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Derivatization of resin acids with a fluorescent label for cyclodextrin-modified electrophoretic separation

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Abstract

A derivatization reaction for resin acids (RAs), commonly found in untreated pulp mill effluents, was performed and evaluated in the separation and sensitive detection of the resin acids by cyclodextrin-modified capillary electrophoresis. The procedure involved the reaction of 4-bromomethyl-7-methoxycoumarin, a fluorescent label, with the carboxyl group of the resin acid in the presence of potassium carbonate to form an ester. Both MS data and HPLC analysis equipped with UV or scanning fluorescence revealed that the derivatization was free from side products and very quantitative. The derivatized esters were very stable and fluoresced optimally at 325 nm, excitation and 400 nm emission, an ideal condition for capillary electrophoresis equipped with He–Cd laser-induced fluorescence. Cyclodextrin-modified capillary electrophoresis using a mixture of negatively charged sulfobutylether-β-CD (SBCD) and neutral methyl-β-CD (MECD) was then optimized for separation and detection of the derivatized RAs. Separation at pH 4.5 and +15 kV applied potential using 42.5 m*M* sulfobutylether–β-CD and 12.5 m*M* methyl-β-CD in 50 m*M* sodium acetate has allowed the achievement of baseline separation of eight very closely related derivatized resin acids. With laser-induced fluorescence, the extrapolated concentration limit of detection (3σ) of the resin acid esters was about 10–20 μg/l, based on capillary electrophoresis analysis of a standard solution containing 500 μg/l of each derivative. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Resin acids (RAs), natural tricyclic diterpenoids with a carboxylic moiety, are released from softwoods in the process waters and then pulp mill effluents during chemical and mechanical pulping. These acids are very resistant to chemical degradation and the main RAs in untreated pulp mill effluents can be classified as either abietanes or pimaranes [1]. The former group of RAs (consisting mainly of dehydroabietic and abietic acids) simply has an isopropyl substituent on the C-13 carbon whereas the latter group (consisting mainly of pimaric or isopimaric acid) possesses two substituents, vinyl and methyl moieties, at this position (Fig. 1). The resin acids with conjugated double bonds can easily undergo isomerization forming thermodynamically more stable isomers with abietic and dehydroabietic acids being the favored final products. Therefore, effluents obtained from pulping processes consist mainly of abietic, dehydroabietic, pimaric and isopimaric acids. Notice also that dehydroabietic acid may account for approximately 20–

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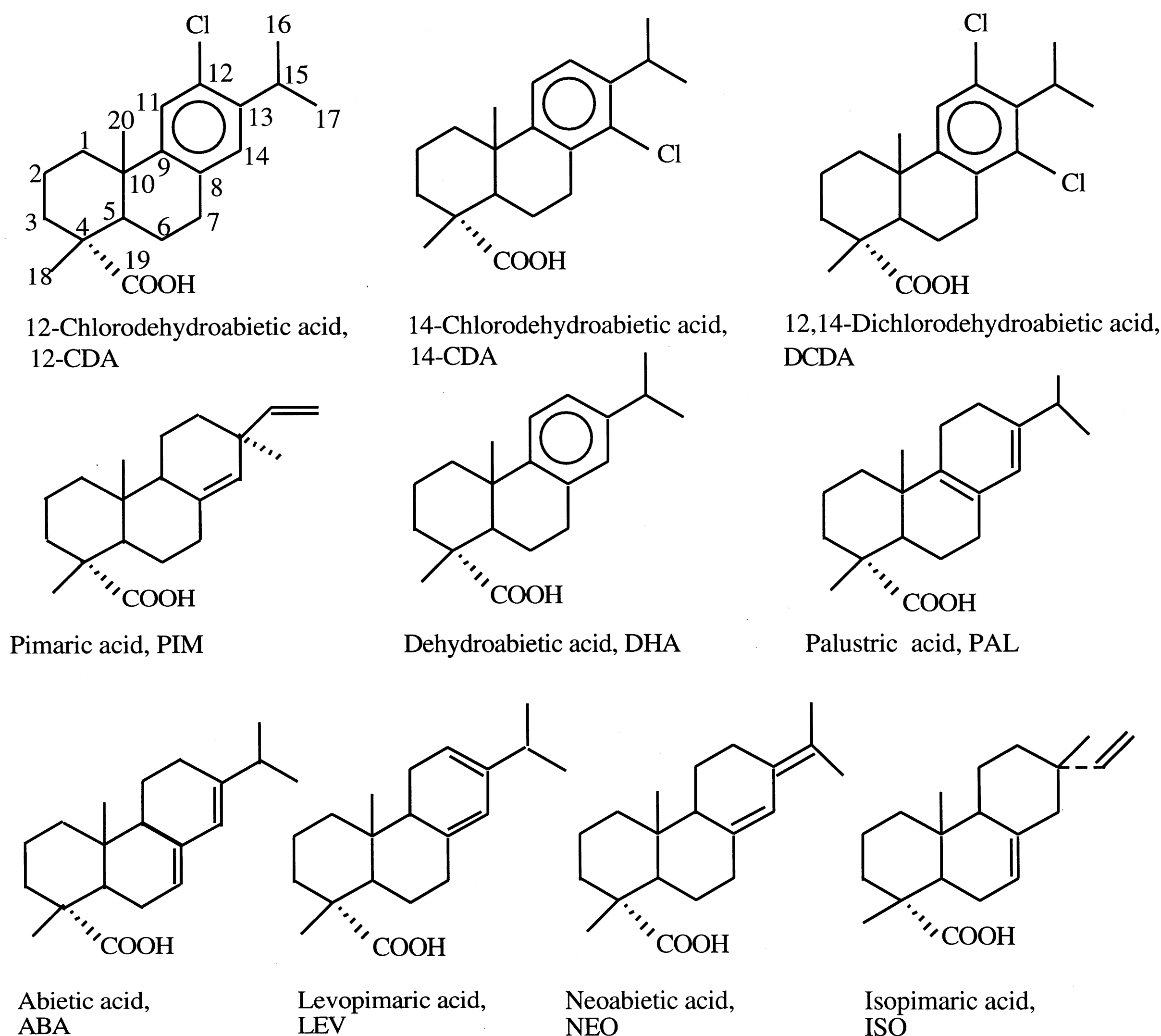


Fig. 1. Chemical structures and abbreviations of the resin acids.

