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Composition of the Cuticular Waxes of *Picea abies* and *P. sitchensis*

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The chemical compositions of the cuticular lipids of the needles of Norway spruce (*Picea abies* (L.) Karst.) and Sitka spruce (*P. sitchensis*) (Bong.) Carr.) were investigated by gas chromatography coupled with mass spectrometry. Fractionation of the wax extracts was carried out using column chromatography. Long chain secondary alcohols, diols and free fatty acids were found as major classes in both species: *n*-alkanes, *n*-alkenes, primary alcohols, α,ω–diols, ketones and ω–hydroxyacids constituted the minor wax classes. The presence of large quantities of estolides (high molecular weight biopolymers) was tentatively confirmed by saponification of the crude extracts which led to the recovery of large quantities of ω–hydroxyacids, α,ω–diols and free fatty acids. Qualitative differences between the two examined species were observed for minor constituents such as long chain aldehydes, methyl- and ethyl-esters and some terpenes. Quantitatively the two wax extracts differed mainly in their proportions of diols and fatty acids.

Keywords: Picea abies; *Picea sitchensis*; cuticular waxes; composition; gas chromatography; mass spectrometry.

INTRODUCTION

Since the early eighties a new type of forest decline affecting essentially forests in non-polluted areas has been observed (Rehfuess, 1981). In order to investigate the possible causes, the alterations of plant tissues after controlled exposure to different stress situations, as well as the plant–environment relationship were studied. For this purpose, a lot of research dealing with the interactions between pollutants, pathogens or insects and the leaf–atmosphere interface have been carried out (Bucovac *et al.*, 1981; Jeffree, 1986; Sauter and Voss, 1986; Tuomisto, 1988). The first barrier between air and plant tissues corresponds to the surface lipids which can be differentiated into the epi- and the intra-cuticular waxes. The former represent the outmost layer often forming crystalline-like structures as observed by scanning electron microscopic (SEM) analysis (Jeffree, 1986). The latter are embedded in the cutin matrix of the cuticle (Riederer, 1989).

Cuticular wax composition changes with the species considered, and it is further modified during leaf development, ageing and by the impact of light, temperature and pollutants (Schütt and Schuck 1973; Franich *et al.*, 1978; Dunstone *et al.*, 1985; Tevini and Steinmüller, 1987; Turunen and Huttunen, 1990; Percy *et al.*, 1993). The cuticular wax of conifers has been studied mainly for the economically most important species such as *Picea abies, Pinus* spp. and *Pseudotsuga menziesii* (Riederer, 1989). However, except for the recent investigations of Percy and Baker (1990), little is known about *Picea sitchensis* which represents one of the most important maritime conifers. In the present study, we report on the identification of wax components of Sitka spruce (*Picea sitchensis*) (Bong.) Carr.) and we re-investigate that of Norway spruce (*Picea abies* (L.) Karst.) in order to compare the two species.

Most of the published studies dealing with the analysis of cuticular wax have involved either analysis of the most important constituents or a selected substance class such as the hydrocarbons (Simini and Leone, 1986; Salasoo, 1987), or detailed studies which require several fractionation procedures. Most of these methods used combinations of liquid–liquid partition, selective crystallisation or thin layer chromatography (TLC) prior to gas chromatography (GC) and mass spectrometric (MS) analysis (Gülz *et al.*, 1987; Günthardt-Goerg, 1991). For the purpose of our research programme, which involved the analysis of a great number of samples, these methods were considered too time-consuming and also required considerable amounts of biological material. The procedure described in this paper involves the use of inexpensive, small chromatographic silica gel columns and adapted eluotropic series, and was developed in order to provide a simple separation of major and minor wax components. As such, it is suitable for measurements in series and for the rapid wax analysis of different coniferous species (Prügel *et al.*, 1994). This work is integrated into a larger research programme directed towards gaining a better knowledge of the modifications of cuticular lipids in response to some pollution treatments (Prügel and Lognay, 1994).

EXPERIMENTAL

Sampling procedure. Current year needles of Norway spruce (collected in the Ardennes, Belgium, the Eifel, Germany, and the Vosges, France) and Sitka spruce (collected in

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Penicuik, Scotland) were sampled from several trees in order to reduce the genetic influences. All samples were taken in late autumn because the waxes are then fully synthesized and no further changes are observed from September to March (Riederer, 1989). After deep-freezing in liquid nitrogen, intact needles were detached from the twigs and were pooled to make 1–1.5 g samples.

