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ORIGINAL ARTICLE

Characterization of two chemotypes of *Pinus pinaster* by their terpene and acid patterns in needles

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Abstract The existence of two chemotypes of *Pinus pinaster*, on the basis of the chemical composition of the resin acids in their needles, is known. An investigation was performed on 54 samples of needles of Spanish *Pinus pinaster* to study the differences between these chemotypes on the basis of monoterpene, sesquiterpene, neutral diterpene, fatty acid, and resin acid composition. One-hundred and twelve compounds were identified by GC–FID and GC–MS. Statistical analysis of the results established the existence of two groups or chemotypes, in the ratio of 5:1. In one chemotype, total acid compounds were more abundant than neutral compounds, whereas in the other the concentrations of both neutral and acid compounds were similar. Distinction of the chemotypes was based on the presence/absence of a sesquiterpene (germacrene D-4-ol acetate), neutral diterpenes (8(14),13(15)-abietadiene, anticopalol, an isomer of anticopalol, and pimarol), fatty acids (10-octadecenoic, 14-hydroxy-10-octadecenoic, and 13-hydroxy-9-octadenoic acids and an unidentified fatty acid), and resin acids (levopimaric + palustric, eperuic, and anticopalic acids, and three isomers of anticopalic acid); and on the different relative percentages of other compounds of these types. This study gives a wide view of the composition of the needles of *Pinus pinaster*, improving the differentiation of chemotypes on the basis of terpene and acid composition.

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Introduction

The natural distribution of the maritime pine (*Pinus pinaster* Ait.) extends over the Western Mediterranean region and the Atlantic zone of Southwest Europe. It is one of the most widely distributed forest species in Spain (MAPA 2003) and the main source of many resinous products obtained from the oleoresin. A research project on genetic improvement of Spanish *P. pinaster* has been carried out. As part of this work, a survey of the neutral terpenes and resin and fatty acids in needles of this species, has been carried out to assess their potential use as molecular markers.

Neutral terpenes are the main components of the essential oil of conifers and are found in concentrations of several milligrams per gram of fresh needle weight. Terpene content and composition are often used as chemotaxonomic tools for identification of conifer subspecies and hybrids, and to study geographical variation (Von Rudlolff 1975; Schaefer and Hanover 1986; Forrest 1987; Hall and Langenheim 1987; Müller-Starck et al. 1992; Lang 1994; Nerg et al. 1994; Gallis and Panetsos 1997). Volatile compounds in the needles of *Pinus pinaster* of several provenances (Table 1), for example monoterpenes (α and β-pinene) and sesquiterpenes (β-caryophyllene and germacrene D), have been reported (Pauly et al. 1973; Kleinhentz et al. 1999; Tiberi et al. 1999; Petrakis et al. 2001; Hmamouchi et al. 2001; Macchioni et al. 2003; Dob et al. 2005). Neutral diterpenes have been reported to a lesser extent (Dominguez et al. 1989; Ottavioli et al. 2008).

On the other hand, the resin acids, dicyclic or tricyclic diterpenes containing abietane, pimarane, or labdane

Table 1 Composition of volatile compounds in *Pinus pinaster* needles of several provenances (percentage of total area of total volatile compounds)

Provenance	α-Pinene	β-Pinene	β-Caryophyllene	Germacrene D	Others	Ref.
Greece	20.9	0.9	14.8	19.2		Petrakis
Algeria	–	0.3	26.6	–	Aromadendrene (12.5)	Dob
Morocco	21.4	2.1	22.2	4.0	γ-Muurolene (6.8) δ-cadinene (6.8)	Hmamouchi
Italy[a]	44.5	22.5	n.d.	n.d.	Myrcene (7.1)	Tiberi
Italy	28.9	21.7	13.2	4.3	Myrcene (4.5)	Maccioni
France	17.0	28.7	10.9	28.1		Kleinhentz
France	10.2	16.0	12.6	11.5	Myrcene (6.1) δ-cadinene (5.0)	Pauly
Corsica	26.8	1.2	11.7	13.5	Abieta-7,13-diene (8.0)	Ottavioli
	8.7	0.5	14.1	20.1	Abieta-7,13-diene (8.5)	
	8.1	0.4	5.4	6.9	Palustradiene (10.9) abieta-7,13-diene (32.3)	
Spain	17.0	26.6	7.3	13.3	Myrcene (5.9)	Domínguez
	19.6	12.6	12.0	15.6	Myrcene (3.1)	

[a] Only monoterpenes are reported (percentage of area of total monoterpenes)

n.d. no data

skeletons, also occur in conifer needles, and in cortex and xylem tissues, usually in resin canals (Croteau and Johnson 1985). Because of their chemical and presumed physiological stability, resin acids are regarded as valuable tools in pine taxonomy and genetic investigations (Zinkel 1977; Tobolski and Zinkel 1982; Gref and Lindgren 1984). Resin acids in *Pinus pinaster* needles have been studied, and evidence of the existence of two chemotypes has been obtained (Walter et al. 1985; Pombeiro et al. 1991; Arrabal and Cortijo 1997). The fatty acid composition of seed oils has also been used in pine taxonomy (Hu et al. 1992; Wolff 1997, 1998, 1999), but similar data for needles were not found in the literature.

The objective of this work was to study the neutral terpenes and fatty and resin acids in the needles of this species.

Materials and methods

Samples

Fifty-four samples of two-year-old needles were collected from ortets and ramets grafted in a clone bank located in Carbonero, Segovia province (Central Spain). The provenance of the ortets was from plus trees (pines with high oleoresin production). Grafting was on rootstock of *P. pinaster* from the same region (Central Spain), where all branches were removed (grafts were six years old). The needles were frozen in liquid nitrogen at the moment of sampling and kept at –70°C until analysis.