30% of the total resin acids in the western hemlock (*Tsuga heterophyli*) pulping effluents [2]. Chlorobleaching of wood pulps using Cl_2 or ClO_2 effects formation of 12-chlorodehydroabietic (CDA), 14-CDA and 12,14-dichlorodehydroabietic (DCDA) acids [1]. The hydrophobic nature of RAs is reflected by their relatively low solubility (a few mg/l) in aqueous systems. However, the concentration of RAs in untreated pulp mill effluents can be significantly higher, particularly at pH levels above their pK_a values (5.7–6.4). Under alkali conditions, the predominantly ionized species may act as surfactants to form micelles and a level of RAs as high as 18% w/v has been reported [3]. As well as being toxic to marine life at low concentrations, with 96-h LC_{50} values ranging from 0.5 to 1.1 mg/l, RAs exert a number of other biological effects including chronic sublethal toxicity, genotoxicity and the potential of bioaccumulation [4]. Resin acids are considered to be the major contributors to the toxicity of pulp mill effluent towards fish and other aquatic organisms. Other wood processing facilities such as sawmills,

log yards, kiln condensate producers, etc. will also need to adopt resin acid reduction and monitoring.

The routine and sensitive analysis of RAs in a variety of matrices is therefore necessary to identify and monitor these chemicals in the environment and in treatment/remediation systems. The rapid separation and quantitation of the RAs is challenging for several chromatographic procedures including capillary electrophoresis (CE) because their respective molecular mass and chemical structure are almost identical. Capillary gas chromatography [5] requires a very lengthy separation time (60 min) while liquid chromatography (LC) can only be used for analysis of abietic and dehydroabietic acid [6]. One of the major barriers to LC development is the difficulty in separating the various RA isomers in a mixture by a C_8 or C_{18} column owing to very similar analyte-column hydrophobic interactions. Of comparable importance in the assay of RAs is the further development of more sensitive detection systems since RAs do not possess strong chromophores in their structures, a condition that precludes their direct detection in the UV at low levels. Due also to their relatively apolar nature and the lack of a strong fluorophore, resin acids are not suitable for direct determination by capillary electrophoresis (CE) equipped with laser-induced fluorescence. So far, there is no attempt on CE separation for resin acids or their derivatives.

The present study has focused on the preparation of fluorescent derivatives of RAs by tagging the resin acids with 4-bromomethyl-7-methoxy coumarin prior to capillary electrophoretic separation. Representatives of ten resin acids including three chlorinated congeners commonly found in untreated pulp mill effluents are chosen. Cyclodextrin modified capillary electrophoresis equipped with laser induced fluorescence [7,8] was then optimized for separation and detection of the derivatized RAs with view of developing a simple method using non-hazardous chemicals and small sample volumes. The procedure employs a mixture of negatively charged sulfobutylether-β-CD and neutral methyl-β-CD to effect differential distribution (partitioning) of the resin acid esters between the buffer and CD phases. This has allowed the achievement of baseline separation of very closely related derivatized resin acids with detection sensitivities down to μg/l levels.

2. Experimental

2.1. Materials

Sulfobutylether-β-cyclodextrin (SBCD, average degree of substitution, ds 4, pK_a of 2) was purchased from CyDex Inc. (Overland Park, KS, USA). Methyl-β-cyclodextrin (MECD) with an average ds of 0.8 methyl groups per cyclodextrin ring and sodium dodecyl sulfate (SDS) were purchased from Aldrich (Milwaukee, WI, USA). The fluorogenic reagent 4-bromomethyl-7-methoxy coumarin (4-BrMMC) with a purity of 97% was purchased from Sigma (St. Louis, MO, USA). The RAs (purity of 95–99% except for pimaric acid, 85–90%, monochlorodehydroabietic acid (CDA), 85–95% and abietic acid, 90–95%) were purchased from Helix Biotechnologies (Richmond, Canada) and used without further purification. CDA acid contains an equal mixture of 12- and 14-chloro isomers. According to the supplier, the RAs come from natural sources so they are already resolved in view of chirality. Fig. 1 presents the chemical structures and abbreviations of the ten RAs investigated in this study. Except for dehydroabietic (M_r 300), CDA (M_r 336) and DCDA acid (M_r 370), the resin acids possess the same molecular mass of 302. Water was purified using a Zenopure Quadra 90 filtration system (Zenon Environmental, Burlington, Canada) with a specific resistivity over 15 MΩ cm.

