TRACE CONSTITUENTS OF NATURAL AND ANTHROPOGENIC ORIGIN FROM NEW ZEALAND *PINUS RADIATA* NEEDLE EPICUTICULAR WAX

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ABSTRACT

Extraction of *Pinus radiata* D.Don needle epicuticular wax and processing of the extract to remove the estolides and fatty and resin acids afforded three minute fractions from silica gel column chromatography, representing less than 0.15% of the wax. The fractions were variously shown to contain a mixture of natural products—viz sesquiterpenes, comprising mainly amorphene, cadinenes, germacrene, calamenene, calacorene, and cadalene; a complex mixture of bisnor, nor, and diterpene hydrocarbons; as well as methyl esters of resin acids and 9,10-seco-dehydroabietanes of unknown ring-A structure. The anthropogenic chemicals identified included polycyclic aromatic hydrocarbons, and a variety of organochlorine compounds, hexachlorocyclohexanes (lindanes), hexachlorobenzene, chlordanes, p,p'-DDE, and pentachlorobiphenyl, hexachlorobiphenyl, and heptachlorobiphenyls. It is suggested that analysis of *P. radiata* needle epicuticular wax would provide one means of biomonitoring in order to assess the quality of the New Zealand environment.

Keywords: Pinaceae; needles; epicuticular wax; monoditerpenes; sesquiditerpenes; diterpenes; alkanes; polycyclic aromatic hydrocarbons; organochlorine anthropogenic compounds; *Pinus radiata*.

INTRODUCTION

The crude epicuticular wax from *Pinus radiata* needles is usually obtained as a hard off-white solid. The colour of the wax varies according to the environment of the tree from which the needles were obtained. For example, wax samples obtained from the needles of trees growing near major roads can be tan to brown in colour. Optical and scanning electron

microscopy of these samples showed they were contaminated with black particulates thought to be from diesel engine exhaust and rubber from tyre wear (J.R.Barnett & Franich unpubl. data).

Pine needle wax has been shown to be an effective absorber of a variety of airborne anthropogenic chemicals (Jensen 1966; Gaggi *et al.* 1985; Thomas *et al.* 1984). Pesticides such as DDT (Eriksson *et al.* 1989; Jensen *et al.* 1992) and polychlorinated dibenzodioxins and dibenzofurans (Safe *et al.* 1992) were detected in pine needle samples from Europe, Scandinavia, and North America. The brush-like anatomy of the foliage of pines (Mirov 1967) and the stable (principally estolide, polyester) nature of the epicuticular wax (Franich *et al.* 1978) confer on pine needles the ability to trap, absorb, and retain lipophilic chemicals transported through the air as gases, aerosols, and particulates, and it is this ability which has been exploited as a means of monitoring atmospheric pollution.

The question of the value of *P. radiata* needles as a potential biomonitor for the New Zealand environment was therefore raised and addressed. An earlier investigation of pollutants absorbed on to pine needles in New Zealand mapped levels of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, DDT, and lindane (Herrman & Baumgartner 1987), although the pollutants were measured by gas chromatography using electron capture detection only, and many compounds were not unequivocally identified.

This report describes the identification, using gas chromatography mass spectrometry (GCMS), of trace-level chemicals of both natural and anthropogenic origin in a composite sample of *P. radiata* needle epicuticular wax from nine sampling points throughout New Zealand, and discusses the value of *P. radiata*, widely distributed throughout New Zealand, as a biomonitor for the environment.

EXPERIMENTAL
Pine Needles

One-year-old needles were sampled from *P. radiata* trees growing in nine sampling regions in the North and South Islands of New Zealand. The sampling areas ranged from coastal with little human habitation through to large plantation forest and farmland to trees growing close to cities with industrial activity.

Needle Extraction

Twenty-five grams of fresh needles from each of the sampling areas were placed in a large test tube and covered with dichloromethane (50 ml) containing 2,3,3,4,4,5,5-heptachlorobiphenyl (20 ng) as internal standard. The test tube was stoppered and shaken with the needles for 3 min, and then left standing for 24 h. After the solution was decanted, another portion of dichloromethane was added to the needles and the test tube shaken for 3 min. The solvent was decanted and the combined solutions were filtered through a dichloromethane-washed filter paper (Whatman No.1) and concentrated to give a hard, off-white, waxy solid, c. 100 mg.