Extraction

The needles were cut into small pieces (2–4 mm). A known weight (1.5–2 g) was extracted for 24 h, at 4°C in the dark, with 5 ml diethyl ether–petroleum ether (1:1) to which 200 µg/ml internal standards (isobutylbenzene for monoterpenes, heptadecane for sesquiterpenes and neutral diterpenes, and heptadecanoic acid for fatty and resin acids) had been added. The extract was then decanted and the neutral terpenes in part of the extract were analyzed by GC, without any further purification. The needle pieces were washed with 2 ml diethyl ether–petroleum ether (1:1), the washing solution was added to the rest of the extract, and the solvent was removed from the final solution by use of a nitrogen stream. The dried extract was redissolved in 1 ml methanol and analyzed by GC after addition of 100 µl tetramethyl ammonium hydroxide, as methylation agent (Song et al. 1993; Galletti et al. 1995; Beverly et al. 1997). The reproducibility of extraction was assessed by analysis of six replicate extracts of the same sample. The coefficients of variation were approximately 5% and always lower than 10% for components with average concentration higher than 5%. Components with average concentrations lower than 5% had high coefficients of variation (between 5 and 40%).

Chemicals

Tetramethyl ammonium hydroxide (Aldrich) was used as methylation agent. Solvents: diethyl ether (Panreac), petroleum ether 40–60° (Panreac), methanol (Merck). For quantitative determination, isobutylbenzene (Fluka), heptadecane (Aldrich), and heptanoic acid (Aldrich) were used as internal standards.

GC–FID

The terpenic compounds and fatty acids were analyzed by gas chromatography with flame-ionization detection (FID).

GC equipment: HP 5890 gas chromatograph. Column: 30 m × 0.25 mm internal diameter, PTE-5 column (0.25 µm film thickness). Chromatographic conditions: helium flow 0.5 ml min^{-1}, oven temperature 60°C (2 min), 4° min^{-1}, 270°C (10 min), injector temperature 260°C, FID temperature, 300°C.

GC–MS

Equipment: HP 5890 gas chromatograph connected to a 5971A mass detector. Column and chromatographic conditions similar to those used for GC–FID.

The compounds were identified by comparing their EI mass spectra at 70 eV with those in the Wiley (1986) and NIST/EPA/NIH (1995) spectral databases and in literature (Enzell and Ryhage 1965; Enzell and Wahlberg 1969; Zinkel et al. 1971; Ekman 1979; Ramaswami et al. 1986; Adams 1989; Lange and Weiβmann 1987, 1989, 1991). Anticopalic, imbricataloic, and epiimbricataloic acid methyl esters were identified by comparing their mass spectra with those of authentic samples provided by Dr Duane F. Zinkel (Forest Products Laboratory, Madison. USA). Quantitative measurements were performed by the internal standard method, with three different standards, using a flame-ionization detector (FID), with the same running conditions.

Statistical analysis

Univariate analysis was carried out by use of BMDP-7D (ANOVA) software (WJ Dixon, BMDP Statistical Software, Software Release, 1990). Mean and standard deviations were calculated for each variable of the two groups of samples, using a single variable model. The Student Newman–Keuls multiple range test was also carried out to determine the significance levels of the differences between the means, at the 95% confidence level. Canonical discriminant analysis was also carried out for all components evaluated, by use of the CANDISC.SAS procedure (SAS Institute, SAS/STAT^{R}, version 6, fourth Edition, 1994).

Results and discussion

As can be seen in Figs. 1 and 2, two types of chromatogram were obtained from analysis of the needles, with regard to both neutral and acid components. A study of the total content of each type of terpene and of the acids also revealed differences between the two groups of needles (Table 2). Samples of chemotype 1 had a higher content of acids than neutral components whereas samples of chemotype 2 had similar contents of neutral and acid components.

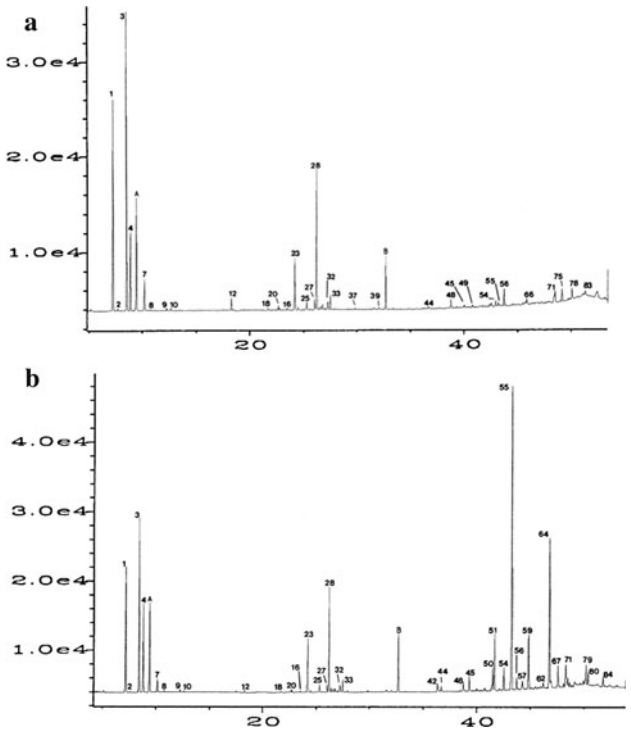

Fig. 1 GC–FID chromatogram obtained from neutral terpenoid components of the needles of *Pinus pinaster*. **a** Chemotype 1, **b** chemotype 2, *A* isobutylbenzene, *B* heptadecane