2.2. Equipment and instrumentation

All the CE experiments were performed on a P/ACE 5500 capillary electrophoresis system (Beckman, Fullerton, CA, USA). An extended light path capillary, known as a "bubble" cell (50 μm I.D., 250 μm I.D. at the detector window and 360 μm O.D., Hewlett–Packard, Mississauga, Canada) was installed in a capillary cartridge. The effective capillary length is 40 cm and the overall length is 47 cm. Capillary temperature was regulated at 25°C using a liquid coolant in a sealed cartridge. Experiments to monitor the separation of derivatized resin acids used the Beckman laser-induced fluorescence (LIF) detector module. The 325 nm, 35 mW output of an Omnichrome series 74 He–Cd laser (Melles Griot, Carlsbad, CA, USA) was coupled to a 100 μm I.D.,

140 μm O.D. fused-silica step-index optical fibre using a laser coupler (OZ Optics, Ottawa, Canada) for transmission to the fluorescence detector. Fluorescence emission was monitored using a 370 nm long-pass filter coupled with a 400 nm bandpass filter before the photomultiplier tube in the LIF detector. Data acquisition and analysis were facilitated by using P/ACE Station software (Version 1.0, Beckman). The resulting signal was fed to a personal Dell Optiplex computer for storage and real-time display of the electropherograms. Sample injection was performed by applying a small pressure (0.5 p.s.i., 3.44 kPa) at the inlet of the capillary for 5 s. The separation voltage was applied over a 1 min ramp to prevent any possible current break-down. On-column UV detection was also performed with a Beckman modular UV detector operated at 214 nm.

The HPLC system consisted of two Waters model 590 pumps, a Waters WISP 710B auto-injector, a Waters LC spectrophotometer at 318 nm (model 481, Waters, Milford, MA, USA) and a Waters scanning fluorescence detector (Model 747). The HPLC column was not thermostated during operation and all injections were made in duplicate using a Waters WISP model 710B autosampler with an injection volume of 15 μl. The stock mobile phase was degassed by helium before use. The resin acid esters were separated using LC-18 Supelco Supelcosil columns (Oakville, Canada): LC-PAH, bonded phase: octadecylsilane, 5 cm×4.6 mm, 3 μm column (5-9133), 15 cm×4.6 mm, 5 μm column (5-8313) or a Supelco Supelcosil LC-8, 15 cm×4.6 mm, 3 μm column (5-8983).

Fluorescence characterization of the derivatized resin acid esters was measured with a Gilford Fluoro-IV spectrofluorometer (Gilford, Oberlin, OH, USA). Unless otherwise stated, the detector photomultiplier tube (PMT) voltage was set to +550 V. The molecular mass of the derivatized esters was determined using a triple quadrupole mass spectrometer (the API III LC/MS/MS system, Sciex, Thornhill, Canada). The instrument has a mass-to-charge (m/z) range of 0 to 2400 and is fitted with a pneumatically-assisted electrospray (also referred to as ionspray) interface. It has a capacity to allow up to 2^{16} (=65 536) data points for each scan. Charged ions were generated by spraying the sample solution (resin acid ester in acetonitrile) through a stainless steel capillary held at a high potential (+4 to +6 kV). The sample solution was delivered to the sprayer by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA) through a fused-silica capillary of 100 μm I.D. A gas curtain formed by a continuous flow (0.6–1.8 l/min) of N_2 in the interface region served to evaporate the aerosol droplets and to break up the cluster formation from supersonic expansion. The potential on the sampling orifice of the instrument was set at +30 V. The instrument tuning, data acquisition and analysis are processed by a MacIntosh IIx computer and further details of the instrument and data acquisition can be found elsewhere [9].

2.3. Procedures

The stock sodium acetate (100 m*M*) and borate (150 m*M*) solutions were buffered to pH 4.5 and 9.25, respectively. Solutions for CE experiments were prepared by diluting appropriately the cyclodextrins with the stock buffers. All the solutions were sonicated and filtered through 0.45 μm Millex-HV filters (Millipore, Bedford, MA, USA) before use. The derivatized resin acid esters were placed in a light-protected vial (5 ml) during the course of electrophoretic separation. New capillaries were conditioned with 1 *M* NaOH for about 30 min and then washed with water and the running buffer, respectively for another 30 min each. Before daily operation, the capillary was cleaned with 0.1 *M* NaOH for about 10 min, and then with deionized water and the running buffer for 10 min each. The capillary was further conditioned by applying +20 kV for about 10 min before the first injection.

Electropherograms were evaluated by determining separation efficiency and peak-to-peak separation. Efficiency, defined as the number of theoretical plates (N) for each peak in the electropherograms, was calculated as $5.54\ (t_R/w_{1/2})^2$, where t_R and $w_{1/2}$ represent the migration time of the analyte and the full peak width at half-maximum, respectively [10]. The theoretical plates number was divided by the effective capillary length (40 cm) to obtain the number of theoretical plates per meter. The resolution (R_s) of the peak from the preceding peak was calculated as $1.18(t_2-t_1)/(w_{1/2,1}+w_{1/2,2})$, where t_i

and $w_{1/2,i}$ are defined as above for the two peaks. The augmentation in peak height from one run to another after spiking with those of standards was used for peak identification.

2.4. Derivatization of the resin acids

Each resin acid (100 mg/l) was derivatized essentially according to the method of Richardson et al. [11] with the exception that 4-BrMMC was used in a two-fold instead of a ten-fold excess since this fluorescent tag is so hydrophobic and interacts strongly with the capillary wall to adversely affect resolution and reproducibility. The resin acid (0.45 ml, 1000 mg/l stock in acetone), 5–6 mg of finely ground potassium carbonate (ca. 100 μm), 4-BrMMC (0.33 ml, 10 m*M* stock in acetone), and 3.72 ml of acetone were placed in a light-protected vial to prevent photolysis. In this reaction, potassium carbonate formed a resin acid salt which in turn reacted with the bromomethyl methoxycoumarin. As reported by Richardson et al. [11] and confirmed in this study, the particle size of potassium carbonate had to be less than 100 μm to accelerate the derivatization.With potassium carbonate used directly as received, over 1 h was required to complete the reaction. The vial was placed in an ultrasonic bath for 25 min at 25°C and kept tightly sealed to ensure no loss of acetone between derivatization and analysis. The need to protect the reaction mixture from visible light was worthy of mentioning since exposure of 4-BrMMC to light caused rapid decomposition of this derivatizing agent as well as the derivatized resin acid ester. The sample was filtered through 0.45 μm Millex-HV filters (Millipore) to remove potassium carbonate before analysis by HPLC or CE.

