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Quantitative analysis of total resin acids by high-performance liquid chromatography of their coumarin ester derivatives

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ABSTRACT

A method for the quantitative analysis of resin acids in effluent and water samples using high-performance liquid chromatography is described. Resin acids in aqueous samples are extracted by passage through C_{18} solid-phase cartridges at pH 9. The (7-methoxycoumarin-4-yl) methyl (MMC) and (7-acetoxycoumarin-4-yl) methyl (MAC) esters are quantitatively formed at room temperature by the reaction of resin acids with 4-bromomethyl-7-methoxycoumarin and 4-bromomethyl-7-acetoxycoumarin respectively, in the presence of potassium carbonate. The effect of potassium carbonate particle size, exposure to light, presence of residual water, and derivatisation reagent: resin acid ratio on their formation are described. The MMC esters of resin acids may be detected by UV absorption at 318 nm at concentrations >20 $\mu g\ l^{-1}$, while the (7-hydroxycoumarin-4-yl) methyl esters, obtained by post-column alkaline hydrolysis of the resin acid MAC (RAMAC) esters, enable the detection of resin acids by fluorescence spectrophotometry to levels below 1 $\mu g\ l^{-1}$. The method has been compared to gas chromatographic analysis of the carboxylic acid methyl esters and shows no evidence of any interferences by other carboxylic acids. The method is routinely used by two newsprint mills for environmental monitoring of resin acids in effluent.

INTRODUCTION

Resin acids are released from softwoods in both mechanical and chemical pulping processes [1]. They are toxic to marine life at low concentrations, having 96-h LC_{50} values ranging from 0.5 to 1.1 mg l^{-1} [2–4], and also accumulate in fish tissue [5,6]. Attempts have been made to estimate effluent toxicity on the basis of chemical composition and LC_{50} values for pure components [2,7–8]. Resin and fatty acids have also been implicated with occurrences of pitch deposits on paper machines and in pulping equipment [9].

The routine monitoring of resin acids in a variety of matrices is therefore necessary and has required the development of analytical techniques for their extraction and analysis. Extraction techniques have been predominantly based on solvent extraction using diethyl ether [10,11], dichloromethane [10,12–13], light petroleum–acetone–methanol [11], and methyl *tert.*-butyl ether [14]. Solid-phase extraction techniques have also been used to isolate resin acids from rosin, paper mill deposits, and process streams [15,16]. The most common instrumental technique used in recent years is that of capillary gas chromatography (GC) of the methyl esters of resin acids [17–19]. More recently, the inherent sensitivity of the electron capture detector has been utilised to measure pentafluorobenzyl esters of resin acids [20].

While high-performance liquid chromatography (HPLC) has been used for qualitative analysis of mixtures containing resin acids [21–26], and for

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quantitative measurement of one resin acid (dehydroabietic acid) [22,27], it has not been used for quantitative analysis of total resin acids in a mixture. The two major barriers to their quantitation by HPLC are the difficulty in separating the various resin acid isomers, and the lack of a suitable chromophore for their detection, particularly for the non-conjugated resin acids [1].

Bromomethyl coumarin compounds containing substituents on the aromatic ring and other bromoalkyl-aromatic compounds have been used to form esters with carboxylic acids [28–35]. The derivatisation procedures have used 4-bromomethyl-7-methoxycoumarin (4BrMMC) [28–31], 4-bromomethyl-6,7-dimethoxycoumarin [32], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone [33], and 4-bromomethyl-7-acetoxycoumarin (4BrMAC) [34,35] to form fluorescent derivatives of carboxylic acids from a variety of matrices. More recently micellar phase transfer catalysis procedures have been developed around 4BrMMC [36] and 9-bromomethylacridine [37] and applied to the analysis of fatty acids in plasma.