Extract Clean-up Procedure

The extract was processed according to the flow chart (Fig. 1). The insolubility of the alkyl esters and estolides in hydrocarbon solvents could be used to efficiently separate the
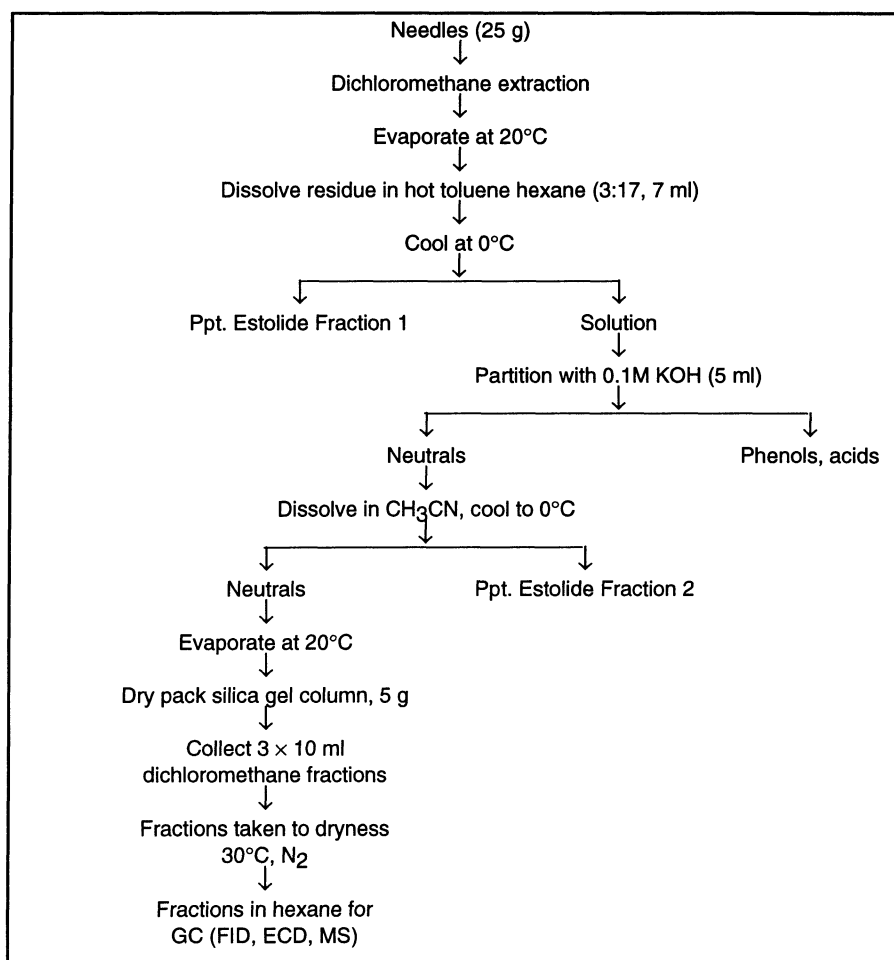
Needles (25 g)
↓
Dichloromethane extraction
↓
Evaporate at 20°C
↓
Dissolve residue in hot toluene hexane (3:17, 7 ml)
↓
Cool at 0°C

Ppt. Estolide Fraction 1   Solution
↓
Partition with 0.1M KOH (5 ml)

Neutrals   Phenols, acids
↓
Dissolve in CH_3CN, cool to 0°C

Neutrals   Ppt. Estolide Fraction 2
↓
Evaporate at 20°C
↓
Dry pack silica gel column, 5 g
↓
Collect 3 × 10 ml
dichloromethane fractions
↓
Fractions taken to dryness
30°C, N_2
↓
Fractions in hexane for
GC (FID, ECD, MS)

FIG. 1–Flow chart for extracting and processing the sample of *Pinus radiata* needles.

bulk of these substances from the rest of the sample. Silica gel used for chromatography was extracted with dichloromethane and then heated overnight at 280°C. The final fractions from the column were transferred to tapered vials and made up to 50 μl with hexane for gas chromatography using electron capture detector. For gas chromatography mass spectrometry analysis, the wax samples from nine sampling areas were pooled.