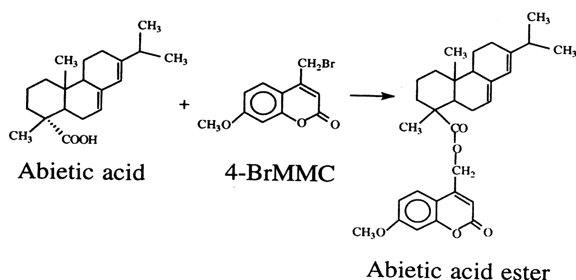
2.5. Safety considerations

Resin acids and their derivatives may be harmful since they have been considered to be the major contributors to the toxicity of pulp mill effluent towards fish and other aquatic organisms. Therefore, special care must be taken to dispose of waste solutions.

3. Results and discussion

3.1. Characterization of derivatized resin acids

Bromomethyl coumarin compounds containing substituents on the aromatic ring and other bromoalkyl-aromatic compounds have been used to form esters with carboxylic acids [12,13]. The derivatization procedures have used 4-BrMMC, 4-bromomethyl-6,7-dimethoxycoumarin, 3-bromomethyl-6,7-dimethoxy-l-methyl(1H)-quinoxalinone as well as 4-bromomethyl-7-acetoxycoumarin. In general, the reaction must be carried out in aprotic dipolar solvents such as acetone to which 18-crown-6 ether and/or potassium carbonate are also added to enhance the reaction rate. However, micellar phase transfer catalysis procedures (using Triton-X and Arkopal N in the presence of tetrahexylammonium bromide) have also been developed for the derivatization of undecylenic acid with 4-BrMMC [14]. In order to facilitate inclusion complexation between the derivatized esters and cyclodextrins, a prerequisite in cyclodextrin modified CE as addressed later, 4-BrMMC was selected in view of its simpler chemical structure. The derivatization of the resin acids was performed with 4-BrMMC in the presence of potassium carbonate to form fluorescent derivatives of the resin acids as follows:



With acetone as a solvent, the derivatization with a two-fold excess of 4-BrMMC was completed within 20–30 min at 25°C. HPLC analysis (with LC-PAH column, 90% acetonitrile–10% water, flowrate of 1 ml/min, UV at 214 nm) of the resulting reaction mixtures confirmed the disappearance of the starting resin acids (retention times of 4–6 min) and the emergence of new peaks between 6–12 min. The new peaks, corresponding to the resin acid deriva-

tives (esters) were detected at 318 nm, whereas the starting resin acid compounds exhibited no absorbance at this wavelength. The peak height corresponding to 4-BrMMC (at 318 nm) was reduced by about half after the derivatization procedure. In all cases, the derivatization was free from side products as only one new peak was observed in addition to the residual 4-BrMMC peak. Peak height of the new peak was very reproducible (>95%) confirming the applicability of the derivatization procedure.

The resin acid derivatives were analyzed to search for their optimal fluorescence condition using the Gilford spectrofluorometer. As exemplified in Fig. 2, the derivatized ester of dehydroabietic acid (1 mg/l) fluoresced optimally at 325 nm, excitation and 400 emission, respectively and this detection condition is ideal for capillary electrophoresis equipped with a He–Cd LIF system. Unreacted 4-BrMMC in the resulting product exhibited little contribution to the fluorescence of these scans as the signal for 1 mg/l 4-BrMMC was less than 5 RFU (relative fluorescence unit). Notice also that the abietic acid derivative exhibited about half the fluorescence intensity of the dehydroabietic acid derivative. The experimental data of Richardson et al. [11] also reported that esters of the conjugated diene acids had a quantum yield of about half of the esters of non-conjugated resin acids. However, the excitation/emission wavelengths (375/475 nm) used by Richardson et al. [11] gave about 2% the fluorescence intensity obtained at 325/400 nm.

At a signal to noise ratio of three, the detection limit of the resin acid esters was as low as 10–20 μg/l using HPLC/LC-8 column equipped with a scanning fluorescence detector. The derivatized esters were confirmed to fluoresce maximally at 325 nm, excitation and 400 nm emission. Notice that the fluorescence due to the 4-BrMMC peak height was reduced by half after the derivatization procedure. The fluorescence quantum yield was observed to depend on resin acid structure since the esters of conjugated diene acids such as abietic, neoabietic, palustric and levopimaric only exhibited a quantum yield about half that of non-conjugated dehydroabietic, pimaric and isopimaric acids. Fluorescence intensity, however, was independent of pH ranging from 4.5 (100 m*M* acetate) to 9.25 (100 m*M* borate). The addition of 15 m*M* MECD or 10% acetonitrile in the mobile phase did not affect the fluorescence intensity of the derivatized resin acids. Such behavior could be explained by the fact that the esterification between the resin acids and 4-BrMMC rendered the derivatized products neutral and hydrophobic. The fluorescent derivatives were stable for

