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Note

Analysis of dehydroabietic acid in paper industry effluent by high-performance liquid chromatography

D. E. RICHARDSON*
Australian Newsprint Mills Limited, Boyer, Tasmania 7400 (Australia)
and
B. V. O'GRADY and J. B. BREMNER
Chemistry Department, University of Tasmania, G.P.O. Box 252C, Hobart, Tasmania 7001 (Australia)
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Diterpene tricyclic resin acids may be found in the effluent of those pulp and paper industries which use softwood species such as *Pinus radiata* in their process. Resin acids are toxic to a variety of aquatic organisms, with the common resin acids such as dehydroabietic, pimaric, abietic, levopimaric, palustric, isopimaric, sandaracopimaric and neoabietic acid all having 96-h LC_{50} values, *i.e.*, concentration of compound which kills half the test species in 96 h, of 0.5–1.0 mg/l when the test species is *Salmo gairdneri* (rainbow trout)[1].

The removal of these toxic compounds from paper industry effluent can be achieved by aerobic secondary treatment processes such as an activated sludge process[2]. The toxicity of both treated and untreated effluents has traditionally been measured by direct bioassay in order to monitor the toxicity reduction achieved by the treatment process. More recently attention has been directed to estimating the probable toxicity of effluents by chemical analysis with some degree of success[1,3], thus reducing considerably the effort required to obtain toxicity data in a routine monitoring situation.

A technique for extracting resin acids from thermo mechanical pulp effluent by adsorption onto XAD resin and subsequent analysis by gas chromatography (GC)[2] has been used to monitor the performance of an activated sludge treatment plant in the first few months of its operation[4]. This technique, by virtue of the large volumes which can be extracted, is capable of detecting very small concentrations of resin acids. However, the sample preparation and analysis time for this technique is quite long. An alternative method capable of producing results for up to six samples within 3 h of collection has been developed using high-performance liquid chromatography (HPLC). As dehydroabietic acid was the most abundant resin acid in both untreated and biologically treated effluent[4], a technique for analysis of this component only has been developed for routine monitoring purposes.

HPLC has been used to separate resin acids in tall oil[5] and Kraft Mill effluents[6] and to monitor the microbial degradation products of resin acids[7–9]. The method of detection in all these cases was by ultraviolet (UV) spectrophotometry.

In the present work a technique that is more sensitive and less susceptible to

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interferences than that described previously, and far quicker than the XAD adsorption–GC method[2,4], will be described. The problem of interferences in the form of co-eluting peaks, has been reduced by the use of fluorescence spectrophotometry as an alternative to UV detection.

EXPERIMENTAL

Equipment

UV spectra were recorded on a Varian DMS 90 UV–visible spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer 650-10S fluorescence spectrophotometer. This instrument was also fitted with an HPLC flow cell (18 μl volume).

HPLC using UV detection was done with a Varian Vista 54 high-performance liquid chromatograph which comprised a Varian 5040 ternary gradient pump, a Varian Vista 401 Data Station, a Varian UV50 variable-wavelength UV detector and a Rheodyne 7125 injection valve fitted with a 20-μl sample loop. HPLC using fluorescence detection was done with a Waters M6000A pump, connected to a Rheodyne 7010 valve fitted with a Rheodyne 7011 loop injector filler port and a 20-μl sample loop, a Perkin-Elmer 650-10S fluorescence spectrophotometer, a Perkin-Elmer Model 56 chart recorder and a Shimadzu E1A Chromatopac integrator.

A Varian MicroPak MCH-5-ncap reversed-phase column (15.0 × 0.40 cm) fitted with a guard column packed with Vydac C_{18} reversed-phase packing was used for most of the chromatographic separations. An Alltech C_{18} 5-μm reversed-phase column was used later in the study to separate an interfering compound from the dehydroabietic acid peak.