Extraction of the cuticular waxes. As recommended in previous works (Franich *et al.*, 1978; Riederer and Schneider, 1989), the needles were extracted for 30 s with 3 × 10 mL pure chloroform. The crude extracts were bulked and filtered through 0.45 μm cellulose nitrate membrane filters (Sartorius, Gottingen, Germany) in order to eliminate dust and other small particles. They were then concentrated under reduced pressure and weighed. This procedure was tested on *P. abies* and showed accurate repeatability (variation coefficient 3%; $n = 10$). Moreover, SEM visualisation of extracted needles showed no further crystalline structures on the surface. The efficiency of the method was therefore judged satisfactory. The saponification of total wax extracts was carried out according to the method of Günthardt-Goerg (1986). The wax samples (or fractions) were stored in stoppered borosilicate vials at −24 °C until required for use.

Fractionation of crude cuticular waxes. Crude wax extracts were fractionated on small chromatographic columns (40 × 5 mm i.d.) filled with heat-activated silica gel (Kieselgel 60 of 230–440 mesh; Merck, Darmstadt, Germany). Aliquots (10–15 mg) of extract were applied to the top of the columns which were then eluted with the following eluotropic series: 10 mL *n*-hexane, 20 mL *n*-hexane:chloroform (1:1 v/v), 20 mL chloroform, 15 mL chloroform:methanol (1:1 v/v) and 10 mL methanol. As observed by TLC (results not shown) this sequence of elution led to the separation of the main chemical classes of cuticular lipids. All fractions were collected separately and concentrated under a gentle stream of nitrogen. With the exception of the hydrocarbons (eluted with *n*-hexane), the recovered substances were silylated for 30 min at 90 °C with 100 μL of *N,N*-bis-trimethylsilylfluoroacetamide:trimethylchlorsilane (99:1 v/v) and 100 μL anhydrous pyridine.

GC and GC–MS analyses. The identifications were performed on a Hewlett-Packard model HP5971 GC–MS (source temperature −170 °C; interface temperature −300 °C; electron impact voltage −70 eV) whereas the GC profiles were established on a Mega 5160 apparatus equipped with a cold on-column injector and a flame ionisation detector (FID) maintained at 300 °C. Other analytical conditions were as follows: columns GC–MS, CP-Sil-5CB (25 m × 0.25 mm i.d.); 0.25 μm film thickness) from Chrompack (Middelburg, The Netherlands); GC–FID, OV-1 (25 m × 0.32 mm i.d.; 0.2 μm film thickness) from Macherey-Nagel (Düren, Germany): carrier gas—helium at 1 mL/min: temperature programme—from 50 to 180 °C at 30 °C/min, then from 180 to 270 °C at 4 °C/min, 270 °C held for 10 min and then from 270 to 300 °C at 6 °C/min.

Quantitation was achieved from the full GC–FID profile recorded for the samples. Pure cholestane was used as internal standard, and the response of the FID-detector was shown to be linear over the range of interest for several selected (but not fully purified) compounds, the amount of each detected compound being determined by reference to cholestane. The results of the total wax weight are reported together with the percentage of each substance class which was established by normalization.

RESULTS AND DISCUSSION

The cuticular waxes of *P. abies* and *P. sitchensis* represent respectively 10 to 15 mg/g and 10 to 12 mg/g dry needles. Similar amounts were reported by Lütz *et al.* (1990) for *P. abies* and by Percy and Baker (1990) for *P. sitchensis*. The separation of wax constituents by column chromatography with the aforementioned solvents led to five major fractions of increasing polarity.

