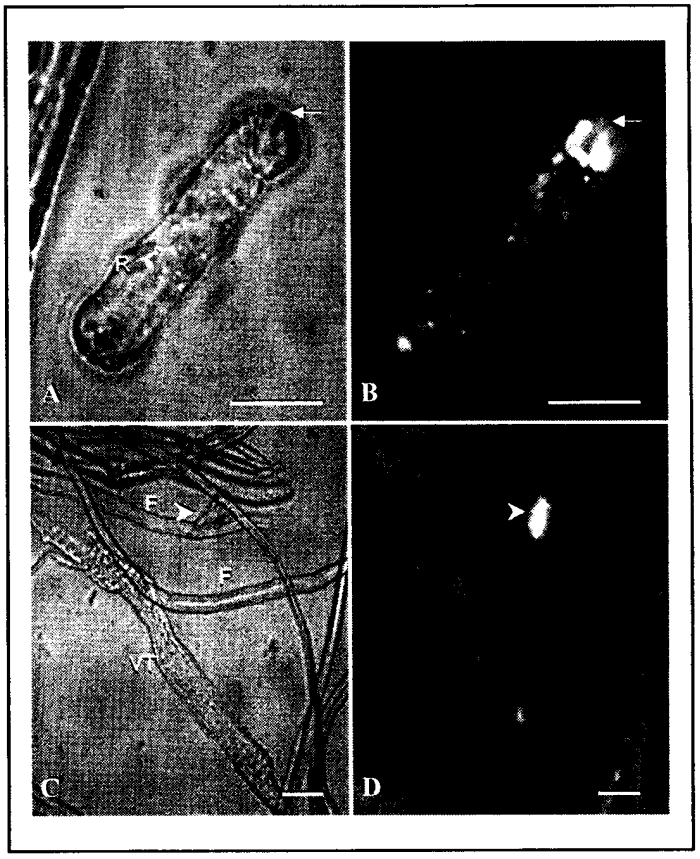


**Fig. 5. TCF-bleached *E. globulus* kraft pulp under phase-contrast (A,C) and ultraviolet microscopy (B,D) showing fluorescent sterol-filipin complexes. A,B) Localization of sterol remainders at the stubby end of ray parenchyma cells (arrow). C,D) pulp fibres and vascular tracheids showing very low fluorescence, whereas some fluorescent aggregates (arrowhead) remained. The scale bars represent 20 μm. F = fibre, R = parenchyma ray cell, VT = vascular tracheid.**

weak fluorescent signals were detected in most bleached fibres and vascular tracheids, and some free fluorescent particles were observed (Fig. 5D).

## DISCUSSION

Free sitosterol is one of the main constituents of the lipophilic fraction from *E. globulus* wood which forms pitch deposits in TCF-bleached kraft pulp. Sitosterol is the most widely distributed plant sterol, being present in heartwood and sapwood of both conifer and deciduous trees [17,26–29] also found in kraft black liquors [30] and hardwood and softwood pulps [31]. In *E. globulus* wood, this sterol is partially esterified with linoleic and oleic acids[6]. Under the alkaline conditions used in kraft cooking, over 75% of the sterol esters are saponified. [14]. However, sitosterol and other minor sterols are not affected by cooking and $H_2O_2$ treatment during TCF bleaching, and they remain in pulp and process waters, and are the origin of pitch deposits [9,23,32]. The situation is different during elemental chlorine free (ECF) bleaching since chlorine dioxide attacks unsaturated sterols (including sitosterol), and only sitostanol together with steroid ketones and hydrocarbons remain in pulps and pitch deposits formed during ECF bleaching [7,32].