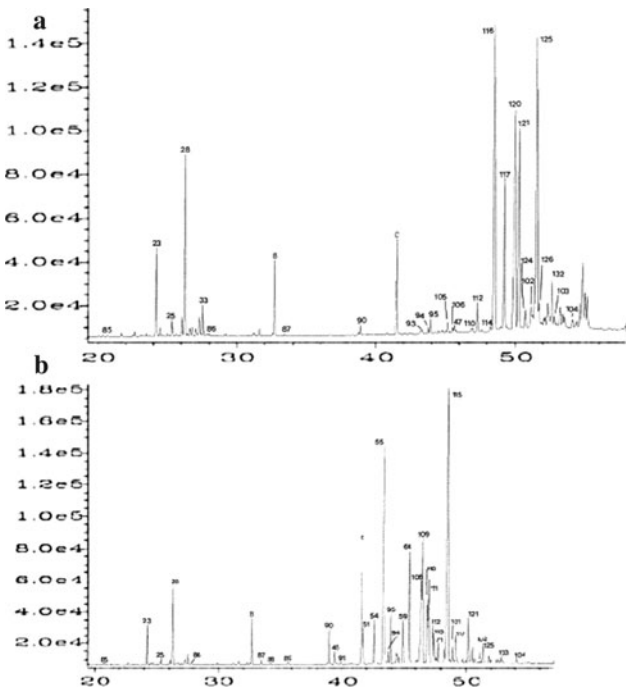

Fig. 2 GC–FID chromatogram obtained from acid components (as methyl esters) of the needles of *Pinus pinaster*. **a** Chemotype 1, **b** chemotype 2, *B* heptadecane, *C* heptadecanoic acid

Neutral diterpenes had the highest correlation with the total canonical structure obtained in discriminant analysis.

Both chemotypes had similar qualitative monoterpene composition (Table 3). We identified 19 compounds: 11 hydrocarbons, 4 esters, 2 alcohols, one ether, and one acid. Three of these compounds are not monoterpenes: *n*-tridecane, methyl eugenol, and phenyl ethyl isovalerate, but are included in this table because they appear close to monoterpenes in the chromatogram. Only five compounds were found in all the samples: α and β-pinene, myrcene and β-phellandrene + limonene. Among these, α and β-pinene were prominent, together accounting for more than 70% of the total monoterpenes in samples of the two groups. The percentage of β-pinene was usually highest, followed by that of α-pinene. The prevalence of β-pinene over α-pinene is in accord with the composition of samples from France (Pauly et al. 1973; Kleinhentz et al. 1999). α-Phellandrene, bornyl acetate, and *n*-tridecane were only detected in some samples of chemotype 1, and at very low percentages. Statistical analysis of monoterpene composition did not enable good separation of the groups of samples; in fact, seven samples were incorrectly classified.

Table 4 shows sesquiterpene composition. Thirty compounds were found: 19 hydrocarbons, 5 alcohols and 6 esters, and 28 of these were identified. As can be seen in Tables 3 and 4, and as was described by Pauly et al. (1973) and Domínguez-Garrido et al. (1988), several acetates are produced in the needles of *P. pinaster*: linalyl, bornyl, geranyl, farnesyl, and, in chemotype 2, also germacrene D-4-ol acetate. In the statistical analysis, the canonical variable obtained for 100% of variance correctly classified all the samples into two groups. The main components in all the samples were germacrene D and β-caryophyllene. Only germacrene D was significantly different in the

Table 2 Total terpenes and acids in needles of *Pinus pinaster* (mg/g needles)

	Chemotype 1		Chemotype 2		C	SL
	x	SD	*x*	SD		
Neutral terpenes	8.30	2.17	14.63	5.55	0.6892	**
Monoterpenes	3.44	1.16	2.26	0.85	−0.4065	**
Sesquiterpenes	3.16	0.89	3.15	1.20	−0.0056	
Diterpenes	1.71	0.60	9.23	3.92	0.9479	**
Acids	32.30	17.58	12.84	3.91	−0.4845	**
Fatty acids	1.88	1.14	1.26	0.48	−0.2424	
Resin acids	30.41	17.01	11.58	3.54	−0.4850	**
Total	40.60	19.18	27.47	5.16	−0.3271	*

x mean, *SD* standard deviation, *C* correlation with total canonical structure, *SL* significance level

* <5%, ** <1%

Table 3 Monoterpenes and other volatile compounds in needles of *Pinus pinaster* (percentage of total area)

Peak component	t_R	Chemotype 1			Chemotype 2			C	SL
		x	SD	*n*	*x*	SD	*n*		
1 α-Pinene	7.110	33.36	5.28	45	35.77	4.83	9	−0.2653	
2 Camphene	7.426	0.27	0.18	28	0.28	0.06	2	0.3228	
3 β-Pinene	8.265	41.95	6.22	45	39.13	4.89	9	0.2679	
4 Myrcene	8.650	12.34	3.55	45	14.56	2.75	9	−0.3643	
5 α-Phellandrene	9.120	0.30	0.11	10				0.3053	
6 δ-3-Carene	9.335	5.04	2.86	13	2.45	1.78	4	0.0347	
7 β-Phellandrene + limonene	10.030	5.99	2.70	45	4.98	2.59	9	0.2155	
8 *trans*-Ocimene	10.589	0.43	0.19	32	0.81	0.62	8	−0.6520	**
9 Terpinolene	12.076	1.34	1.58	40	1.30	0.78	9	−0.0445	
10 Linalool	12.451	0.40	0.16	30	0.41	0.26	5	0.0818	
11 Cuminic acid	15.794	0.26	0.12	8	0.26		1	0.0901	
12 Linalyl acetate + geraniol	18.048	1.44	1.33	40	0.48	0.10	5	0.4542	*
13 Bornyl acetate	19.631	0.34	0.22	13				0.3138	
14 *n*-Tridecane	21.053	0.70	0.60	8				0.2144	
15 Geranyl acetate	22.755	0.38	0.22	25	0.50	0.20	3	0.0637	
16 Methyl eugenol	23.089	0.38	0.14	25	0.37	0.10	4	0.1244	
17 Phenyl ethyl isovalerate	26.164	1.03	0.66	41	1.70	2.09	8	−0.3174	

t_R retention time, *x* mean, *SD* standard deviation, *n* number of samples in which it was detected, *C* correlation with total canonical structure, *SL* significance level