Chemicals

Water was distilled from glass before use. Acetonitrile was either Waters HPLC grade, or Ajax Unichrom grade. Dichloromethane was either Waters HPLC grade, or redistilled Ajax AR grade. Dehydroabietic acid was supplied by courtesy of Dr. D. Zinkel, U.S. Department of Agriculture, Forest Service, Forest Products Laboratory, Madison, WI, U.S.A. Solvents for the Waters HPLC pump were pre-mixed and filtered under vacuum. A solvent composition of 75% acetonitrile, 24.9% water and 0.1% acetic acid was used with the Waters pump. A solvent composition of 65% acetonitrile, 34.9% water and 0.1% acetic acid was used with the Varian system.

Sample extraction

The pulping process from which the effluent samples in this work were taken used *Pinus radiata* exclusively. The effluent was treated by an activated sludge treatment plant which has been described elsewhere[10]. The effluent samples were adjusted to pH 12, filtered through Whatman 541 filter-paper, and the conductivity adjusted to 2 mS/cm with sodium chloride immediately after collection. The samples were then stored at 4°C prior to analysis, or alternatively shipped to the analytical laboratory within 24 h of collection. Just prior to analysis, 500 ml of each sample were adjusted to pH 3 with 1 *M* hydrochloric acid. The sample was then extracted twice for 2 min with 2 × 60 ml of dichloromethane. The combined extracts were evaporated to 1 ml on a rotary evaporator at a temperature less than 40°C, quantitatively transferred to a washed 3-ml vial and evaporated to dryness with a stream of dry

nitrogen gas. The extract was then reconstituted with 1.00 ml of acetonitrile prior to injection for HPLC. Quantitation was achieved by the use of external standards.

RESULTS AND DISCUSSION

Spectroscopic properties of dehydroabietic acid

Published UV spectra data for methyl dehydroabietate give absorption maxima at 276 nm (ε = 750 l mol^{-1} cm^{-1}), 267.9 nm (ε = 645 l mol^{-1} cm^{-1}) and 199.8 nm (ε = 57,000 l mol^{-1} cm^{-1}) in isooctane[11]. The spectra obtained on the free acid[12] gave absorption maxima at 276 nm (ε = 155 l mol^{-1} cm^{-1}) and 268 nm (ε = 132 l mol^{-1} cm^{-1}). The spectrum of dehydroabietic acid in acetonitrile was measured in this laboratory and found to be similar to the methyl ester, giving absorption maxima at 276 nm (ε = 740 l mol^{-1} cm^{-1}), 267.5 nm (ε = 740 l mol^{-1} cm^{-1}) and 198 nm (ε = 59,700 l mol^{-1} cm^{-1}). A previously reported method[6] for measuring dehydroabietic acid by HPLC monitored the response of dehydroabietic acid at 220 nm where the extinction coefficient is only 9700 l mol^{-1} cm^{-1}. A significant improvement in detection limit was therefore to be expected by monitoring at 200 nm.

The fluorescent properties of dehydroabietic acid have not been reported previously. Fig. 1a shows the excitation spectrum of dehydroabietic acid in methanol. (These spectra have not been quantum corrected, and therefore no significance should be given to the relative intensities.) Three excitation bands were observed at 224, 269 and 276 nm when the emission at 292 nm was monitored. Fig. 1b shows the emission spectra of dehydroabietic acid in methanol at each of the excitation wavelengths. The middle excitation band, 269 nm, together with the emission at 292 nm were chosen to monitor the HPLC separation. This combination gave reasonable sensitivity without too much effect from interferences such as the Rayleigh scattering peak associated