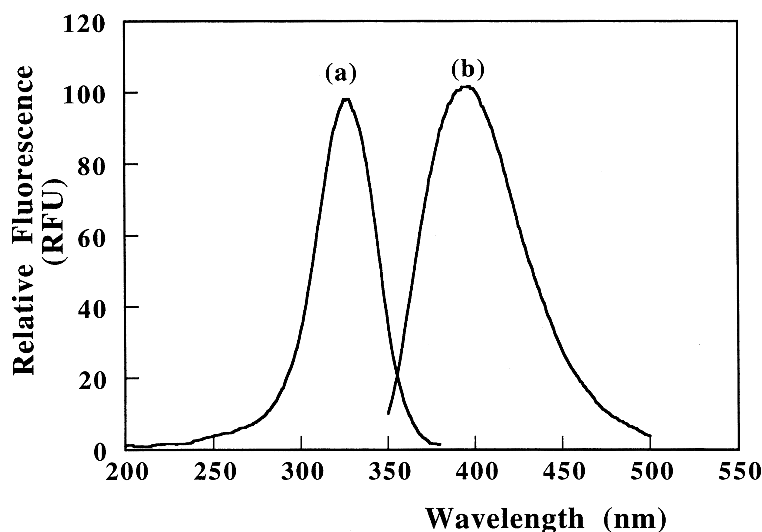


Fig. 2. Excitation (a) and emission (b) wavelength scans of 1 μg/l 7-methoxycoumarin 4-yl-methyldehydroabietate diluted in acetone at a PMT of 550 V. In (a), the emission wavelength was fixed at 400 nm as displayed whereas in (b), the excitation wavelength was fixed at 325 nm.

several weeks if not months, as manifested by the constancy of the fluorescent signal obtained during the course of repeated measurements as long as they were well protected from visible light.

The identity of an ester formed between abietic acid and 4-BrMMC was also confirmed by mass spectroscopy. As shown in Fig. 3, the derivatized abietic acid ester displayed a major peak at 491 m/z (mass-to-charged ratio) as anticipated. The absence of the abietic acid peak (M_r 302) in the spectrum clearly indicated that the derivatization proceeded to completion and yielded no side products. The remaining or unreacted 4-BrMMC (used two-fold in excess) showed a doublet at 269 and 271 m/z, respectively: one peak should correspond to ^{79}Br (50.54%) while the other peak corresponds to ^{81}Br (49.48%).

As reported by Richardson et al. [11] and confirmed in this study, the resin acids could also be derivatized using 4-bromomethyl-7-acetoxycoumarin in the presence of potassium carbonate. The derivatized esters, however, did not fluoresce but could be hydrolyzed to the fluorescent (7-hydroxy-methylcoumarin-4-yl) methyl ester of the resin acid by the addition of 0.2 M potassium hydroxide. This fluorescent product was very unstable, therefore, except for post-column alkali hydrolysis as well as detection, the esters derivatized from 4-bromomethyl-7-acetoxy coumarin were not suitable for on-line detection by CE-laser induced fluorescence.