* <5%, ** <1%

Table 4 Sesquiterpenes in needles of *Pinus pinaster* (percentage of total area of all sesquiterpenes)

Peak component	t_R	Chemotype 1			Chemotype 2			C	SL
		x	SD	*n*	*x*	SD	n		
18 α-Cubebene	21.499	1.01	0.31	45	0.48	0.15	6	0.6545	**
19 α-Ylangene	22.280	0.41	0.12	30	0.37		1	0.3984	**
20 α-Copaene	22.437	1.64	0.38	45	1.10	0.32	9	0.4906	**
21 β-Cubebene	22.908	0.61	0.08	39				0.7098	**
22 Longifolene	23.499	0.69	0.49	13				0.2015	
23 β-Caryophyllene	23.968	20.65	6.57	45	16.54	2.39	9	0.2507	
24 β-Gurjunene	24.258	1.02	0.28	44	0.82	0.20	8	0.3107	*
25 α-Humulene	25.086	3.64	0.94	45	2.94	0.31	9	0.2902	*
26 α-Amorphene	25.385	0.43	0.11	17	0.36	0.17	2	0.1437	
27 γ-Muurolene	25.824	4.86	1.28	45	2.97	1.31	9	0.4945	**
28 Germacrene D	26.024	37.16	8.72	45	45.85	6.53	9	−0.3707	**
29 Isolongifolene	26.390	2.17	0.56	45	1.38	0.48	9	0.4829	**
30 α-Muurolene	26.565	1.83	0.43	45	1.47	0.30	9	0.3241	*
31 Sesquiterpene hydrocarbon	26.794	0.86	0.21	44	0.67	0.21	9	0.2714	
32 γ-Cadinene	27.036	6.14	1.19	45	3.92	1.05	9	0.5927	**
33 δ-Cadinene	27.296	7.02	1.75	45	4.90	1.83	9	0.4200	**
34 1,4-Cadinadiene	27.602	0.75	0.20	26	0.56	0.14	3	0.2362	
35 α-Bisabolene	27.772	0.92	0.34	42	0.82	0.39	7	0.1984	
36 Germacrene D-4-ol	28.993	1.00	0.63	29	0.94	0.76	5	0.0652	
37 Guaiol	29.646	1.08	0.50	26	0.77	0.32	7	0.0622	
38 T-Cadinol	30.989	0.62	0.16	34	0.59	0.16	4	0.2488	
39 α-Cadinol	31.381	1.26	0.64	43	1.18	0.53	8	0.0821	
40 Sesquiterpene hydrocarbon	32.285	0.91	0.58	4	2.05	1.61	2	−0.2766	*
41 (*E*,*E*)-Farnesol	33.269	1.11	0.87	34	0.36	0.05	2	0.3353	*
42 Germacrene D-4-ol acetate	36.137				5.08	1.64	9	−0.9628	**
43 (*Z*,*E*)-Farnesyl acetate	36.392	0.32	0.14	20	0.45	0.11	6	−0.2959	*
44 (*E*,*E*)-Farnesyl acetate	36.538	1.14	0.78	45	1.27	0.62	7	0.0551	
45 (*Z*,*E*)-Farnesyl propionate	39.129	1.39	0.86	37	5.72	1.51	9	−0.8688	**
46 (*E*,*E*)-Farnesyl propionate	39.839	0.74	0.51	39	0.95	0.65	9	−0.2081	
47 (*E*,*E*)-Farnesyl isovalerate	45.501	2.63	1.08	45	0.41	0.17	4	0.6921	**

t_R retention time, *x* mean, *SD* standard deviation, *n* number of samples in which it was detected, *C* correlation with total canonical structure, *SL* significance level

* <5%, ** <1%

comparison of means tests, but its correlation with the total canonical structure was low. Amounts of the other sesquiterpenes were always below 10%. Amounts of the hydrocarbons α-humulene, γ-muurolene, and γ and δ-cadinene in the two types of sample were ~3% or higher. The average concentrations of these constituents were higher in chemotype 1 than in chemotype 2, with significant differences and good correlation with total canonical structure. Moreover, for germacrene D-4-ol acetate and (*Z*,*E*)-farnesyl propionate concentrations were higher than 5% in chemotype 2 samples only, with significant differences between means and the highest correlation with total canonical structure. In fact, germacrene D-4-ol acetate was only found in chemotype 2 samples, and the presence/absence of this compound can be regarded as a criterion for characterization of this group of samples.

Forty neutral diterpenes were found (Table 5): 11 hydrocarbons and 29 oxygenated diterpenes. Among the oxygenated diterpenes alcohols, aldehydes, and methyl esters (naturally present in needles) were identified. Alcohols were the largest group, and consisted of four of the labdane type, two of the pimarane type, two of the abietane type, and two not fully identified. Significant differences for thirty-three components were found in the comparison

Table 5 Neutral diterpenes in needles of *Pinus pinaster* (percentage of total area of all neutral diterpenes)