Analysis

The final purified extracts were analysed by gas chromatography using an HP-5 fused silica capillary column (25 m × 0.2 mm, 0.25 μm phase film thickness) and helium as the carrier gas. The column was programmed from 50°C to 300°C at 5°C/min. Flame ionisation and electron capture detectors were used, as well as a quadrupole filter mass spectrometer operating under electron ionisation mode with 70 eV 300 μA electron energy. To determine

the presence of organochlorine compounds in Fraction 1, the sample was analysed using gas chromatography negative ion chemical ionisation mass spectrometry, obtaining full-scan mass spectra. The presence of organochlorine compounds in each of the fractions was confirmed by monitoring ions m/z 35 and 37 only. A gas chromatogram peak was assigned to contain chlorine if the ratio of the ion current for the m/z 35 and 37 peaks was approximately in the range $3.0 \pm 20\%$: 1.

RESULTS

After a series of selective precipitations to remove the bulk of the estolides and alkyl esters, followed by liquid-liquid partitions to isolate the neutral compounds, the remaining chemicals were divided into three fractions on a silica gel column. The amount of substance from all three fractions from the silica gel column (which was estimated from the gas chromatography flame ionisation detector response for each fraction) was less than 150 μg, or less than 0.15% of the composite sample of needle epicuticular wax. The residue therefore comprised 0.017% (170 ppm) of the oven-dry needle weight.

The compounds identified in the three purified fractions are summarised in Table 1. For the sesquiterpenes, in particular, not all the mass spectra acquired could be easily assigned to a particular structure, and so the number of unidentified sesquiterpenes, recognised as such

TABLE 1–Composition of the neutral trace constituents from *Pinus radiata* needle epicuticular wax.

Class	R_t (min)	Identity	No. of each class	Silica gel column fraction
Monoterpene	13.4	Borneol		3
	14.1	α-terpineol		
	16.7	Thymol		
	12.2–14.6	Aldehyde/ketone (M+·152)	5	
Sesquiterpene hydrocarbon	16.5	α-amorphene		1*
	16.9	α-cadinene		
	17.5	δ-cadinene		
	18.8	Germacrene		
	19.7	Cadina-1,4-diene		
	15.8–20.0	Others (M+·204)	12	
	19.4	Calamenene		
	19.8–20.4	Others (M+·200)	2	
	20.2	Calacorene		
	21.7	Unsaturated cadalene (M+·196)		
Sesquiterpene alcohol/ketone	21.6–30.0	Unknowns (M+·220 and 222)	6	3
Diterpene hydrocarbon	24.2–25.3	Bisnor mono-unsaturated	5	1*
	25.1–25.5	Bisnor-saturated (M+·248)	3	
	26.3–28.2	Nor mono-unsaturated (M+·260)	5	
	28.3	Abietene		
	28.5–28.8	Mono-unsaturated (M+·274)	4	
	28.4	Abietadiene		
	21.7–31.3	Di-unsaturated (M+·272)	6	
	29.7	6-18-nordehydroabietane		
	30.5	18-nordehydroabietane		