This paper describes the application of these derivatisation techniques to the analysis of resin acids in effluents as an alternative to existing methods of analysis with a view to developing a simple method with low detection limits and using small sample volumes. Both UV and fluorescence properties of the coumarin-4-yl methyl esters of resin acids were investigated based on the reactions shown in Fig. 1. A procedure based on the formation of coumarin-4-yl methyl esters of resin acids from 4BrMMC and 4BrMAC has been developed and used successfully for environmental monitoring of two newsprint mills' effluent for over two years. Factors affecting the quantitative formation, spectrophotometric properties, and HPLC separation of coumarin-4-yl methyl esters of resin acids from 4BrMMC and 4BrMAC are discussed in this paper.

EXPERIMENTAL

Materials and reagents

The resin acids (dehydroabietic, isopimaric, neoabietic, palustric, abietic, levopimaric and sandaracopimaric) were obtained from Helix Biotech (Vancouver, Canada) and used without further pu-

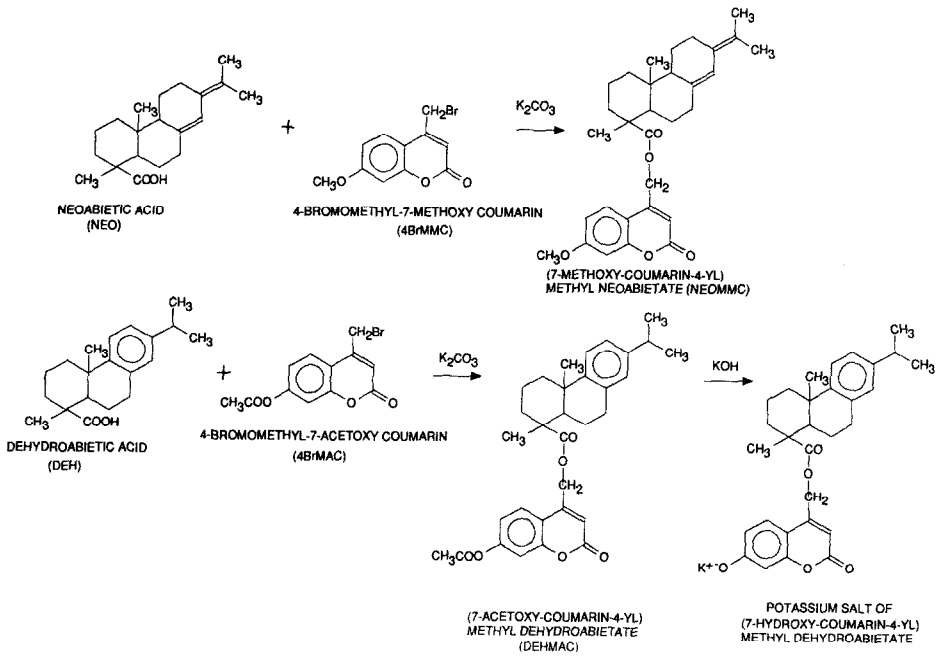

Fig. 1. Reaction schemes for formation of (7-methoxycoumarin-4-yl) methyl neoabietate and (7-hydroxycoumarin-4-yl) methyl dehydroabietate.

rification. Dehydroabietic (DEH), isopimaric (ISO), and neoabietic (NEO) acid were found by GC assay to be >98% pure, while the remainder contained 5%–15% of other resin acids. 4BrMMC was obtained from Sigma (St. Louis, MO, USA), while 4BrMAC was synthesised from 4-methylumbelliferone according to the method of Tsuchiya *et al.* [34]. HPLC-grade acetonitrile, dichloromethane and methanol, and spectroscopic grade acetone were used throughout. Water was obtained from a Millipore Milli-Q water purification system. All other reagents were analytical-reagent grade. Analytichem C_{18} 3-ml Bond Elut solid-phase extraction columns (part. No. 607303) were used to extract effluent samples. Samples were placed in a Branson B-220 ultrasonic bath for the derivatisation process.

Spectrophotometric analyses

Quantum yields of the resin acid derivatives of 4BrMMC and 4BrMAC were measured according to an established method [38] using a Perkin-Elmer LS-5 fluorescence spectrophotometer connected to a Perkin-Elmer 3600 data station with quinine sulfate (Sigma) and 9,10-diphenylanthracene (Aldrich) as reference standards. A Varian DMS 90 UV–VIS spectrophotometer was used to record UV spectra.