After identifying sitosterol as one of the main lipophilic compounds in eucalypt pulps and pitch deposits during TCF bleaching, a method for its specific localization was developed using filipin fluorescent staining. Filipin is a polyene antibiotic formed by a mixture of pentaenes [33], used as a probe to detect cholesterol in aortic deposits and animal membranes, as well as to detect ergosterol in fungal membranes [22,34]. It has been reported that filipin reacts specifically with those 3$\beta$-hydroxysterols including an 8–10 carbon side-chain and a flat tetracyclic nucleus, and not with 3$\alpha$-hydroxysterols or esterified sterols [21–35]. The filipin staining can be adapted as an alternative to other methods for sterol ester localization [36,37], by including a pretreatment of samples with sterol esterase before staining [37,38]. The reaction of filipin with sterols is more efficient when a double bond is present at $C_5$ [22]. In ECF pulps only sitostanol survives chlorine dioxide treatment, and this saturated sterol does not form stable fluorescent complexes with filipin. The complexes formed with filipin have absorption and emissions bands at 357 and 480 nm, respectively; therefore, they can be localized after ultraviolet excitation using epifluorescence microscopy. Since these complexes are formed in a 1:1 stoichiometric reaction, sterol presence and distribution can be quantitatively analyzed using different techniques, such as fluorescence and freeze-fracture electron microscopy (in combination with image analysis), flow citometry, and spectroscopic methods [22,39–41]. This could be applied for rapid sterol analysis during wood pulping after establishing a statistical correlation between filipin reaction and sitosterol content, estimated through GC of lipophilic extracts, as shown for resin acid estimation in pulping effluents using enzyme-linked immunosorbent assay [42].

Fluorescence microscopy has been already applied to study resin behaviour during sheetforming, using an ester of cholesterol with a fluorescent dye and confocal laser scanning microscopy [43]. Moreover, an alternative to the conventional haemocytometer method for counting colloidal pitch particles has been reported using a fluorescent dye in combination with flow citometry [44]. However, in contrast to filipin staining, these methods are not specific for the localization of problematic compounds from wood extractives. Recently, immunolocalization techniques have been assayed for the first time for the specific localization of extractives in pulps, but they are still to be optimized [45].

The results from filipin staining showed a differential distribution of sitosterol in eucalypt wood cells. The highest sterol amount was located in the ray parenchyma, which in *E. globulus* wood is predominantly uniseriate and more abundant than the longitudinal parenchyma. Filipin staining shows that sitosterol in eucalypt parenchyma is associated with cell walls, with the highest concentration in pits, which are involved in liquid movement into the wood. Filipin staining showed the presence of sitosterol in some of the spherical deposits found inside ray cells, but other deposits are formed mainly by anthocyanins and other polar extractives as revealed by specific staining [13]. Pit diameter in the *E. globulus* parenchyma cells is up to 30 μm, enabling tylose development for occlusion of vessels during heartwood formation (in woods with smaller pits, vessel occlusion is caused by secretion of lipophilic gum droplets) [46].

Despite the fact that the young fast-growing *E. globulus* trees used for kraft pulp production show limited heartwood development, tyloses were observed in some heartwood vessels. Filipin staining showed the presence of sitosterol in these heartwood structures, as well as in pits in vessel cell walls. Vascular tracheids are present in eucalypt wood [47], but they are difficult to recognize in wood sections and show low sitosterol content as revealed by filipin staining. The eucalypt fibres are of the fibre–tracheid type, characterized by the abundant presence of conspicuous bordered pits [47] which, as found for pits in parenchyma and vessels, exhibited distinct fluorescent signals. The eucalypt bordered pits include a canal and a chamber where sitosterol and other extractives can be deposited [48]. Despite the fluorescence level in eucalypt fibres being lower than in parenchyma cells, a significant percentage of sitosterol can be located in fibres walls because of the higher relative volume of fibres (64%) compared with ray (16%), longitudinal parenchyma (7%) and vessels (13%) in *E. globulus* wood [47].