TABLE 1–continued

Class	R_t (min)	Identity	No. of each class	Silica gel column fraction
	31.0	Δ-6-dehydroabietane		
	31.4	Dehydroabietane		
Diterpene ketone	35.5	7-keto dehydroabietane		3
Diterpene acid methyl ester	33.7	Methyl pimarate	2	
	34.1	Methyl isopimarate		
	35.6	Methyl abietate		
	36.1	Methyl dehydroabietate		
	36.3	Methyl Δ-6-dehydroabietate		
Stilbene derivatives	26.0	Benzoin		3
	25.8–30.3	9,10-seco-dehydroabietanes	14	2*
Alkane	32.1	Heneicosane		1
	33.7	Docosene		
	33.8	Docosane		
	35.8	Tricosane		
	37.2	Tetracosane		
	38.8	Pentacosane		
PAH	13.2	Methyl naphthalene		1
	23.2	Dimethyl isopropyl-naphthalene (cadalene)		
	25.5	Phenanthrene		
	28.7	Methyl tetrahydro-phenanthrene		
	29.0	Methyl phenanthrene		
	30.9	Dimethylphenanthrene		
	31.7	Benzfluorene		
	32.6	Pyrene		
	33.0	Methylethylphenanthrene		
	34.7	Retene		
	38.0	Dibenzacenaphthene		
Organochlorine	24.2	α-hexachlorocyclohexane		1
	24.9	Hexachlorobenzene		
	25.0	Pentachloroanisole		
	27.4	β-hexachlorocyclohexane		
	27.9	Δ-hexachlorocyclohexane		
	28.2	δ-hexachlorocyclohexane		
	29.1	Tetrachloronaphthalene		
	33.2	cis-chlordane		
	33.4	trans-chlordane		
	33.6	δ-chlordane		
	34.1	trans-nonachlor		
	34.6	p,p'-DDE		
	34.8	Dieldrin		
	35.6	Pentachlorobiphenyl		
	36.1–40.0	Hexachlorobiphenyl	3	
	37.5	p,p'-DDT		
	40.8	Heptachlorobiphenyl		
	43.1	Heptachlorobiphenyl (internal standard)		

* Silica gel column fraction in which this class predominates

by their characteristic mass spectra (Ramaswami *et al*. 1986) is reported. The majority of the non-polar compounds such as the sesquiterpenes, the polycyclic aromatic hydrocarbons, the diterpene hydrocarbons, and the organochlorine compounds of interest were eluted in the first 10-ml dichloromethane fraction from the silica gel column. The second and third fractions also contained lesser amounts of numerous other organochlorine compounds according to their m/z 35 and 37 ion chromatograms. No attempt was made to identify these organochlorine compounds other than to recognise that they were more polar than the polychlorinated biphenyls and chlordanes.

The second fraction from the silica gel column contained lesser amounts of sesquiterpene hydrocarbons, but contained the majority of the diterpene acid methyl esters together with a series of 9,10-secoditerpenes. These latter compounds gave characteristic mass spectra having m/z 104, 118, 132, and 146 base peaks for styrene, methyl, ethyl, and isopropyl styrene fragments arising from electron ionisation-induced rearrangement of the octahydrostilbene structure (cf. Zinkel *et al*. 1971).

The third fraction contained monoterpene, sesquiterpene, and diterpene alcohols and ketones or aldehydes. Only two of the monoterpene alcohols and one of the diterpene ketones could be identified. The others showed characteristic mass spectra, but could not be assigned unequivocal structures. The stilbene derivative benzoin was identified in this fraction.

Using the heptachlorobiphenyl internal standard, the organochlorine compound content of the composite sample of needle wax was: (i) for combined hexachlorocyclohexanes and hexachlorobenzene, 0.3 ng/g; (ii) for all chlordane compounds, 1.9 ng/g; (iii) for DDT and DDE, 0.1 ng/g; and (iv) for all polychlorobiphenyls, 1.7 ng/g. These values are comparable with those found for samples of needle wax from *P. sylvestris* L. growing in Scandinavia and Europe (Jensen *et al*. 1992).

DISCUSSION

Among the mixture of compounds identified at the trace level in *P. radiata* needle epicuticular wax were the methyl esters of the resin acids. Resin acid methyl esters have been identified in the gum rosin neutral fraction from *P. sylvestris* (and other pine species) (Lange & Weissman 1987), but have not been identified in extracts of needles. One possibility is that needle surface micro-organisms may be able to methylate the resin acids (Orpiszewski *et al*. 1990). Methyl abietate was the dominant resin acid ester.

One source of the diterpene hydrocarbons could be the resin acids in the needle wax (Franich *et al*. 1978), which have undergone decarboxylation and disproportionation reactions, resulting in the formation of mono-unsaturated diterpenes and aromatic diterpenes. Recognised in the mixture was a series of mono-unsaturated diterpene hydrocarbons with distinct molecular ions of m/z 274 and characteristic fragments in their mass spectra. Another series of compounds with molecular ions m/z 272, characteristic for di-unsaturated diterpene hydrocarbons was observed, and m/z 270 for the aromatic ring-C hydrocarbon dehydroabietane. An unsaturated dehydroabietane having molecular ion m/z 268, possibly with the double bond at carbon 6, was also identified.