3.2. Separation of resin acid esters using HPLC

Two different columns (Supelco's LC-PAH and

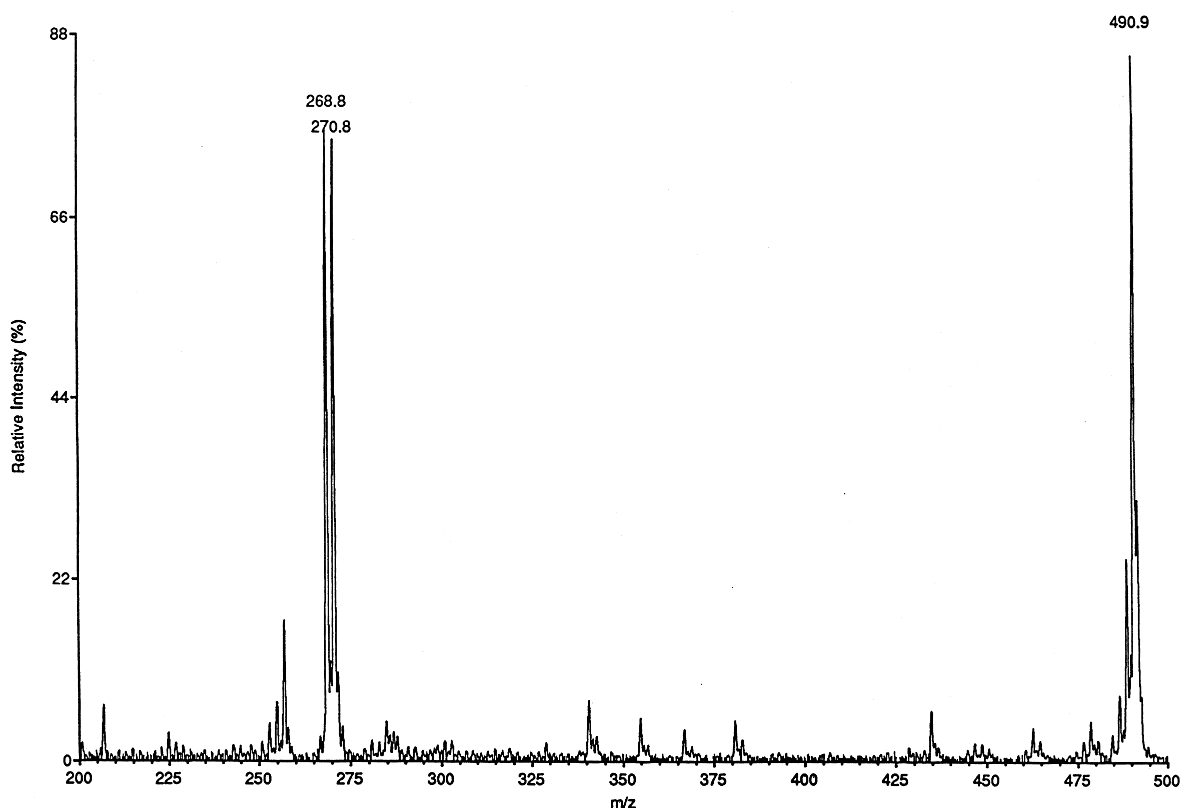


Fig. 3. Electrospray ionization mass spectrometry (ESI-MS) spectrum of the resulting derivatization mixture containing abietic acid ester (M_r 491) and 4-bromomethyl-7-methoxy coumarin (4-BrMMC) in acetonitrile. The derivatization was performed with a two-fold excess of 4-BrMMC.Unreacted 4-BrMMC was denoted as the peak 269 and 271 m/z, respectively corresponding to ^{79}Br (50.54%) and ^{81}Br (49.48%). The abietic acid peak (M_r 302) disappeared in the spectrum, an indication of complete derivatization.

LC-8) were used to separate ten resin acid esters using various mobile phases. Regardless of the column used, the resolution of the (7-methoxy-coumarin-4-yl) methyl esters was better with acetonitrile compared to methanol and to tetrahydrofuran which showed very poor resolution of the esters. With the LC-PAH column, only seven peaks were observed as compared to eight peaks when the LC-8 column was used. Both DCDA and neoabietic acid esters were baseline separated on the LC-8 column as opposed to the LC-PAH column. Using the latter, only dehydroabietic and CDA acid esters were baseline separated using mixtures of acetonitrile and water. Addition of up to 15% (w/v) MECD, in the mobile phase, did not improve the separation.

The best separation was obtained using the LC-8 column with a mobile phase consisting of a mixture of acetonitrile–water (55:45, v/v, flow-rate of 1.5 ml/min) since eight discernible peaks were observed and five resin esters were baseline resolved (Fig. 4). After the system's peak (combined acetone and 4-BrMMC), the DHA ester eluted as the first peak whereas the DCDA ester interacted strongly with the LC-8 column and only eluted after 100 min of separation. The CDA esters (denoted as a and b in Fig. 4) presented a baseline resolved doublet and this pair was also baseline resolved from the neoabietic acid ester. However, isopimaric and palustric acid esters were not separated and co-eluted as a single peak. Similar behavior was also observed for the levopimaric and abietic acid esters. Although not baseline resolved, the pimaric acid ester displayed a single peak. Exhaustive attempts to modify the mobile phase including the addition of several organic modifiers did not resolve the above unresolved peaks. The poor resolutions were owed to the similar chemical structures of the resin acids/esters having differed only by the position of a double bond, thereby making it very difficult for them to be separated using reverse phase liquid chromatography.

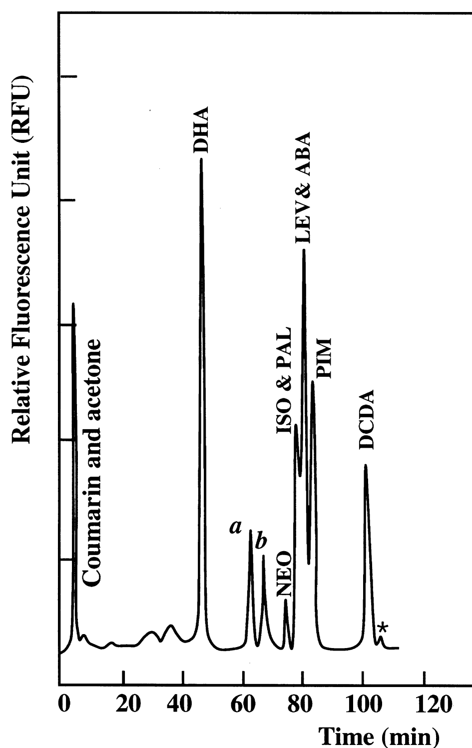


Fig. 4. Chromatogram of the resin acid esters using the LC-8 (15 cm×4.6 mm, 3 μm particle) column. The mobile phase consisted of acetonitrile: water (55:45%, v/v%) with a flow-rate of 1.5 ml/min. Abbreviations for peak identification are given in Table 1. The esters of 12-chlorodehydroabietic and 14-chlorodehydroabietic acids are denoted as peaks *a* and *b*. The concentration of the resin acid esters is 10 μg/l each, except for abietic and levopimaric esters (50 μg/l). The asterisk denotes the impurity from the resin acids. Fluorescence condition: 325 nm for excitation and 400 nm for emission.

3.3. Separation of resin acid esters using CE–LIF

Some initial CE experiments equipped with UV detection involving different SBCD and MECD concentrations indicated that the concentration of MECD in the running buffer had to be somewhat equivalent to SBCD to effect good separation. Without SBCD in the separation buffer, all of the resin acid esters co-migrated as a single peak which was very close to electroosmotic flow (EOF). On the other hand, in the absence of MECD, all resin acid esters formed strong inclusion complexes with SBCD and did not appear in the electropherogram, even after 25 min of separation (data not shown). On the basis of such findings, a series of experiments was conducted to optimize the SBCD/MECD concentration ratios, pH as well as the separation potential for the resin acid esters.