Extraction and derivatisation

The volume of sample to be extracted, normally within the range of 2–100 ml, was chosen so that it contained no more than 50 μg of resin acids. This was to ensure that the ratio of derivatisation reagent to resin acid was sufficient to obtain quantitative conversion to the RAMMC or RAMAC ester. The pH of samples containing more than 10 mg l^{-1} of suspended solids was adjusted to 11 with solid potassium hydroxide. The samples were then filtered through Whatman No. 4 filter paper. The pH was then reduced to 9 with dilute H_2SO_4 and the conductivity increased to >2 mS cm^{-1} with solid NaCl. The Bond Elut cartridges were conditioned by rinsing successively with 2 ml acetone, 3 ml methanol and 3 ml water. The effluent sample was then passed through the solid phase extraction cartridge at 20 cmHg vacuum. Excess water was removed from the extraction cartridge by applying vacuum for at least 15 min after all sample had passed through. The adsorbed resin acids and other organic material were eluted into a light-protected 2-ml vial by adding sequentially 0.5 ml acetone, 2 × 0.5 ml dichloromethane and 0.5 ml methanol. The solvent was then removed under a gentle stream of nitrogen. The derivatisation was performed *in situ* in the vial in the presence of 4–5 mg of finely ground potassium carbonate (>100 μm) by adding 0.50 ml of 0.0074 *M* 4BrMMC or 0.01 *M* 4BrMAC (in acetone), and 1.00 ml of acetone, and placing it in an ultrasonic bath for 10 min at 25°C. The potassium carbonate was prepared by grinding with a mortar and pestle for 10 min and measuring the particle size by optical microscopy. The vial was kept tightly sealed with a PTFE-lined septum to ensure no loss of solvent between derivatisation and analysis. The potassium carbonate was allowed to settle before presenting the sample for HPLC analysis.

Standards were prepared by placing a known quantity of dehydroabietic acid and isopimaric acid in a vial before addition of the derivatising reagent. The method of external standards was used for calibration.

HPLC analysis

Both the (methoxycoumarin-4-yl) methyl (MMC) esters and the (acetoxycoumarin-4-yl) methyl (MAC) esters were separated using a Rainin Dynamax C_8 5-μm 25 cm × 4 mm reversed-phase column (80-325-C5) fitted with a Rainin Dynamax guard column (80-300-G5) and a 2-μm in-line filter to remove any traces of particulate potassium carbonate. A Varian 5060 ternary gradient HPLC system (fitted with a Rheodyne 7126 injection valve (20-μl loop) and a Varian Model 9090 autosampler) was used to deliver an acetonitrile–water (70:30, v/v), solvent mix at 1.5 ml min^{-1}. A linear gradient to acetonitrile–water (90:10, v/v) was generated at 2.5% min^{-1} where it was held for 5 min before returning to the initial solvent conditions.

The MMC esters were detected using a Varian UV100 variable-wavelength detector set at 318 nm. The MAC esters did not fluoresce but were hydrolysed post-column to the fluorescent (7-hydroxymethylcoumarin-4-yl) methyl ester of the resin acid by the addition of 0.2 *M* potassium hydroxide in methanol–water (80:20, v/v) at a flow-rate of 0.5 ml min^{-1}. The fluorescence signal was measured with a Perkin-Elmer Model LS4 fluorescence spectrophotometer with the excitation monochromator set

at 375 nm (slit width 15 nm), and the emission monochromator set at 475 nm (slit width 20 nm). The detector signal was processed by a Varian DS654 data system.

The composition of hydrolysis products from the addition of alkali to resin acid MAC (RAMAC) esters was determined using a Varian 9060 Polychrom photodiode array detector.

Gas chromatography analysis

The evaporated extracts from the Bond Elut cartridges were made to react with diazomethane (in diethyl ether) to form resin acid methyl esters. Heptadecanoic acid was added to the sample, prior to methylation, as an internal standard. The methylated extracts were reconstituted in acetone for injection onto a Varian 3600 gas chromatograph fitted with a 30 m × 0.32 mm HP-1 column (Hewlett-Packard) film thickness 1.05 μm and a Varian 1075 split/splitless injector (used in the splitless mode). Data were processed on a Varian Vista 402 data system.