A series of 18-norditerpene hydrocarbons was also easily recognised, i.e., nordehydroabietane with molecular ion m/z 256, and two unsaturated nordehydroabietanes with molecular ion m/z 254.

Of the sesquiterpene hydrocarbons, which were the most abundant of the trace natural compounds recovered from the extracts, only the cadinenes, amorphene, germacrene, calamenene, calacorene, and cadalene were unequivocally identified. At least 12 other GCMS peaks showed distinct mass spectra for sesquiterpenes, with molecular ion m/z 204, and characteristic fragments (Zinkel *et al.* 1971), but could not be identified.

The other major compounds in the mixture were the alkanes and polycyclic aromatic hydrocarbons. The alkanes comprised only C_{21} to C_{25} chain lengths which suggests a specific source, either the needle itself or needle surface micro-organisms. Previously, long-chain constituents identified in *P. radiata* needle wax had chain lengths of 25 to 31 carbons (Franich *et al.* 1979). The absence of longer-chain hydrocarbons in the fraction may be due to their removal during the CH_3CN precipitation step (Fig. 1).

Among the polycyclic aromatic hydrocarbons were two dimethyl isopropyl naphthalenes which could either be derived from the sesquiterpenes by processes such as allylic hydroxylation and dehydration, or could be deposited from the air, as alkylnaphthalenes are known to be produced during combustion of firewood and brown coal (Hubble *et al.* 1982). Similarly, the phenanthrenes may be derived by diagenic-like and microbial metabolic processes from the diterpenes in the needle epicuticular wax, but could also be derived from coal and released into the air during combustion (Hayatsu *et al.* 1978). However, in this composite sample, there appeared to be few of the common four-ring and none of the five-ring polycyclic aromatic hydrocarbons expected from wood and coal combustion emissions. Either these typical compounds were absent from the sample, or possibly they were selectively removed from the sample during the wax processing procedure, probably at the estolide precipitation stage (Fig. 1).

Many of these phenanthrenes derived from resin acids have been identified in the earth's troposphere (Simoneit & Mazurek 1982) and in forest soils (LaFlamme & Hites 1978) and therefore it could be expected that they would be found absorbed into pine needle wax.

The organochlorine compounds could only be readily detected and identified using negative ion chemical ionisation mass spectrometry, as they were present in concentration orders of magnitude less than the terpenes and polycyclic aromatic hydrocarbons. The chlordane compounds identified in this sample of pine needle wax probably have as their source the wood preservation and processing industry in New Zealand, where chlordane has been used for many years as an insecticide in the glueline of plywood (a manufacturing process which involves heating, and possibly volatilisation of the insecticide into the factory exhaust system, and therefore into the atmosphere). Pentachloroanisole is probably derived from microbiological alkylation of pentachlorophenol (Cserjesi & Johnson 1972), another chemical which has been widely used in the New Zealand wood preservation industry. The PCBs are common pollutants worldwide.

The mass spectra of eight other compounds showed that they contained chlorine, but structures could not be assigned to them.

The presence of the norditerpene and diterpene hydrocarbons and the phenanthrenes in the needle epicuticular wax raises the question whether the living pine needles with their phylloplane microbiological consortia and exposure to air, sunlight, and water are indeed a source of these compounds, or whether they are a sink for those compounds derived from forest soils and fossil fuel combustion and deposited on to the needles through eolian redistribution.

The detection of polycyclic aromatic hydrocarbons and organochlorine compounds in these New Zealand samples of *P. radiata* needle wax suggests that this pine species, which is widely distributed throughout the country, would be valuable for biomonitoring to enable a quantitative assessment of the quality of the New Zealand environment to be made. The potential value of the biomonitoring system would be to enable detection of changes in pollutant content of pine needles as an indicator of local or regional pollutant release, similar to DDT levels monitored using *P. sylvestris* in Scandinavia and Europe after release of the insecticide in forestry operations in former East Germany (Jensen *et al.* 1992).

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