Peak component	t_R	Chemotype 1			Chemotype 2			C	SL
		x	SD	*n*	*x*	SD	*n*		
48 8(17),12,14-labdatriene	38.562	7.66	3.52	45	0.98	0.56	9	−0.6165	**
49 19-Nor-4,8,11,13-Abietatetraene	40.554	1.59	0.57	44	0.46	0.13	9	−0.5937	**
50 7,13-Abietadiene	41.322	0.60	0.14	6	4.68	4.14	9	0.7304	**
51 8(14),12-Abietadiene	41.486	1.23	0.64	23	8.47	5.02	9	0.8185	**
52 Oxygenated diterpene	41.918				0.36	0.08	9	0.9751	**
53 19-Nor-6,8,11,13-abietatetraene	42.143	1.74	0.59	44	0.18	0.08	8	−0.7044	**
54 8,11,13-Abietatriene	42.322	2.73	1.00	45	3.05	0.67	9	0.1245	
55 8,13-Abietadiene	43.167	7.00	1.73	45	34.32	8.62	9	0.9408	**
56 Isoabienol	43.537	15.93	12.52	44	1.66	1.03	9	−0.4145	**
57 Anticopalol isomer	44.049				2.09	1.31	9	0.8389	**
58 Abienol	44.280	1.95	0.92	42	0.41	0.28	4	−0.5522	**
59 8(14),13(15)-Abietadiene	44.656				5.51	1.85	9	0.9446	**
60 8,15-Pimaradien-18-al	45.230	4.30	1.15	45	0.41	0.19	9	−0.8132	**
61 8(14),11,13(15)-Abietatriene	45.779				0.72	0.17	9	0.9701	**
62 Diterpene alcohol	45.981				0.70	0.33	9	0.8937	**
63 Isopimaral	46.379	2.78	1.04	42				−0.6591	**
64 Anticopalol	46.594				15.03	5.32	9	0.9391	**
65 Diterpenic alcohol	46.649	1.09	0.37	3				−0.1041	
66 Levopimaral	47.044	5.91	2.99	45	1.40	0.23	7	−0.5525	**
67 Pimarol	47.297				4.02	2.06	9	0.8647	**
68 Dehydroabietal	47.367	1.10	0.75	35				−0.4019	**
69 Diterpene hydrocarbon	47.581				0.25	0.08	6	−0.6905	**
70 Oxygenated diterpene	48.071	1.04	0.46	32	0.60	0.23	9	−0.0931	
71 Abietal + methyl levopimarate[a] + methyl palustrate[a]	48.271	15.63	4.17	45	3.37	0.98	9	−0.7701	**
72 Diterpene hydrocarbon	48.505				0.98	0.28	9	0.9596	**
73 Isopimarol	48.648	0.62	0.35	11	1.08	0.58	9	0.6864	**
74 Oxygenated diterpene	48.714				0.37	0.14	8	0.8643	**
75 Methyl dehydroabietate	49.260	9.66	3.62	45	1.17	1.36	9	−0.6905	**
76 Neoabietal + methyl imbricataloate[a]	49.506	3.47	3.69	45	0.42	0.12	9	−0.3308	*
77 Oxygenated diterpene	49.716				0.32	0.13	6	0.7313	**
78 Methyl abietate	50.001	8.43	2.50	45	0.44	0.40	7	−0.7996	**
79 Abietol	50.269	0.99	1.54	17	3.15	2.42	9	0.6154	**
80 Oxygenated diterpene	50.323				1.40	0.62	9	0.9098	**
81 Oxygenated diterpene	50.537	1.37	0.70	42	0.36	0.15	8	−0.4602	**
82 Oxygenated diterpene	50.687	2.80	2.06	34	0.17	0.09	6	−0.3577	**
83 Methyl neoabietate	51.107	5.42	3.53	45	1.82	0.98	9	−0.3855	**
84 Neoabietol	51.641	1.59	1.86	7	0.65	0.29	9	0.1812	

t_R retention time, *x* mean, *SD* standard deviation, *n* number of samples in which it was detected, *C* correlation with total canonical structure, *SL* significance level

* <5%, ** <1%

[a] Only detected in chemotype 1

of means tests, at level of 1% for thirty-two of the compounds. In the canonical discriminant analysis, the canonical variable obtained for 100% of variance classified correctly all samples into two groups. The samples of chemotype 1 were characterized by their high content of oxygenated diterpenes, specially isoabienol (13(16), 14-labdadien-8-ol) and the mixture abietal + methyl levopimarate + methyl palustrate; average concentrations of these were 15%, and correlation with total canonical structure was good. Other oxygenated diterpenes can be

Table 6 Fatty acids (as methyl esters) in needles of *Pinus pinaster* (percentage of total area of all methyl esters)

Peak component	t_R	Chemotype 1			Chemotype 2			C	SL
		x	SD	*n*	*x*	*SD*	*n*		
85 Decanoic $C_{10:0}$	20.654	1.38	0.96	34	2.92	0.97	9	0.595	**
86 Lauric $C_{12:0}$	27.706	2.81	2.48	13	1.07	0.75	4	−0.073	
87 Myristic $C_{14:0}$	33.263	4.67	3.81	44	4.85	0.90	9	0.044	
88 Unidentified	35.298				2.76	1.81	9	0.845	**
89 Pentadecanoic $C_{15:0}$	36.135	1.58	0.81	22	3.55	0.55	9	0.776	**
90 Palmitic $C_{16:0}$	38.642	11.54	7.43	45	30.23	5.71	9	0.728	**
91 Unidentified	39.636	1.77	1.12	26	4.04	1.53	9	0.690	**
92 Linoleic $C_{18::2\ (9,12)}$	42.688	3.03	2.56	44				−0.435	**
93 Octadecenoic $C_{18:1\ (10)}$	42.947	10.13	4.50	45				−0.693	**
94 Oleic $C_{18:1\ (9)}$	43.036	13.26	13.46	40	10.98	5.78	9	−0.015	
95 Stearic $C_{18:0}$	43.649	23.73	9.69	45	22.83	6.38	9	−0.018	
96 14-hydroxy-10-octadecenoic	44.154	5.86	3.13	45				−0.626	**
97 13-hydroxy-9-octadecenoic	44.372	3.81	2.26	45				−0.584	**
98 Nonadecadienoic $C_{19:2\ (9,12)}$	44.737	0.16	0.18	45				−0.447	**
99 Nonadecenoic $C_{19:1\ (9)}$	45.454	0.96	0.56	45				−0.596	**
100 Nonadecanoic $C_{19:0}$	45.537	0.97	0.75	43	3.16	1.07	5	0.320	*
101 Eicosanoic $C_{20:0}$	48.689	4.81	2.18	44	5.49	2.82	9	0.146	
102 Heneicosanoic $C_{21:0}$	50.849	5.74	3.47	17	2.12	1.27	8	−0.027	
103 Behenic $C_{22:0}$	53.612	8.19	6.04	45	3.35	1.76	9	−0.311	*
104 Lignoceric $C_{24:0}$	56.413	5.01	4.10	45	4.88	1.79	9	−0.003	

t_R retention time, *x* mean, *SD* standard deviation, *n* number of samples in which it was detected, *C* correlation with total canonical structure, *SL* significance level