Table 1. Relative abundance of different substance classes in the chromatographically resolvable cuticular lipids of *Picea abies* and *P. sitchensis*

Eluent[a]	Substance class	*Picea abies* Area (%)[b]	*Picea abies* Weight (%)[c]	*Picea sitchensis* Area (%)[b]	*Picea sitchensis* Weight (%)[c]
1. Hexane	Alkanes	<2	<0.5	<2	<0.7
	Alkenes	<0.2		<0.2	
2. Hexane:chloroform	Primary alcohols	1	0.2	1	0.3
	Secondary alcohols	47	10	47	14
(1:1 v/v)	Fatty acid methyl esters	2	<0.5	ND	
	Fatty acid ethyl esters	<1	<0.2	ND	
	Long chain aldehydes	ND		<1	<0.3
	Ketones	<0.5	<0.1	<0.1	<0.03
3. Chloroform	Diols	13	3	25	7.5
	n-Fatty acids	20	4	4	1,2
4. Methanol:chloroform	*n*-Fatty acids				
(1:1 v/v)	ω-Hydroxy fatty acids	1	0.2	1	0.3
5. Methanol	ω-Hydroxy fatty acids				
Total		88	19	81	24

[a] For details of the column chromatographic system used see Experimental section.
[b] Area % derived from GC–FID profile.
[c] Weight % of the total wax amount.
ND, None detected.

Table 2. Identified constituents in the soluble cuticular lipids of *Picea abies* and *P. sitchensis*: A hydrocarbons and alcohols

Substance class	MW[a]	Characteristic fragments[b]	*Picea abies*	*Picea sitchensis*
n-alkanes	212–492	57, 71, 85, 99, . . . , $[M]^+$	C16–C35, C16 and C34 traces	C15–C35
n-alkenes	238–448	55, 69, 83, 97, . . . , $[M]^+$	C18–C32	C17–C31
Primary alcohols	186–438 (258–510)	75, 103, 147, $[M-15]^+$	C12–C28, even	C12–C30, even
Secondary alcohols		73, 103, 147, $[M-15]^+$		
3-Docosanol	326 (398)	131, 369	+	ND
10-Pentacosanol	368 (440)	313, 229	+	+
6-Pentacosanol	368 (440)	173, 369	+	ND
3-Hexacosanol	382 (454)	131, 425	+	ND
10-Heptacosanol	396 (468)	341, 229	+	+
8-Heptacosanol	396 (468)	201, 369	+	ND
4-Heptacosanol	396 (468)	145, 425	+	+
10-Octacosanol	410 (482)	355, 229	+	ND
10-Nonacosanol	424 (496)	369, 229	+	+
6-Nonacosanol	424 (496)	173, 425	+	+
4-Nonacosanol	424 (496)	145, 453	+	+
11-Triacontanol	438 (510)	369, 243	+	ND
10-Triacontanol	438 (510)	383, 229	+	ND
12-Hentriacontanol	452 (524)	369, 267	+	ND
10-Hentriacontanol	452 (524)	397, 229	+	+
10-Tritriacontanol	480 (552)	425, 229	+	+
Diols		73, 103, 147, $[M-15]^+$		
5,10-Heptacosanediol	412 (556)	159, 341, 317, 499	+	+
4,10-Heptacosanediol	412 (556)	145, 341, 317, 513	ND	+
5,10-Octacosanediol	426 (570)	159, 355, 317, 513	ND	+
10,13-Nonacosanediol	440 (584)	229, 327, 359, 457	+	+
7,10-Nonacosanediol	440 (584)	187, 369, 317, 499	+	+
10,16-Nonacosanediol	440 (584)	229, 285, 401, 457	+	+
6,10-Nonacosanediol	440 (584)	173, 369, 317, 513	+	+
5,10-Nonacosanediol	440 (584)	159, 317, 369, 527	+	+
4,10-Nonacosanediol	440 (584)	145, 317, 369, 541	+	+
3,10-Nonacosanediol	440 (584)	131, 369, 317, 555	+	ND
Triterpenic alcohols				
24-Methylenecycloartanol	440 (512)	73, 379, 407, 422, 497, 512	+	ND

[a] Molecular weight of TMS derivative in parentheses.
[b] Fragmentation pattern of TMS derivatives; base peak underlined.
+, Present; ND, not detected.