It has been reported that the cell wall in eucalypt fibres has enough free space for penetration of low molecular weight extractives [49]. Infiltration by lipophilic extractives should be completed during heartwood formation, when parenchyma cells die and their cellular content, including starch and other reserve substances, transforms into extractives [50,51]. A characteristic of sitosterol location in *E. globulus* wood is its high concentration in pits found in parenchyma cells, vessels and fibres. The biosynthesis of some extractives in the vicinity of the half-bordered pit between tracheids and ray cells has been described [52]. Filipin staining of *E. globulus* kraft pulps showed that a significant amount of sitosterol is located in lumen deposits and cell wall regions of ray parenchyma cells showing strongly fluorescent signals. However, a considerable amount of this sterol was found also in kraft fibres. During kraft cooking, the pulping liquor penetrates into the fibres through vessels and parenchyma cells, where the extractives are concentrated. This is facilitated by the increase of pit diameter during kraft cooking, probably due to material solubilization and mechanical modification under cooking conditions. A fraction of sitosterol from ray parenchyma cells, including intracellular deposits, is released into the black liquor and then reprecipitates onto the fibre surface, together with residual lignin and xylans [53]. In subsequent washing, sitosterol is partially removed from the brown pulp and can be recovered in the wash waters [23]. The presence of sitosterol in both wash waters and eucalypt pulps is the origin of pitch troubles in the final products, especially in modern mills where water recirculation is increased to approach zero liquid effluent operation, resulting in an increased concentration of lipophilic compounds in mill circuits.

The filipin staining described here can be useful also for pitch-control strategies during manufacturing of TCF-bleached kraft pulps from other wood types containing significant amounts of free or esterified sitosterol. This is the case for birch (*Betula verrucosa*) and aspen (*Populus tremuloides*) hardwoods, the extractives fractions of which are dominated by free and esterified sterols which can cause pitch problems [17,27]. Moreover, filipin staining can be used to investigate sitosterol behaviour during TCF bleaching of some softwoods, such as European spruce (*Picea abies*), which contain sterols together with high amounts of triglycerides [29], since triglycerides are saponified during kraft cooking but sterols remain as one of the most problematic compounds for pitch deposit formation [54].

## CONCLUSIONS

Several conclusions can be drawn from the filipin staining results presented here.

- Sitosterol is one of the main lipophilic compounds in *E. globulus* wood and TCF-bleached kraft pulp, and forms pitch deposits both in pulp and on mill equipment.
- This and other $C_5$-unsaturated wood sterols form fluorescent complexes with the polyene antibiotic filipin.
- Filipin staining is a specific, simple and rapid method to localize sitosterol during TCF pulp manufacturing, which can assist other methods for the chemical mapping of wood fibres.
- In *E. globulus* wood, sitosterol was located mainly in the cell walls and lumen deposits of ray parenchyma cells, whereas lower levels were found in fibres and vessels.
- In eucalypt kraft pulps, parenchyma cells retained the high fluorescence found in wood. However, sitosterol deposition was observed also on the surface of unbleached fibres. A strong decrease in fibre fluorescence was found after TCF bleaching, revealing sitosterol removal.
- In both wood and pulps, distinct fluorescence was observed at the pit level (in parenchyma, vessels and fibres), revealing sitosterol accumulation in these cell wall regions.
- Filipin staining is described for the first time during the manufacture of *E. globulus* pulp, but the method can be also of utility for pitch control during kraft pulping and during TCF bleaching of other sitosterol-containing woods.


## ACKNOWLEDGEMENTS

The authors thank Prof. W.E. Hillis, CSIRO, Australia, for critical reading of the manuscript. M. Ollacarizqueta is acknowledged for assistance in fluorescent microscopy, and J. Romero, ENCE, Pontevedra, Spain, for wood, pulps and water samples. This work has been supported by the European contract QLK5-99-1357, the Spanish project 1FD97-742, and the Comunidad de Madrid. The first author acknowledges a fellowship from the Spanish Agency for International Cooperation.


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**REFERENCE:** SPERANZA, M., MARTÍNEZ, M.J., GUTIÉRREZ, A., del RÍO, J.C. and MARTÍNEZ, A.T., Eucalypt Wood and Pulp Localization of Sterols Involved in Pitch Deposition Using Filipin Fluorescent Staining, Journal of Pulp and Paper Science, 28(9):292–297 September 2002. Paper offered as a contribution to the Journal of Pulp and Paper Science. Not to be reproduced without permission from the Pulp and Paper Technical Association of Canada. Manuscript received April 4, 2001; revised manuscript approved for publication by the Review Panel March 27, 2002.

**KEYWORDS:** EUCALYPTUS GLOBULUS, PHYTOSTEROLS, PITCH, KRAFT PULPS, BLEACHED PULPS, CHLORINE FREE BLEACHING, STAINING, FLUORESCENCE.