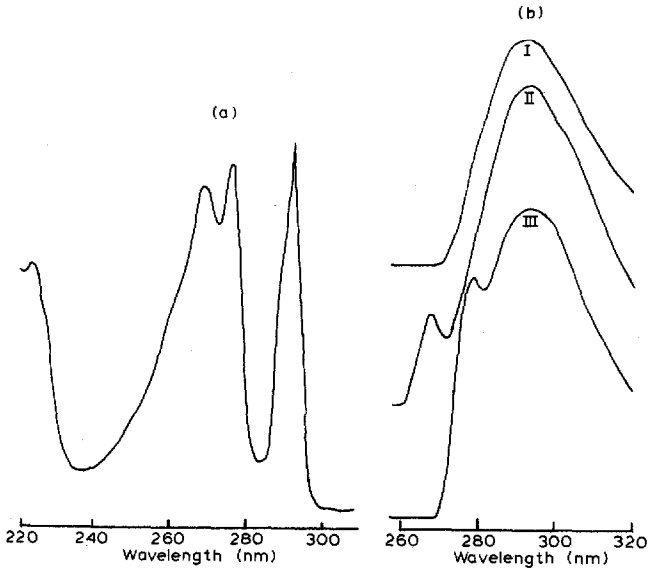

Fig. 1. Fluorescent properties of dehydroabietic acid (10 μg/ml in methanol): (a) excitation spectrum measured by monitoring emission at 292 nm; (b) emission spectra obtained by excitation at 224 nm (I), 269 nm (II) and 276 nm (III).

with excitation and emission wavelengths being close together. However, as pointed out later, excitation at 224 nm results in a better detection limit because of the much lower noise level found when excitation and emission wavelengths are quite separate.

Analysis of effluents samples

Chromatograms using UV detection and fluorescence detection for the same sample are shown in Fig. 2. The concentration of dehydroabietic acid in the original sample was 38 μg/l and the amount injected onto the chromatographic column for both methods was 0.38 μg.

It should be noted that the other main resin acids, none of which have an aromatic ring, do not have any fluorescent activity. When fluorescence detection is used, injection of the next sample can therefore be made as soon as the dehydroabietic acid peak has eluted. However for UV detection the other resin acids such as abietic and pimaric acid, which absorb at 200 nm, elute after dehydroabietic acid, thus preventing injection of the next sample until they have eluted. The greater selectivity of fluorescence detection enables a larger proportion of organic solvent to be used in the eluent and sample throughput is increased by a factor of 3 compared to UV detection.

The quantitative recovery of the technique was demonstrated by analysing six 0.5-l samples of biologically treated effluent containing from 11 to 70 μg/l of dehydroabietic acid. The samples were spiked with 58.5 μg of dehydroabietic acid and an average recovery of 95 ± 7% was obtained.

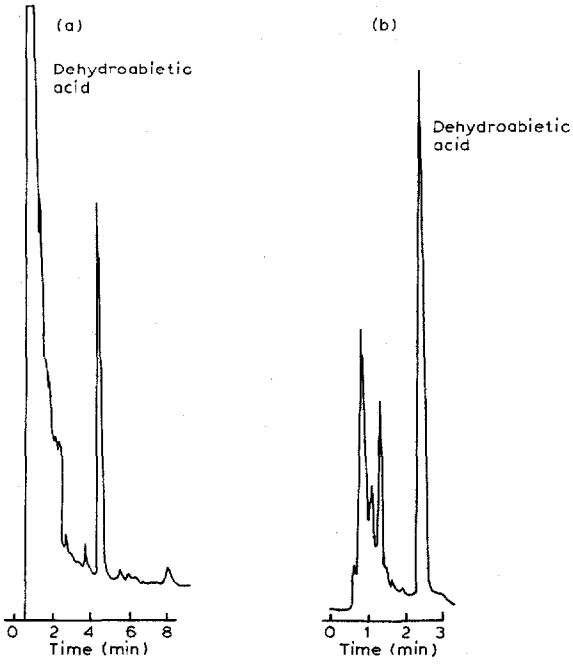

Fig. 2. Chromatograms of effluent extracts showing elution of dehydroabietic acid. (a) UV detection at 200 nm: 0.05 a.u.f.s.; flow-rate 2.0 ml/min; solvent composition, 65% acetonitrile, 34.9% water and 0.1% acetic acid. (b) Fluorescence detection: λ_{Ex} = 269 nm, λ_{Em} = 292 nm; flow-rate 2.0 ml/min; solvent composition, 75% acetonitrile, 24.9% water and 0.1% acetic acid.