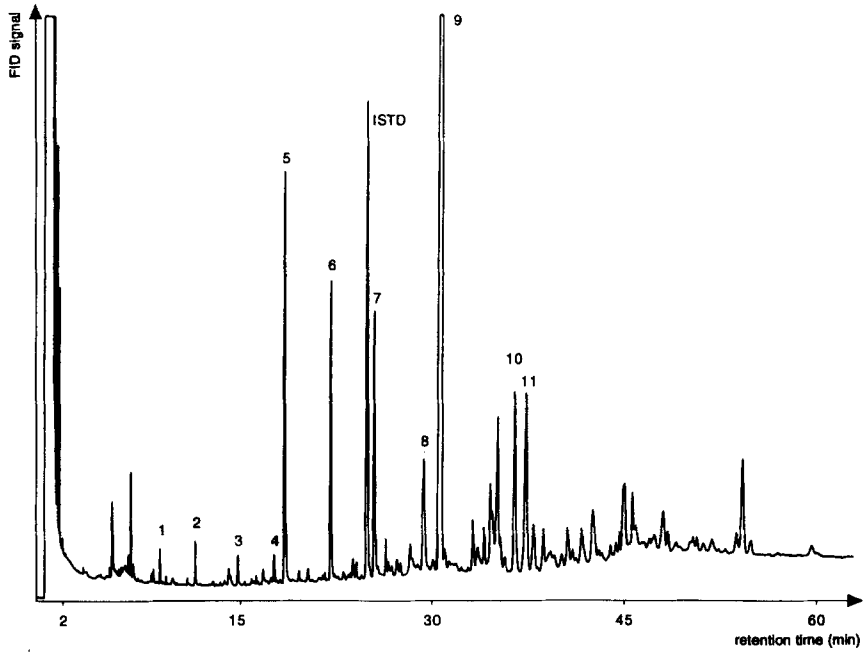

Figure 1. Typical GC–FID profile of *Picea abies* total wax extract. Key to major peaks: **1** tetradecanoic acid; **2** hexadecanoic acid; **3** octadecanoic acid; **4** 16-hydroxyhexadecanoic acid; **5** eicosanoic acid; **6** docosanoic acid; **7** tetracosanoic acid; **8** hexacosanoic acid; **9** 10-nonacosanol; **10** 5,10-nonacosanediol; **11** 4,10-nonacosanediol; ISTD internal standard (cholestane). (For chromatographic protocol see Experimental section).

Table 2 continued. Esters, acids and carbonyl compounds

Substance class	MW[a]	Characteristic fragments[b]	*Picea abies*[c]	*Picea sitchensis*[c]
Methyl esters		74, 87, 143, $[M-43]^+$, $[M]^+$		
Methyl tetracosanoate	382	382	+	ND
Methyl hexacosanoate	410	410	+	ND
Methyl octacosanoate	438	438	+	ND
Methyl triacontanoate	466	466	+	ND
Methyl dotriacontanoate	494	494	+	ND
Methyl tetratriacontanoate	522	522	+	ND
Ethyl esters	284–396	88, 101, 143, $[M-43]^+$, $[M]^+$		
Ethyl hexadecanoate	284	284	+	ND
Ethyl octadecanoate	312	312	+	ND
Ethyl docosanoate	368	368	+	ND
Ethyl tetracosanoate	396	396	+	ND
Aldehydes		52, 82, 96, 110, $[M-18]^+$		
Docosanal	324	306	ND	+
Triacosanal	338	320	ND	+
Tetracosanal	352	334	ND	+
Pentacosanal	366	348	ND	+
Hexacosanal	380	362	ND	+
Heptacosanal	394	376	ND	+
Octacosanal	408	390	ND	+
Nonacosanal	422	404	ND	+
Triacontanal	436	418	ND	+
Hentriacontanal	450	432	ND	+
Dotriacontanal	464	446	ND	+
Ketones				
10-Nonacosanone	422	71, 155, 171, 295	+	+
Saturated fatty acids	200–452 (272–524)	73, 117, 129, 132, 145, $[M-15]^+$, $[M]^+$	C12–C30; all excepted C27 and C29	C12–C26 even, C23
Unsaturated fatty acids	254 (326)	73, 117, 129, 132, 145, 311, 326	C16	C16
ω-Hydroxy fatty acids		73, 75,		
12-Hydroxydodecanoic acid	216 (360)	255, 329, 345, 360	+	+
14-Hydroxytetradecanoic acid	244 (388)	283, 357, 373, 388	+	+
16-Hydroxyhexadecanoic acid	272 (416)	311, 385, 401, 416	+	+

[a]Molecular weight of TMS derivative in parentheses.
[b]Fragmentation pattern of TMS derivatives; base peak underlined.
+, Present; ND, not detected.

The different components were identified by GC–MS and determined (in terms of area percent of the chromatographically detectable components) by GC–FID. The results are presented in Tables 1 and 2; typical chromatograms of total cuticular wax extracts of the two species are shown in Figs. 1 and 2.