RESULTS AND DISCUSSION

Spectophotometric properties

Resin acid MMC (RAMMC) esters were prepared and isolated as pure compounds [1] so that their spectral properties could be examined. While they were all found to have a molar absorptivity of 13600 ± 260 l mol^{-1} cm^{-1} at 318 nm, their fluorescence quantum yield was found to be dependent on resin acid structure, with the RAMMC esters of conjugated diene acids (abietic, neoabietic, palustric, and levopimaric) having a quantum yield about half that of the RAMMC esters of non-conjugated resin acids (dehydroabietic, isopimaric, and sandaracopimaric). Furthermore the quantum yield was affected by the water content of the HPLC solvent mixtures, in a similar fashion to that reported for fatty acid MMC esters [31]. It was concluded therefore that RAMMC esters could only be measured by UV detection.

RAMAC esters had a molar absorptivity of 8200 ± 150 l mol^{-1} cm^{-1}at 310 nm, and therefore could also be detected quantitatively by UV detection. However, because the molar absorptivity was lower than that for the RAMMC esters, UV detection was not used for RAMAC derivatives. RAMAC esters did not fluoresce until they were hydrolysed to the (7-hydroxycoumarin-4-yl) methyl esters. The fluorescence quantum yields of hydrolysed DEHMAC, ISOMAC, and NEOMAC (representing aromatic, non-conjugated diene, and conjugated diene resin acid structures, respectively) were 0.28 ± 0.01, 0.30 ± 0.02, and 0.26 ± 0.02 respectively. In contrast to the RAMMC esters, their quantum yields were the same regardless of resin acid structure, and did not vary significantly over the range of water–solvent proportions encountered in their HPLC separation. The fact that the fluorescence response was independent of resin acid structure enabled the use of a single resin acid to represent the response of all resin acids, and therefore measure quantitatively the total resin acid concentration, even though they co-eluted when analysed by HPLC. Investigation of the products of hydrolysis using a photodiode array detector revealed that at room temperature the predominant hydrolysis product was the (7-hydroxycoumarin-4-yl) methyl ester. Comparison of sample spectra with authentic compounds revealed that no significant free resin acid was formed. This finding is in contrast to the alkaline hydrolysis of fatty acid MAC esters in which it has been claimed that both the acetoxy and carboxylate ester linkages are hydrolysed [34].

HPLC separation

Measurements of the capacity factors (k') of both the MMC and MAC esters on C_{18} and C_8 columns showed that, while DEHMMC (and DEHMAC) eluted with a lower k' than the non-aromatic RAMMC (and RAMAC) esters, the latter had almost identical k' regardless of resin acid structure, and eluted with a narrower distribution of k' on a C_8 column than on a C_{18} column. The resin acid, pimaric acid, was not available as a pure standard, and hence the k' of its MMC and MAC esters could not be measured. However, GC analysis of effluent from a mechanical pulping process containing pimaric acid (10–15% of total resin acid content) showed good agreement between results for the two types of measurement. The k' values of chlorinated resin acids were not measured as only effluent from mechanical pulp mills was evaluated by this technique.

Fig. 2 is a chromatogram of the MMC esters of several fatty acids ($C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and