* <5%, ** <1%

also pointed out in samples of chemotype 1: the methyl esters of dehydroabietic, abietic, and neoabietic acids, and levopimaral. For all of these, average concentrations were >5%, and correlation was good. In samples of chemotype 2, anticopalol (8(17),13-labdadien-15-ol) was the main neutral oxygenated diterpene (15%), and, in discriminant analysis, this also correlated very well with total canonical structure. However, the largest differences between the two groups of samples were those of hydrocarbons. Thus, 8,13-abietadiene was the main neutral diterpene in samples of chemotype 2, with an average concentration of 34%, with significant differences at the 1% level and very high correlation with total canonical structure. The other main hydrocarbons were: 8(14),12-abietadiene, 8(14),13(15)-abietadiene, and 7,13-abietadiene, with percentages higher than 4.5% and correlations also very high.

Only a few of neutral diterpene compounds have previously been identified in needles of *Pinus pinaster*: levopimaradiene, neoabietadiene, palustradiene, 7,13-abietadiene and 8,11,13-abietatriene (Dominguez et al. 1989; Pombeiro et al. 1991; Ottavioli et al. 2008).

In Table 6, the results obtained for the fatty acids can be seen. Twenty fatty acids were found, and eighteen of these were identified. In chemotype 1, the main fatty acid was stearic, followed by oleic which, in some samples, was the main fatty acid. Average concentrations of palmitic and octadecenoic acids were also high. In chemotype 2, palmitic acid was the main fatty acid, followed by stearic and oleic. In the statistical analysis of this type of compound, seventeen were significantly different in comparison of means tests. The canonical variable obtained in discriminant analysis enabled good separation of the two groups of samples. The compounds with the highest correlation with total canonical structure were an unidentified component (peak number 88), pentadecanoic acid, and palmitic acid. The presence/absence of 10-octadecenoic acid, 14-hydroxy-10-octadecenoic acid, 13-hydroxy-9-octadecoic acid, and an unidentified fatty acid, with the different relative percentages of pentadecanoic and palmitic acids could be used as chemotaxonomic values.

There was great variability in the resin acid composition of the two groups of samples, and significant differences

Table 7 Resin acids (as methyl esters) in needles of *Pinus pinaster* (percentage of total area of all methyl esters)

Peak component	t_R	Chemotype 1			Chemotype 2			C	SL
		x	SD	*n*	*x*	SD	*n*		
105 Seco I[a]	44.477	0.16	0.17	45				−0.3605	**
106 Seco II[b]	44.849	0.21	0.22	45				−0.3683	**
107 Secodehidroabietic isomer	45.110	0.17	0.17	45				−0.3705	**
108 Anticopalic isomer	46.059				6.98	3.06	9	0.9112	**
109 Eperuic	46.174				11.04	2.44	9	0.9751	**
110 Pimaric	46.584	0.78	0.64	45	7.89	5.13	9	0.7898	**
111 Anticopalic isomer	46.635				5.83	2.60	9	0.9028	**
112 Sandaracopimaric	47.004	1.39	0.19	45	2.69	0.88	9	0.7879	**
113 Anticopalic isomer	47.150				1.79	0.89	9	0.8893	**
114 Isopimaric	47.937	0.35	0.23	45	0.85	0.44	9	0.5763	**
115 Anticopalic	48.206				47.20	6.37	9	0.9906	**
116 Levopimaric + palustric	48.215	26.67	8.25	45				−0.8007	**
117 Dehydroabietic	48.872	6.02	1.69	45	2.95	1.06	9	−0.5860	**
118 Resin acid (M^+316)	48.999	0.24	0.15	45				−0.2460	
119 8,12-abietadien-18-oic	49.223	0.17	0.13	45				−0.3617	**
120 Imbricataloic	49.470	11.71	5.55	45	0.87	0.77	8	−0.6310	**
121 Abietic	49.928	12.11	2.93	45	4.94	2.92	9	−0.6817	**
122 Resin acid (M^+318)	50.202				0.39	0.14	9	0.9388	**
123 Resin acid (M^+314)	50.402	0.63	0.31	45				−0.6366	**
124 Epiimbricataloic	50.550	0.61	0.47	45				−0.4507	**
125 Neoabietic	51.262	22.99	6.44	45	4.07	3.85	9	−0.7621	**
126 Dihydroagathic	51.380	0.93	0.49	34	0.84	0.37	8	−0.4700	**
127 Pinifolic	51.545	0.42	0.39	40				0.3384	*
128 Oxoresin acid (M^+330)	51.639	0.47	0.27	42				−0.3721	**
129 Hydroxyresin acid (M^+334)	51.817	0.89	0.59	39				−0.4862	**
130 Oxohydroxydehydroabietic	52.139	0.77	0.49	30	0.34	0.10	8	−0.1980	
131 Methoxyresin acid (M^+346)	52.318	0.98	0.95	31				−0.3554	**
132 Oxohydroxydehydroabietic	52.471	3.32	1.71	36				−0.5686	**
133 Hydroxyabietic (M^+332)	52.632	0.82	0.67	34				-0.3943	**
134 19-Nor-12-oxo-3,5,8-abietatrienoic	52.749	0.43	0.34	43				−0.3248	*
135 Hydroxyresin acid (M^+330)	52.827	0.24	0.23	43				−0.2994	*
136 Hydroxydehydroabietic (M^+330)	53.840	0.50	0.42	43				−0.3608	**
137 Dihydroxyresin acid (M^+348)	54.202	0.40	0.19	35				−0.4570	**
138 Oxoresin acid (M^+328)	54.325	1.09	0.51	21				−0.6177	**
139 15-Hydroxydehydroabietic	54.474	2.46	1.75	15				−0.4779	**
140 Dihydroxyresin acid (M^+348)	54.793	1.42	0.87	6				−0.5282	**
141 Dihydroxyresin acid (M^+348)	55.780	1.39	0.81	35				−0.4355	**
142 Unidentified compound	63.272	1.31	0.77		1.18	0.53			