Hexane fraction

The hexane fraction contained essentially straight-chain hydrocarbons (*n*-alkanes and *n*-alkenes) which represented only about 2% of the two spruce waxes. These molecules were identified by comparison of their fragmentation patterns with those of commercial reference substances and with the Wiley Mass Spectral Library (MacLafferty and Stauffer, 1989). The odd numbered homologues dominated the *n*-alkane profile whereas for the *n*-alkenes the even numbered molecules were predominant (the unsaturated molecules were eluted before their saturated counterparts). For *P. abies*, hydrocarbons ranging, respectively, from 17 to 35 (*n*-alkanes) and from 17 to 31 carbon atoms (*n*-alkenes) were found; traces of the saturated C16 homologue were also detected. For *P. sitchensis*, C15 to C35 *n*-alkanes, and C17 to C31 *n*-alkenes were identified. The present results are in line with those of several authors (Corrigan *et al.*, 1978; Euteneuer *et al.*, 1988; Berteigne *et al.*, 1988; Kerfourn and Garrec, 1992) but to the author's knowledge the occurrence of *n*-alkenes in waxes of *Picea* spp. has not yet been reported.

Hexane–chloroform fraction

Primary and secondary alcohols (constituting 48% of the total chromatographically resolvable waxes) were eluted within this fraction. For the two examined species, the chromatograms were characterized by the major peak of 10-nonacosanol. Minor quantities of 10-pentacosanol, 10- and 4-heptacosanol, 6- and 4-nonacosanol, 10-hentriacontanol and 10-tritriacontanol were also detected. Some additional secondary alcohols, detailed in Table 2a, were found in the wax of *P. abies*. The mass spectra of the silylated alcohols consisted essentially of the trimethylsilyl (TMS) fragment ion ($m/z = 73$) and the two fragments resulting from the cleavage on each side of the hydroxyl group (Fig. 3a). 10-Nonacosanol, 10-heptacosanol and 10-hentriacontanol have already been reported for the two spruce waxes (Schulten *et al.*, 1986; Günthardt-Goerg, 1986) but all other secondary alcohols were identified for the first time in the present study.