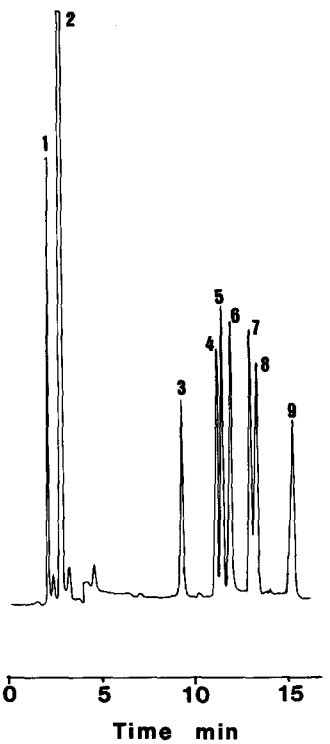

Fig. 2. Chromatogram showing response of 200 ng (each) of MMC esters of resin and fatty acids. HPLC conditions: Column, Rainin Dynamax C_8 5-μm 25 cm × 4 mm (80-325-C5) + Rainin Dynamax guard column (80-300-G5); initial solvent acetonitrile–water (70:30, v/v), to acetonitrile–water (90:10, v/v) at 2.5% min^{-1}, UV detection at 318 nm. Peaks: 1 = acetone; 2 = 4BrMMC; 3 = DEHMMC; 4 = non-aromatic resin acid MMC esters; 5 = $C_{16:1}$ MMC; 6 = $C_{18:2}$ MMC; 7 = $C_{16:0}$ MMC; 8 = $C_{18:1}$ MMC; 9 = $C_{18:0}$ MMC.

$C_{18:2}$), dehydroabietic acid and isopimaric acid. Resolution of these components in particular is important as this range of fatty acids is commonly found in extracts and pulping effluents from softwoods such as *Pinus radiata*. Baseline resolution of $C_{16:1}$ MMC from ISOMMC was almost achieved with the solvent program and column shown. A similar separation was achieved for RAMAC esters.

Reaction conditions

The main purpose of this technique was to develop a method that was robust enough to be used in a paper mill by technical staff for routine environmental monitoring. It was also for this reason that the procedure had to be as simple as possible and not involve exposure to hazardous chemicals. Several published methods for formation of fatty acid MMC and MAC esters require the use of the highly toxic crown ether 18-crown-6 [29,34,35]. A key factor in eliminating the crown ether catalyst was to reduce the particle size of the potassium carbonate used to form the resin acid salt which reacted with the bromomethyl coumarin. The effect of reducing the potassium carbonate to a small particle size (< 100 μm) was to bring about quantitative formation of the RAMMC ester in less than 10 min compared to > 1 h for potassium carbonate used directly as received.

Some procedures highlight the need to protect the reaction mixture from visible light due to the photodecomposition of both 4BrMMC and 4BrMAC [29,35]. Exposure of either of these reagents to light causes rapid decomposition to products that either cannot form derivatives with carboxylic acids, or form adducts that interfere in the analysis of the target compounds [1]. The effect of exposing resin acids to light whilst performing the derivatisation was quite dramatic for the conjugated diene resin acids (neoabietic, abietic, levopimaric and palustric), and was manifested by degradation of the resin acid coumarin ester after about 20 min exposure time. This phenomenon was not observed for the non-conjugated diene resin acids such as isopimaric acid, nor for dehydroabietic acid.

The extraction of aqueous samples using solid-phase extraction cartridges can result in traces of water being eluted along with the resin acids unless great care is taken to dry the cartridges by drawing air through them for some time after all sample has been adsorbed. The tolerance of the procedure to the presence of traces of water was determined by addition of known amounts of water to the reaction mixture. The maximum amount of water that could be tolerated in the reaction was 4% (v/v) or 60 μl in a total of 1.50 ml. An amount of water greater than 4% resulted in a significant decrease in yield, approaching 0 at 20% water.

An important consideration in a derivatisation reaction is to have a sufficient excess of reagents to ensure that quantitative conversion to the desired product occurs at all sample concentrations. The excess required to achieve this for both 4BrMMC and 4BrMAC was investigated by allowing isopi-

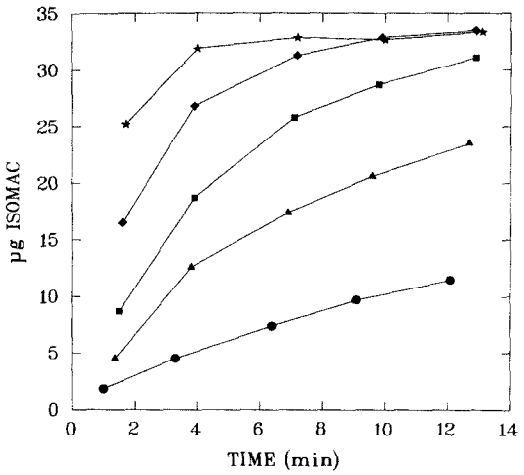

Fig. 3. Effect of BrMAC:ISO molar ratio on yield of ISOMAC. BrMAC:ISO ratio: ● = 1:1; ▲ = 2.5:1; ■ = 5:1; ◆ = 10:1; ★ = 20:1. Theoretical yield, 34.4 µg.

maric acid to react with 4BrMAC and 4BrMMC over a range of molar excesses of bromomethylcoumarin to isopimaric acid. Fig. 3 shows that a 10-fold excess was sufficient to ensure that quantitative conversion occurred after 10 min.