t_R retention time, *x* mean, *SD* standard deviation, *n* number of samples in which it was detected, *C* correlation with total canonical structure, *SL* significance level

* <5%, ** <1%

[a] Seco 1 2α-[2'(*m*-isopropylphenyl)ethyl]-1β.3α-dimethyl-cyclohexanecarboxylic acid

[b] Seco 2 2β-[2'(*m*-isopropylphenyl)ethyl]-1β.3α-dimethyl-cyclohexanecarboxylic acid

were found in comparison of means tests for all the components except two (Table 7). Canonical discriminant analysis correctly classified all samples into two groups. In samples of chemotype 1, thirty-three compounds were found, seventeen of them fully identified. The mixture levopimaric + palustric acids (only found in this

Table 8 Compounds discriminating chemotypes in needles of *Pinus pinaster*

Peak component	Chemotype 1			Chemotype 2			C	SL
	x	SD	*n*	*x*	SD	*n*		
Sesquiterpenes								
27 γ-Muurolene	4.86	1.28	45	2.97	1.31	9	0.4945	**
28 Germacrene D	37.16	8.72	45	45.85	6.53	9	−0.3707	**
32 γ-Cadinene	6.14	1.19	45	3.92	1.05	9	0.5927	**
33 δ-Cadinene	7.02	1.75	45	4.90	1.83	9	0.4200	**
42 Germacrene D-4-ol acetate				5.08	1.64	9	−0.9628	**
45 (*Z*,*E*)-Farnesyl propionate	1.39	0.86	37	5.72	1.51	9	−0.8688	**
Neutral diterpenes								
48 8(17),12,14-Labdatriene	7.66	3.52	45	0.98	0.56	9	−0.6165	**
50 7,13-Abietadiene	0.60	0.14	6	4.68	4.14	9	0.7304	**
51 8(14),12-Abietadiene	1.23	0.64	23	8.47	5.02	9	0.8185	**
55 8,13-Abietadiene	7.00	1.73	45	34.32	8.62	9	0.9408	**
56 Isoabienol	15.93	12.52	44	1.66	1.03	9	−0.4145	**
57 Anticopalol isomer				2.09	1.31	9	0.8389	**
59 8(14),13(15)-Abietadiene				5.51	1.85	9	0.9446	**
60 8,15-Pimaradien-18-al	4.30	1.15	45	0.41	0.19	9	−0.8132	**
63 Isopimaral	2.78	1.04	42				−0.6591	**
64 Anticopalol				15.03	5.32	9	0.9391	**
66 Levopimaral	5.91	2.99	45	1.40	0.23	7	−0.5525	**
67 Pimarol				4.02	2.06	9	0.8647	**
71 Abietal + methyl levopimarate + methyl palustrate	15.63	4.17	45	3.37	0.98	9	−0.7701	**
75 Methyl dehydroabietate	9.66	3.62	45	1.17	1.36	9	−0.6905	**
78 Methyl abietate	8.43	2.50	45	0.44	0.40	7	−0.7996	**
79 Abietol	0.99	1.54	17	3.15	2.42	9	0.6154	**
83 Methyl neoabietate	5.42	3.53	45	1.82	0.98	9	−0.3855	**
Fatty acids								
88 Unidentified				2.76	1.81	9	0.845	**
89 Pentadecanoic $C_{15:0}$	1.58	0.81	22	3.55	0.55	9	0.776	**
90 Palmitic $C_{16:0}$	11.54	7.43	45	30.23	5.71	9	0.728	**
92 Linoleic $C_{18:2\ (9,12)}$	3.03	2.56	44				−0.435	**
93 Octadecenoic $C_{18:1\ (10)}$	10.13	4.50	45				−0.693	**
96 14-hydroxy-10-octadecenoic	5.86	3.13	45				−0.626	**
97 13-hydroxy-9-octadecenoic	3.81	2.26	45				−0.584	**
Resin acids								
108 Anticopalic isomer				6.98	3.06	9	0.9112	**
109 Eperuic				11.04	2.44	9	0.9751	**
110 Pimaric	0.78	0.64	45	7.89	5.13	9	0.7898	**
111 Anticopalic isomer				5.83	2.60	9	0.9028	**
113 Anticopalic isomer				1.79	0.89	9	0.8893	**
115 Anticopalic				47.20	6.37	9	0.9906	**
116 Levopimaric + palustric	26.67	8.25	45				−0.8007	**
117 Dehydroabietic	6.02	1.69	45	2.95	1.06	9	−0.5860	**
120 Imbricataloic	11.71	5.55	45	0.87	0.77	8	−0.6310	**
121 Abietic	12.11	2.93	45	4.94	2.92	9	−0.6817	**
125 Neoabietic	22.99	6.44	45	4.07	3.85	9	−0.7621	**
132 Oxohydroxydehydroabietic	3.32	1.71	36				−0.5686	**
139 15-Hydroxydehydroabietic	2.46	1.75	15				−0.4779	**