The primary alcohols, previously reported in Norway

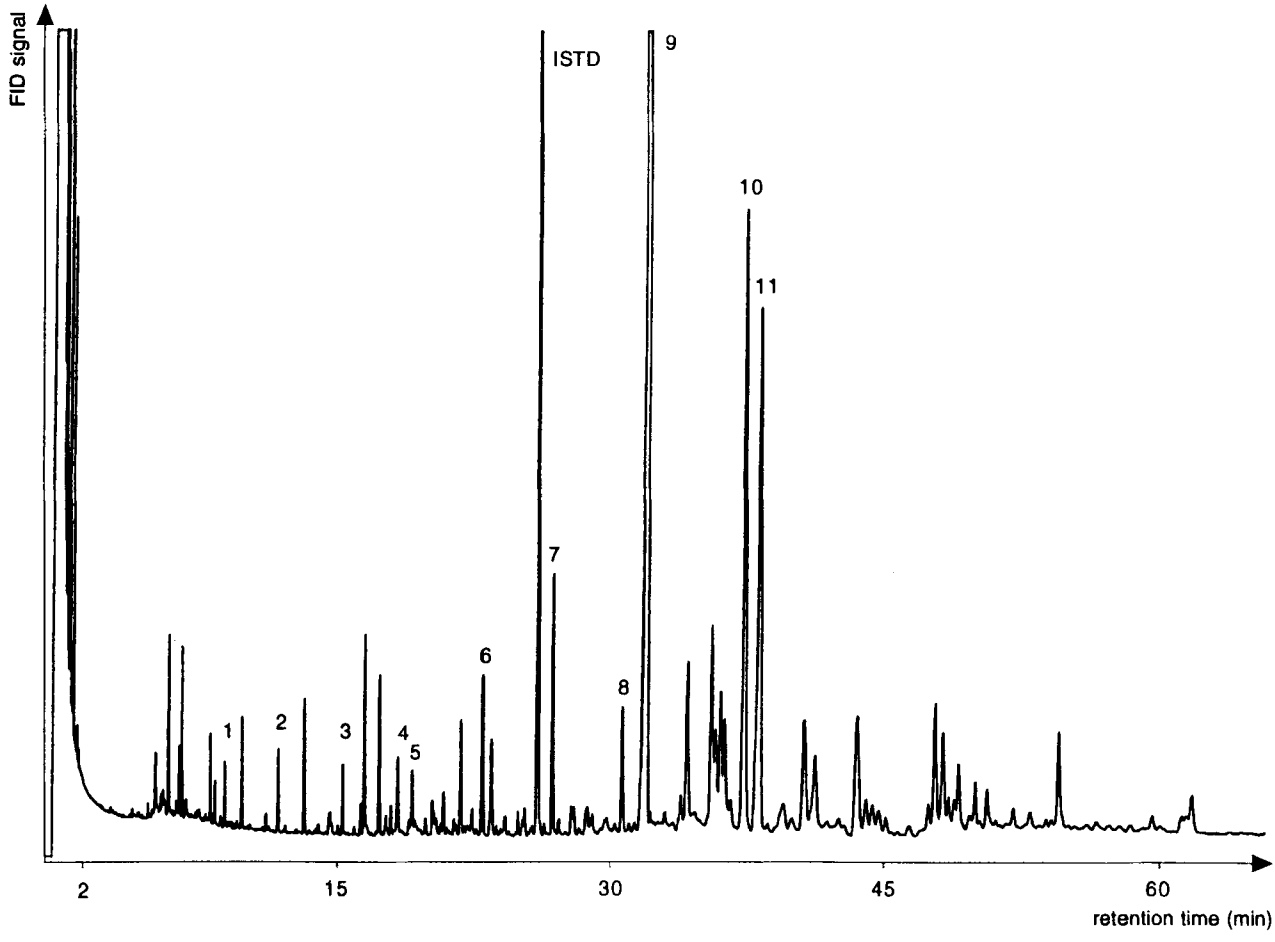

Figure 2. Typical GC–FID profile of *Picea sitchensis* total wax extract. Key to major peaks as in legend to Fig. 1. (For chromatographic protocol see Experimental section.)

spruce (Günthardt-Goerg, 1986) but not in Sitka spruce, are minor wax components (1% in both *Picea* species). Only the even numbered homologues (C12 to C28) were found here; however, traces of primary triacosanol were detected in some samples. The mass spectra are characterized by intense ions at M-15 [M-CH_3] and at $m/z = 75$ (dimethylsilanol), a typical fragment of primary alcohols (Holloway *et al.*, 1976).

The hexane–chloroform fraction also contained methyl- and ethyl esters, *n*-alkanals and ketones (Table 2b). Ethyl esters of straight chain fatty acids (C16 to C24) were observed as a minor fraction (<1%) exclusively in *P. abies*. They are characterized by a base peak at $m/z = 88$ (McLafferty rearrangement) and by fragments resulting from the aliphatic chain breakdown. The mass spectra of the methyl esters of fatty acids from C24 to C34 (total level of 2% in Norway spruce) showed similar fragmentation patterns with a base peak at $m/z = 74$ also indicating a typical McLafferty rearrangement. The long chain esters (C36 to C50) reported in *P. glauca* (Tulloch, 1987) and *P. rubens* (Percy *et al.*, 1992) were not detected under our analytical conditions. A series of even numbered long chain aldehydes (C22 to C32) were identified for the first time in *P. sitchensis* waxes (<1%) (Table 2b and Fig. 3b). 10-Nonacosanone was the only aliphatic ketone seen in the chromatographic profile of the species analysed (<0.5% in *P. abies*; <0.1% in *P. sitchensis*): the molecular ion (M^+) and fragments resulting from two cleavages relative to the oxo-group were observed. *p*-Hydroxyacetophenone ($M^+ = 208$ plus other ions at $m/z = 193$ [M–CH_3] and 73 [TMS]) was also revealed, but it is not established whether this molecule is a real wax constituent. These fragments are in line with those published previously (MacLafferty and Stauffer, 1989).