With capillary electrophoresis equipped with laser-induced fluorescence, separation at pH 4.5 and +15

kV applied potential using 42.5 m*M* SBCD and 12.5 m*M* MECD in 50 m*M* sodium acetate was very satisfactory since eight resin acid esters were baseline separated within 26 min (Fig. 5). Notice that at 40 m*M* SBCD-5 m*M* MECD, only a few peaks were observed. Increasing MECD concentration to 10 m*M* in the separation buffer resulted in eight discernible peaks, however, the abietic acid and levopimaric acid esters were not fully resolved. The separation of levopimaric and abietic acid esters as well as isopimaric and palustric acid esters was noteworthy since these two pairs were not separated by HPLC–LC-8 column (Fig. 4). The separation of palustric and levopimaric acid esters was also of significance since these two resin acid esters are not separated by GC. Notice that the heaviest resin acid ester derived from DCDA eluted first while the ester of neoabietic acid migrated as the last peak. The elution pattern was quite different from that of reversed-phase HPLC with LC-8 column and such behavior was not completely unexpected because the separation of the resin acid esters in HPLC was entirely based on the hydrophobic interaction between the analytes and the LC-18 or LC-8 phase. As described earlier, the most hydrophobic resin acid ester derived from DCDA interacted strongly with the LC-8 column and eluted as the last peak (Fig. 4). On the other hand, the migration order of the resin acid esters in Fig. 5 is related to a pattern of distribution between the cyclodextrins, with earlier emerging peaks favoring complexation with MECD and later emerging peaks favoring SBCD. Surprisingly, the heaviest and most hydrophobic DCDA ester emerged as the first peak in the electropherogram. Although the guest–host interaction was attributed to effective separation, it was not possible to decipher whether the analyte would form an inclusion complex with either MECD or SBCD through the coumarin or resin acid moiety. With respect to the internal cavity size of either MECD or SBCD (ca. 0.60–0.64 nm), it was very unlikely that the resin acid ester could be completely included in such a hydrophobic cavity. Reproducibility for the migration times and peak heights of the eight resin acid derivatives, respectively was 1.9–2.4% and 4–10% RSD (from six repeated electropherograms).

Separation was also attempted at pH 9.25 to shorten the analysis time using a separation buffer containing 42.5 m*M* SBCD and 12.5 m*M* MECD in 50 m*M* sodium borate. At +15 kV applied potential, the electroosmotic flow was much faster and separation was completed within less than 17 min with an appreciably narrower separation window (figure not shown). The elution pattern at this pH condition was identical to that of pH 4.5. Except for the palustric/dehydroabietic acid ester pair ($R_s = 0.8$ in compari-

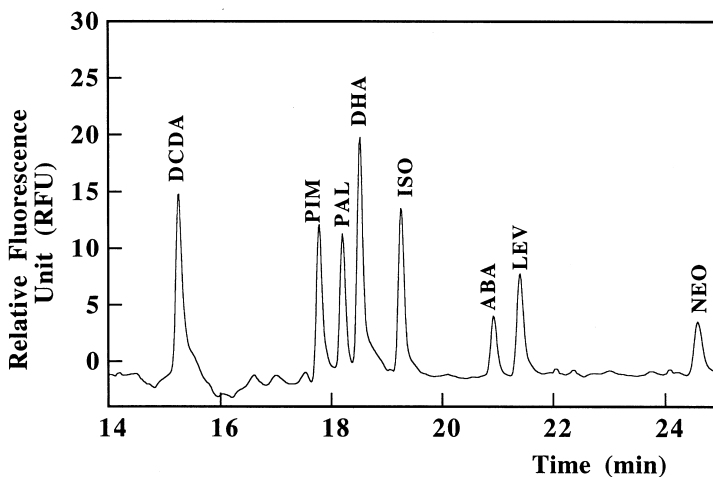


Fig. 5. Separation of the eight resin acid esters at pH 4.5 using a separation buffer containing 50 m*M* sodium acetate, 12.5 m*M* MECD and 42.5 m*M* SBCD. The potential applied is +15 kV with a resultant current of 85 μA. The concentration of the resin acid esters is 500 μg/l each. Abbreviations used for peak identification are given in Table 1.

son to $R_s = 1.7$ at pH 4.5), the remaining resin acid derivatives were baseline separated. The separation of the pimaric and palustric acid esters was still considered satisfactory since the resolution was about 1.5 (compared to $R_s = 2.2$ at pH 4.5). Decreasing the applied potential from 15 kV to 10 kV did not improve the separation efficiency of the unresolved peaks, but rather prolonged the separation time to 27 min. Altering the MECD/SBCD concentration ratio as well as adding organic modifiers such as methanol, acetonitrile, γ-cyclodextrin, hydroxypropyl-β-cyclodextrin, sodium dodecyl sulfate (SDS) to the running buffer failed to improve the overall quality of separation.

Separation was then extended for the analysis of the esters derivatized from 12-chloro and 14-chlorodehydroabietic acids. Regardless of the separation pH, the most remarkable result obtained with these CD systems in comparison to HPLC (Fig. 4) was the extreme separation of the esters derived from 12-CDA and 14-CDA isomers. The CDA acid used in this derivatization study contains an equal mixture of 12- and 14-chloro positional isomers and neither 12-nor 14-form is commercially available. Therefore, the peak assignment for either 12-chloro or 14-CDA was not possible. As shown in Fig. 6, at pH 9.25, one (peak *b*) of these two esters was baseline separated from the levopimaric acid ester peak, whereas the other (peak *a*) was barely split from the DCDA ester peak. A similar result was also attained for the separation performed at pH 4.5 using 42.5 m*M* SBCD and 12.5 m*M* MECD in 50 m*M* sodium acetate (Fig. not shown). Again, altering the MECD/SBCD concentration ratio and applied potential as well as adding several organic modifiers including SDS failed to separate the DCDA ester from one positional ester isomer (peak *a*) derived from monochlorodehydroabietic acid.