x mean, *SD* standard deviation, *n* number of samples in which it was detected, *C* correlation with total canonical structure, *SL* significance level
** <1%

chemotype) and neoabietic acid were the main components, and were those with the highest correlation with total canonical structure. Other components in these samples were abietic, imbricataloic, and dehydroabietic acids, for which average concentrations (between 5 and 15%) were higher than in chemotype 2 samples; correlation with total canonical structure was good. Six resin acids (levopimaric, palustric, neoabietic, abietic, imbricataloic, and dehydroabietic) together accounted for more than 79% (average value) of total resin acids, and, in some samples, more than 85%. Five of these, levopimaric, palustric, dehydroabietic, abietic, and neoabietic acids, have previously been described in needles of *P. pinaster* (Walter et al. 1985; Arrabal and Cortijo1997) and in many other *Pinus* species as main resin acids. However, no data were found in the literature about the presence of imbricataloic acid in needles of *P. pinaster*. Amounts of all of the resin acids were significantly different at the 1% level. Other minor resin acids identified by us in needles of chemotype 1 only were 15-hydroxydehydroabietic, epiimbricataloic, 19-nor-12-oxo-3,5,8-abietatrienoic, pinifolic, 8,12-abietadien-18-oic, and seco I and seco II acids.

In samples of chemotype 2, only fifteen resin acids were found, nine of them fully identified. The main component was anticopalic acid, for which the average concentration was 47.2%. Three other peaks, with identical mass spectra, were tentatively identified as isomers of anticopalic acid. For two of these (peaks 108 and 111) average concentrations were >5%. The second main component was another labdane-type acid, eperuic acid, for which average amounts were close to 11%. These five compounds were found in chemotype 2 samples only, and correlation with total canonical structure was very high, especially for anticopalic acid. Among all the compounds found in these samples, anticopalic acid was the one with the highest correlation (0.9906). Dehydroabietic, imbricataloic, abietic, and neoabietic acids together accounted for less than 15% (average value).

Average concentrations of the other resin acids identified in the samples of this chemotype were lower than those found in the samples of chemotype 1, except for pimaric and sandaracopimaric acids. Correlation with total canonical structure was good.

Pombeiro et al. (1991) also identified two chemotypes by study of Portuguese *P. pinaster* needles: one was rich in anticopalic acid, the other was rich in two diterpene hydrocarbons, 7,13-abietadiene and 8,11,13-abietatriene, but differences between mono and sesquiterpene composition were not found. Walter et al. (1985) identified two chemotypes from the resin acid pattern of the needles of French *P. pinaster*, depending on the origin of the trees (Landes/Atlantic or Corsican/Mediterranean). Arrabal and Cortijo (1997) found the same resin acid patterns in needles of *P. pinaster* from Central Spain (Segovia province).

Although all the samples studied by us were collected in a small geographical region, this region can be regarded as an overlapping zone of two provenances, Atlantic and Mediterranean. In fact, they could be classified into two groups clearly differentiated by their terpenic composition, especially in relation to the presence or absence of anticopalic, eperuic, and levopimaric + palustric acids, in agreement with Walter et al. (1985). Besides anticopalic and eperuic acids, three isomers of anticopalic acid characterized the resin acid composition of needles of chemotype 2, together with percentages of pimaric and sandaracopimaric acids higher than in the needles of chemotype 1. The percentages of anticopalic and eperuic acids found in our samples, were similar to those described by Arrabal and Cortijo (1997) in needles of *P. pinaster*, but lower than those of anticopalic acid obtained by Walter et al. (1985) and higher than those of eperuic acid obtained by the same authors.

A compilation of compounds discriminating both chemotypes in needles of *Pinus pinaster* is shown in Table 8.

Conclusions

Of 54 samples of needles of *Pinus pinaster* studied, 83.3% and 16.6% were classified as the main and second chemotypes, respectively. A total of 112 compounds were identified. The geographical distribution of the Atlantic and Mediterranean provenances of *P. pinaster* overlaps in some areas of Central Spain. In these overlapping zones, specimens of both provenances can be found. With a simple and rapid procedure for determination of monoterpenes, sesquiterpenes, neutral diterpenes, fatty acids, and resin acids, the different chemotypes can be studied. On the whole, in the most abundant chemotype acids were present at higher concentrations than neutral compounds whereas in the other chemotype concentrations of neutral and acid compounds were similar. In the characterization of these chemotypes, study of individual monoterpenes, similar in both chemotypes, did not enable correct classification of the samples. However, study of detailed sesquiterpenes, neutral diterpenes, resin acids and fatty acids, enabled correct classification of all samples into the two chemotypes. The presence/absence of the sesquiterpene germacrene D-4-ol acetate; of the neutral diterpenes 8(14),13(15)-abietadiene, anticopalol, anticopalol isomer, and pimarol; of the fatty acids 10-octadecenoic, 14-hydroxy-10-octadecenoic, and 13-hydroxy-9-octadenoic acids and an unidentified fatty acid; and of the resin acids levopimaric + palustric, eperuic, and anticopalic acids, and three

anticopalic acid isomers; together with the different relative percentages of sesquiterpene (*Z*,*E*)-farnesyl propionate; of the neutral diterpenes 8,13-abietadiene, 8(14),12-abietadiene, 8,15-pimaradien-18-al, methyl abietate, abietal + methyl palustrate + methyl levopimarate, 7,13-abietadiene, and methyl dehydroabietate, of the fatty acids pentadecanoic and palmitic acids, and of the resin acids pimaric, sandaracopimaric, abietic, dehydroabietic, imbricataloic, and neoabietic acids were the criteria for characterization of samples of these two chemotypes.

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