Chloroform fraction

The chloroform fraction contained mainly the long-chain diols and small amounts of fatty acids. Higher quantities of diols were found in *P. sitchensis* (25%) than in *P. abies* wax (13%). As shown in Fig. 3c, the mass spectra of the diol-di-TMS-ethers gave important fragments due to the α-scission on each side of the two TMS–ether groups. The loss of trimethylsilanol (TMSOH) was also observed. In Norway spruce, seven C29 isomers and one C27 diol were identified. They eluted in the following order: 5,10-heptacosanediol, 10,13-, 7,10-, 10,16-, 6,10-, 5,10-, 4,10- and 3,10-nonacosanediol. It seems, therefore, that the retention time of the positional isomers increases as a function of the distance between the two hydroxyl groups. All of the above diols except 3,10-nonacosanediol (which was absent in all *P. sitchensis* samples) were present in Sitka spruce waxes, and these also contained 5,10-octacosanediol. The two major diols (5,10- and 4,10-nonacosanediol) have already been reported in several spruce and pine species (Franich *et al.*, 1978; Riederer, 1989), whereas the five other nonacosanediols were not identified in the studied species. Percy and Baker (1990) reported 10,13-, 7,10-, 5,10-, and 4,10-

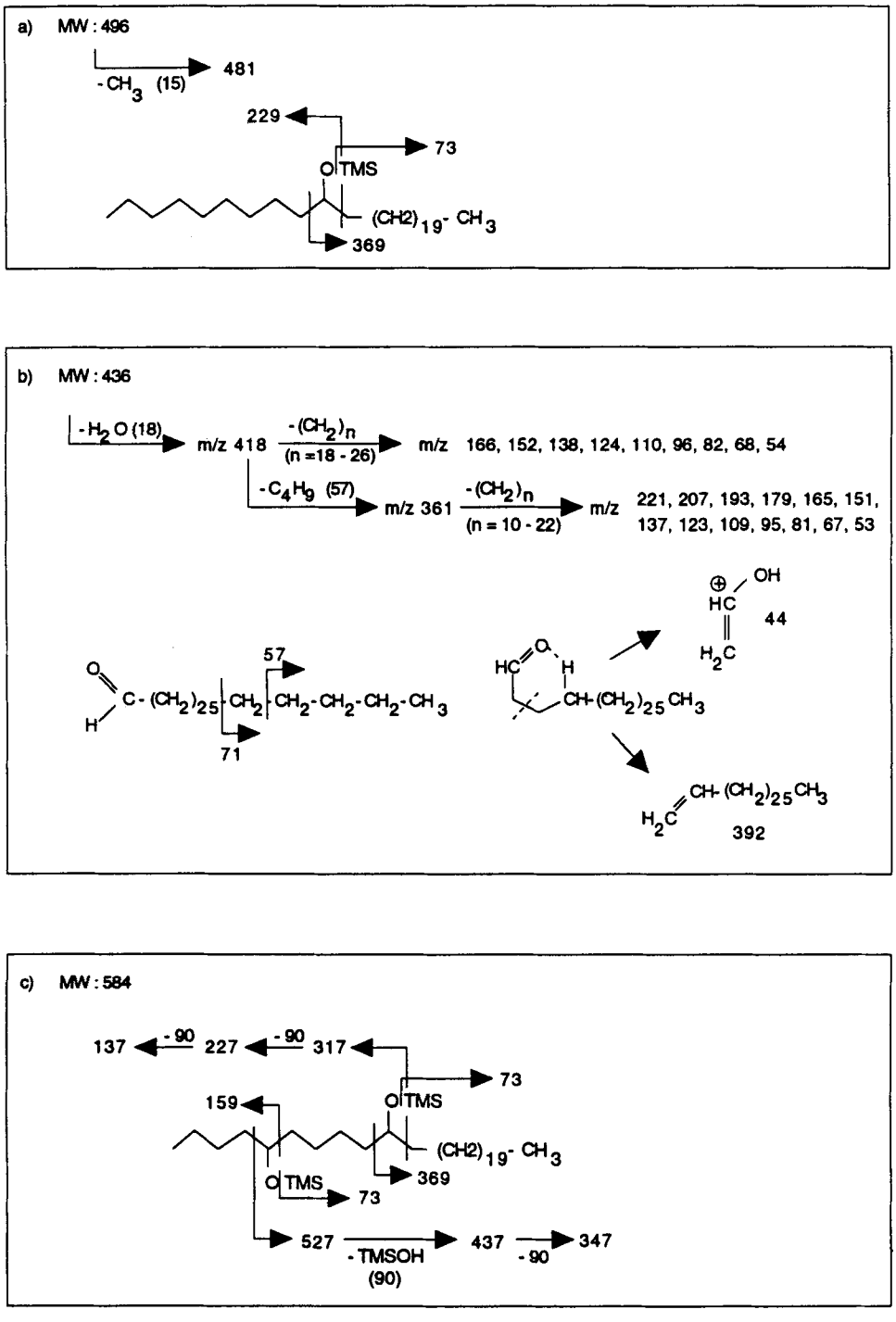

Figure 3. Typical fragmentation patterns of some wax constituents: **a)** secondary alcohol (10-nonacosanol); **b)** long chain aldehyde (*n*-triacontanal); **c)** diol (5,10-nonacosanediol). (For spectroscopic conditions see Experimental section.)

nonacosanediols in *P. sitchensis*, but did not mention the other isomers. Franich *et al.* (1978) found 5,10-heptacosanediol and 10,13-nonacosanediol in the needle lipids of *Pinus radiata*. Recently, Percy *et al.* (1992), identified 10,16-, 7,10- and 3,10-nonacosanediol in the red spruce (*Picea rubens* Sarg).

Chloroform–methanol fraction

The major part of the fatty acids was recovered in the methanol–chloroform eluent. Norway spruce samples showed higher amounts of fatty acids (20%) than did Sitka spruce (about 4%). For both species, the even-numbered homologues dominated the profile with the C20 to C26 being the most abundant. All fatty acids from C12 to C30 (except C27 and C29) were identified in *P. abies* whereas the C12 to C26 series was encountered in *P. sitchensis*. Günthardt-Goerg (1991) has already analysed fatty acids with a chain length from C12 to C32 in Norway spruce waxes. However, so far only the hexadecanoic homologue has been identified as the main fatty acid in the cuticular lipids of Sitka spruce (Percy and Baker, 1990).

Three ω-hydroxyacids having 12, 14 and 16 carbon atoms were also present partly in this fraction (about 1% for the two species).