Acetonitrile was investigated as an alternative solvent to acetone in which to perform the derivatisation but it was found to be inferior due to the faster reaction in acetone compared to acetonitrile. The enhanced rate in acetone has been ascribed to the greater dispersion of charge in the transition state complex in a solvent with medium dielectric constant (such as acetone, ε (25°C) = 20.7) than in a solvent with a larger dielectric constant, (acetonitrile ε (25°C) = 36.2) [39].

Application to effluent and water samples

Some important factors in developing a reliable extraction and chromatographic technique for routine trace organic analysis are quantitative recovery of spiked samples, freedom from interference, reproducible chromatographic separation over the range of sample conditions likely to be encountered, correlation with other established methods, and ease of use. Recoveries of DEH and non-aromatic resin acids (added to effluent samples at the 1 mg l^{-1} level) using the solid-phase adsorption method and analysis of their MMC esters, averaged 95±8%, and 91±11%, respectively, over a six-month period. It was found that a slightly alkaline extraction (pH 9) was necessary to obtain good recoveries, due to adsorption of resin acids on glassware and plasticware when these are used to manipulate samples at pH < 7.

Use of the solid phase extraction technique for resin acid extraction has several advantages over solvent extraction methods, including elimination of emulsions, lower solvent consumption, and the ability to handle large sample volumes. The loss of analyte by breakthrough, or preferential adsorption of other extractives, are two potential disadvantages of the procedure. Elimination of interfering compounds in solid-phase extraction of pulp and paper effluents has been achieved by the addition of 15% (v/v) methanol to the sample [16]. Presumably competition for adsorption sites between resin acids and other components was reduced by minimising the adsorption of lignin material [16]. The high recoveries of resin acids from effluent containing very little lignin was achieved in this work without methanol addition.

GC mass spectrometric analysis of samples of effluent containing extractives from both *Pinus radiata* and various *Eucalyptus* species showed that the only carboxylic acids present which were likely to interfere with either RAMMC or RAMAC esters were fatty acids of chain length 16 carbons or greater. The HPLC conditions chosen were able to resolve satisfactorily both the RAMMC and RAMAC esters from the nearest fatty acid ($C_{16:1}$) MMC or MAC ester. The amount of $C_{16:1}$ fatty acid to resin acid can, in effluent, vary significantly depending upon whether the effluent has been biologically treated. Excellent separation of the resin acid (RAMMC or RAMAC) from the $C_{16:1}$ fatty acid ester can be obtained over a wide range of resin acid: $C_{16:1}$ fatty acid ratios. Fig. 4 shows the separation obtained in the case of an untreated effluent when the ratio is high, whereas Fig. 5 shows the separation when the amounts of resin and fatty acids are approximately equal.

The choice of which derivatisation reagent to use is dependent on the concentration of resin acid present in the sample and the type of detector available to the analyst. Formation of RAMMC esters combined with UV detection enabled a concentration of resin acid >20 µg l^{-1} to be measured using effluent volumes of 50 ml. RAMAC esters, however, could