In addition to poorer resolution, except for the first two peaks (DCDA and PIM), separation at pH 9.25 also resulted in lower theoretical plates (Table 1). Notice that at pH 4.5, all components resulted in peaks with $N > 1 \times 10^5$ to 2×10^5 (or 2×10^5 to 4×10^5 theoretical plates/m; effective capillary length, 40 cm). The number of theoretical plates obtained in this study is in the order of those obtained by Brown et al. [8] for polycyclic aromatic hydrocarbons with four and five rings such as benzo[*ghi*]perylene, pyrene, indeno[*1,2,3-cd*]pyrene, pyrene, etc. using also a mixture of SBCD and MECD in electrophoretic separation. Such a result thus illustrated that the separation was reasonably efficient with respect to the bulky structure of the target analytes.

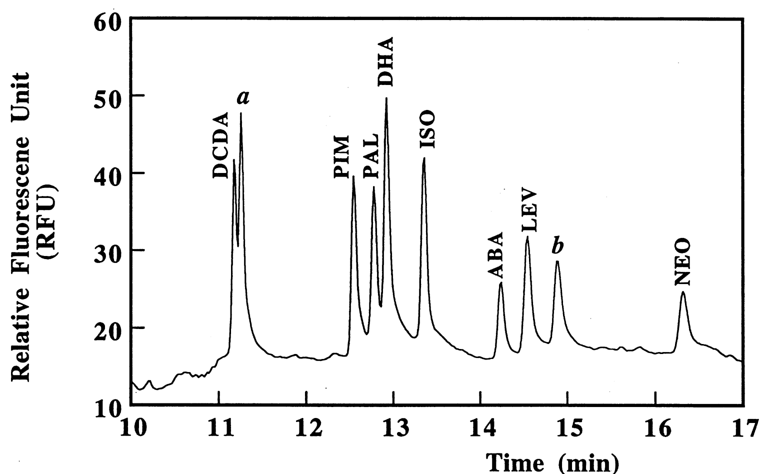


Fig. 6. Separation of the ten resin acid esters at pH 9.25 using a separation buffer containing 50 m*M* sodium borate, 12.5 m*M* MECD and 42.5 m*M* SBCD. The concentration of the resin acid esters is 500 μg/l each and abbreviations for peak identification are given in Table 1. The esters of 12-chlorodehydroabietic and 14-chlorodehydroabietic acids are denoted as peaks *a* and *b*. The potential applied is +15 kV with a resultant current of 91 μA.

Table 1
Resolution (R_s) and theoretical plates per meter of the resin acid esters resolved by cyclodextrin modified capillary electrophoresis equipped with laser-induced fluorescence[a]

Peak identification	pH 4.5		pH 9.25	
	R_s	Plates/m	R_s	Plates/m
Dichlorodehydroabietic (DCDA)	nd	nd	nd	nd
Pimaric (PIM)	11.3	280 000	6.1	290 000
Palustric (PAL)	2.2	410 000	1.5	260 000
Dehydroabietic (DHA)	1.7	300 000	0.9	250 000
Isopimaric (ISO)	3.5	340 000	2.7	270 000
Abietic (ABA)	7.9	390 000	5.4	310 000
Levopimaric (LEV)	2.1	300 000	1.8	290 000
Neoabietic (NEO)	8.0	310 000	6.9	230 000
Monochlorodehydroabietic (CDA)				
* Peak *a*	nd	nd	nd	nd
* Peak *b*	3.1	230 000	1.8	210 000

[a] The number of theoretical plates for the DCDA peak was not determined since this resin acid ester migrated very close with one of the positional resin acid ester isomers derived from the monochlorodehydroabietic acid as described in the text. The value determined for this peak from an electropherogram using only eight resin acid esters in the absence of the esters derived from monochlorodehydroabietic acid at pH 4.5 and 9.25 was 200 000 and 200 000 plates/m, respectively.
nd. not determined.

To establish the detection limit for each resin acid ester, a standard sample containing each derivatized resin acid was analyzed by cyclodextrin modified CE with 5 s injection time. The separation buffer used for this analysis contained 42.5 m*M* SBCD and 12.5 m*M* MECD in 50 m*M* borate, pH 9.25. A calibration plot of peak area versus concentration was constructed for each derivatized resin acid by running electropherograms of the standard mixture diluted in acetone at several ratios. The detection limit was estimated as the concentration at which the peak area was equal to three-times the standard deviation of the peak area versus concentration plot. The detection limit of the esters of conjugated diene acids such as abietic, neoabietic, palustric and levopimaric was about 20 to 40 μg/l whereas that of the esters of non-conjugated dehydroabietic such as pimaric and isopimaric acids was 10 to 20 μg/l. Enhancement in detectability was, therefore, pushed down by about three orders of magnitude since without derivatization, the detection limit of the underivatized resin acids ranged from 5–10 mg/l with CE–UV detection. Notice that the detection limit obtained by capillary electrophoresis/laser-induced fluorescence is also very competitive to that of HPLC/scanning fluorescence detection (10–20 μg/l) as mentioned previously.

4. Conclusion

In brief, this study has illustrated the suitability of derivatization of the resin acids with a coumarin fluorescing tag at their carboxylic acid group to improve the detectability. The resin acids were readily reacted with 4-bromomethyl-7-methoxy coumarin to form fluorescent resin acid esters which fluoresced optimally at 325 nm, excitation and 400 nm emission, respectively. The derivatization reaction has several advantages including (i) relatively rapid reaction with extremely high yields, (ii) stability of the derivatized resin acid esters, and (ii) no sample clean up. Together with MECD, SBCD was selected as the pseudo-stationary phase to baseline separate nine out of ten esters derivatized from resin acids which are commonly found in pulp mill effluents.

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