The presence of interferences in the form of co-eluting peaks can be checked by either varying the chromatographic parameters within a run (such as temperature, stationary phase, solvent composition and solvent programming), or by using selective detectors. A number of samples were analysed for dehydroabietic acid by HPLC using fluorescence and UV detection and by GC using a flame ionisation detector. Linear regression analyses were then performed on the data, the results of which are summarised in Table I. The most precise results were obtained on comparing the two HPLC detectors, but a 1:1 ratio was not observed. The data indicates that the UV detector gave a larger answer, suggesting the presence of an interfering compound. A column from a different manufacturer (Alltech C_{18} 5-μm reverse phase) showed that in some samples there was a small peak eluting just after the dehydroabietic acid peak which was not observed when using the Varian column. This second compound has not as yet been identified. The use of the fluorescence detector was quite valuable in detecting this interference.

Comparison of UV detection in HPLC with the GC technique revealed further information. A 50-m capillary column coated with 1,4-butanediolsuccinate was used for the GC analyses. The chromatographic conditions employed using GC[4] resulted in an unresolved shoulder eluting on the dehydroabietic acid peak in many of the samples, thus inflating the dehydroabietic acid concentration. This positive interference in both the GC and HPLC (using UV detection) techniques resulted in a purely chance 1:1 correlation. Linear regression analysis of data from GC and HPLC using fluorescence detection further illustrated the presence of an interference in the GC method compared with the fluorescence method by giving a slope greater than 1 as shown in Table I. The high 95% confidence intervals for regression analyses involving GC data were due to the co-elutant peak in the gas chromatogram varying in size significantly compared to the dehydroabietic acid peak. In some samples it was non-existent, while in others it formed almost 50% of the total peak area.

The detection limit of the technique was assessed and Table II summarises the minimum detectable quantity (MDQ) of dehydroabietic acid in a 0.5-l sample using different detection methods and different excitation wavelengths for fluorescence detection. The main contribution to the noise in UV detection is baseline drift as a result of minor impurities in the mobile phase. Fluorescence is more selective and far less sensitive to this type of noise, the main contribution being from the instrument itself. This is particularly evident in the increase of the MDQ on increasing the excitation wavelength with resultant interference from the Rayleigh scattering peak. There are further ways of reducing the MDQ such as by increasing the volume of

TABLE I

LINEAR REGRESSION ANALYSIS OF DEHYDROABIETIC ACID CONCENTRATION DETERMINED BY DIFFERENT TECHNIQUES

Data based on 27 samples containing from 3 to 606 μg/l of dehydroabietic acid.

Y Parameter	*X Parameter*	*Slope ± 95% confidence interval*	*Intercept ± 95% confidence interval*	*Variance due to regression (%)*
Fluorescence	UV	0.872 ± 0.043	0.006 ± 0.007	99
XAD-GC	UV	1.02 ± 0.12	0.014 ± 0.016	94
XAD-GC	Fluorescence	1.19 ± 0.21	−0.003 ± 0.019	93

TABLE II

COMPARISON OF MINIMUM DETECTABLE QUANTITY FOR THE DIFFERENT ANALYTICAL METHODS

Extraction technique	*Instrument analysis*	*Minimum detectable quantity**	
		ng per injection	*μg/l in sample*
XAD-2	GC	0.05	0.1
Solvent	HPLC/UV	7	0.7
Solvent	HPLC/Fluorescence Excitation 224 nm	1	0.1
Solvent	HPLC/Fluorescence Excitation 269 nm	3	0.3
Solvent	HPLC/Fluorescence Excitation 276 nm	5	0.5

* That quantity of material that produces a signal twice the size of the noise.

the injection loop, decreasing the volume to which the final extract is made up and by increasing the sample size.

Some samples of untreated effluent have been analysed by direct injection of 20 μl of a filtered sample onto the HPLC column. A comparison between direct injection and solvent extraction was made and the two methods were found to give similar results (7.91 mg/l by solvent extraction and 7.83 mg/l by direct injection). Analysis of samples by direct injection has not caused any visible deterioration in column life or performance after about 50 injections.

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