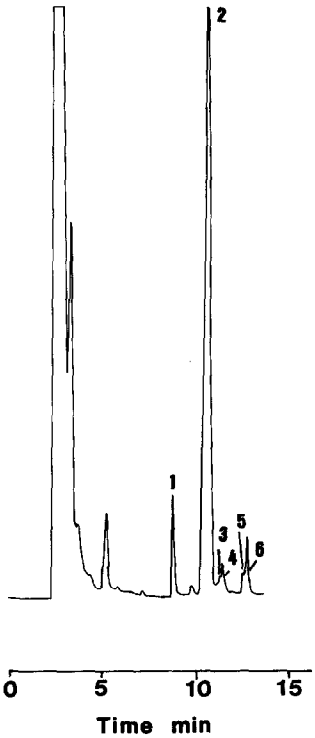

Fig. 4. Chromatogram of total resin acids in softwood pulping effluent, containing 3 mg l^{-1} of resin acids. Peaks: 1 = DEHMMC; 2 = non-aromatic resin acid MMC esters; 3 = $C_{16:1}$ MMC; 4 = $C_{18:2}$ MMC; 5 = $C_{16:0}$ MMC; 6 = $C_{18:1}$ MMC. HPLC conditions as in Fig. 2.

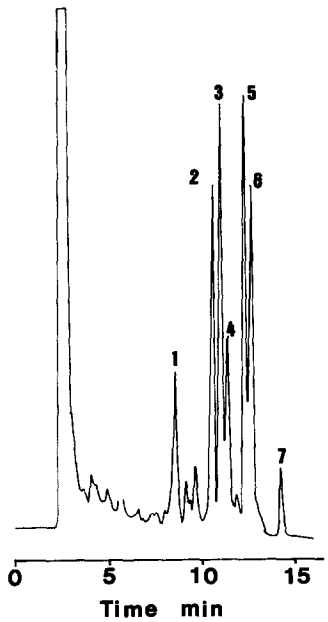

Fig. 5. Chromatogram of total resin and fatty acids in biologically treated effluent containing 20 $\mu g\ l^{-1}$ resin acids. Peaks: 1 = DEHMAC; 2 = non-aromatic resin acid MAC Esters; 3 = $C_{16:1}$ MAC; 4 = $C_{18:2}$ MAC; 5 = $C_{16:0}$ MAC; 6 = $C_{18:1}$ MAC; 7 = $C_{18:0}$ MAC. HPLC conditions as in Fig. 2, except that fluorescence detection at excitation wavelength 375 nm and emission wavelength 475 nm was used instead of UV detection.

be formed and their hydroxycoumarin esters detected by fluorescence detection where resin acid concentrations down to 1 $\mu g\ l^{-1}$ were to be measured.

Correlation of the total resin acid results obtained by HPLC with those obtained by analysis of the methyl esters by capillary GC was undertaken to ensure that good agreement was obtained. The correlation coefficients, slopes and intercepts obtained by linear least-squares analysis of the data (Table I) showed that, within experimental error,

TABLE I

CORRELATION COEFFICIENTS FOR TOTAL RESIN ACID MEASUREMENTS BY HPLC *vs.* GC

HPLC values determined using resin acid esters, GC values determined using resin acid methyl esters (RAME).

HPLC assay (*x*-axis), using	GC assay (*y*-axis), using	R^2	Slope	95% C.L.[c]	Intercept	95% C.L.
RAMMC	RAME	0.9936	0.9518	0.054	0.0220	0.0478
RAMAC[a]	RAME	0.9890	1.011	0.075	0.0438	0.0641
RAMAC[b]	RAME	0.8110	0.9855	0.287	0.0068	0.0089

[a] High concentration samples (0.20–1.60 mg l^{-1})
[b] Low concentration samples (0.005–0.080 mg l^{-1})
[c] C.L. = Confidence level.

the correlation between the HPLC method (using both derivatives) and the GC method, was excellent.

CONCLUSIONS

Resin acids reacted readily with both 4BrMMC and 4BrMAC at 25°C, without the need for a crown ether catalyst, to form RAMMC and RAMAC derivatives, respectively. They were separated from the esters of other carboxylic acids such as fatty acids present in pulp and paper effluents (derived from mechanical pulping processes), by reversed-phase HPLC using a C_8 stationary phase and an acetonitrile–water gradient. The high quantum yield of the (7-hydroxycoumarin-4-yl) methyl esters (obtained by post-column alkaline hydrolysis) enabled total resin acids to be measured at levels down to 1 $\mu g\ l^{-1}$ in water using solid-phase extraction at a slightly alkaline pH.

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