Methanol fraction

The methanol fractions were composed mainly of the three ω-hydroxyacids described above representing ca. 1% for both species.

Various amounts of terpenic molecules were found in both waxes. The 24-methylenecycloartanol–TMS ($M^+ = 512$, plus other fragments at 73 (base peak), 245, 353, 379, 407, 422, 497) was found only in *P. abies*. It is likely, but not proven, that this component is excreted in the cuticular waxes. Dehydroabietic ($m/z = 73$, 239 (base peak), 255, 357 and 372) and abietic acid ($m/z = 73$, 185, 213, 241, 256 (base peak), 359 and 374) were identified in both waxes. However, pimaric acid ($m/z = 73$, 121 (base peak), 257, 359 and 374) and isopimaric acid ($m/z = 73$, 227, 241 (base peak), 256, 359 and 374) were detected only in Sitka spruce wax extracts. The presence of such molecules might be related to a pollution of the samples by small amounts of resins (Lorbeer and Zelman, 1988).

CONCLUSION

In the chromatographic conditions described herein, about 20 to 30% of the total wax extracts can be recovered for both species. 10-Nonacosanol represented about 45% of the chromatographically detectable wax in Norway spruce, whereas it accounted for only about 9% of the total wax amount. This might be explained by the temperature limits of the capillary columns and of the apparatus which were insufficient to analyse compounds with very high molecular weight such as long-chain esters and estolides (bio-polymers). This assumption is supported by additional saponification experiments. High quantities of ω–hydroxyacids (C12 to C16), α,ω–diols (C12 to C16) and fatty acids (C14 to C24), which are the components of estolides, were recovered. However, the original composition cannot be clarified here because the long-chain esters that were probably present would have been saponified together with the estolides.

In conclusion, about 85% of the chromatographically resolvable waxes of *P. abies* and *P. sitchensis* were identified. Cuticular lipids of both species were mainly composed of secondary alcohols, diols and free fatty acids: those of *P. abies* were characterized by a high content of fatty acids and a relatively low level of diols, whilst wax of *P. sitchensis* showed the inverse. Significant qualitative differences between the two species were revealed by minor components. Indeed methyl- and ethyl-esters and 24-methylenecycloartanol were exclusively present in *P. abies*, whereas *n*-aldehydes occurred only in *P. sitchensis*. Apart from some quantitative differences, other classes of compounds such as alkanes, alkenes and ω-hydroxyacids were common in both waxes.

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