CHEMOMETRICS IN CHEMOTAXONOMY AND FOREST DECLINE MONITORING

II. CHEMOSYSTEMATIC STUDIES OF *PINUS SILVESTRIS* L. AND *PINUS NIGRA* ARNOLD SPECIES

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The multivariate data of *Pinus silvestris* and *Pinus nigra* terpenic GC/MS profiles were treated by the exploratory data analysis. The computer approach shows the separation of the species by means of essential oil compositions; the main distinctive feature is content of Camphene, α-Pinen, Δ-3-Karen, Spathulenol, several sesquiterpenes, Dodecanoid acid ethyl ester. It became apparent that the more careful analysis of the terpenoid compounds content is desirable (also after considerable reduction of mumber of variables, in the case of the applied headspace technique) in diagnosis of wood species and for evaluation of their resistance or reaction to the changed environmental conditions.

INTRODUCTION

Although a few Species of *Pinus* genus [1, 2, 3], had been systematically studied particularly by Zavarin et al and several other authors [4, 5, 6, 7, 8], the variation in *Pinus silvestris* and *Pinus nigra* has not been described in details. For the future programme in pine forest decline monitoring it is of interest to point out the risk of the chemotaxonomic approaches by mentioning terpenes and terpenoids [6].

Turpentine composition has proved its special value in the study of hybridization between *Pinus contorta* Dougl. and *Pinus banksiana* Lamb. *Pinus banksiana* consists mostly of α- and β-Pinen and of *Pinus contorta* is mostly β-Phellandrene plus small but consistent amounts of 3-Karen and the two pinenes [13]. Association of turpentine constituents (percent) of α-Pinen and β-Phellandrene for *Pinus banksiana* and *Pinus contorta* hybrids presented in the diagram [3] represents the first „chemometric approach" in such

discussion and this is a remarkable progress compared with previously published papers, including those beginning from 1929 [1]. For the pure populations of *Pinus* species a number of terpenes percentages could be used for identification. A definite intermediacy, rather limited in extent, could be demonstrated for the mixed stand, however [8]. In the related type of study [7] the 95 trees of *P. concorta* have been analyzed and compared. The effects of two localization have been discussed.

The composition of essential oils can be very variable even in one tree. The composition of shoots from various parts of one-year-old shoots, particularly various parts of the crown of (40 years old) *P. silvestris* was studied [8].

The terpenic profiles for 8 major monoterpenes were determined on 10 grafted clones of *P. silvestris* grown in the three different sites in Sweden. A strong, broad-sense heritability was determined for 3-Karen, Myrcene, Limonen and β-Phellandrene. Both sites and clones have a small effect on the overall phenotypic variation. Pinenes and sabinenes seem to be much more dependent on environmental factors [2]. The terpenes profile can be completed for taxonomic evaluation by amounts of isopimaric and further resin acids [4]. In a population of Scots Pine, the two flavonoid chemomorphs coexist [6]. One is rich in prodelphinidin and lacks taxifolin ($=T^-$ morph); the other exhibits more o-dihydroxylated flavonoids, including quercetin and taxifolin ($=T^+$ morph). The latter situation occurs only at low elevations, whereas the high altitude or latitude populations are almost entirely made up of the T^- morph.

The solution of such chemosystematic problems is usually complicated and requires the treatment of large data sets. Therefore an empirical, inductive, chemometric approach is promising for investigating and detecting such a relationship in dedicated problems [10]. A set of species (characterized by properties) – relevant for a defined problem – is the basis for generating multivariate data. The multivariate space is mapped onto a suitable plane. Depending on the problem, different mapping methods (principal component projection, discriminant component plot, PLS) can be used to visualize the data [11, 12]. The human ability for recognizing clusters in scatter plots is advantageoulsy used for the interpretation.

The aim of the paper is to present the multivariate data of essential oils of *Pinus silvestris* L. and *Pinus nigra* Arnold. by the exploratory data analysis (principal component analysis – PCA), and to show the discrimination of both species by easy-to-use computer technique. *P. silvestris* and *P. nigra* have been selected for our study for several reasons:

– the are the most common *Pinus* species in central Europe;

– although the hybridization of both species is seldom observed in mixed forest, their intentional breeding enables one to gain the well-growing and resistant hybrid,

– monitoring and modelling of forest decline for both species is in programme.

MATERIAL AND METHODS

The samples of needles were taken from 2-5 year old trees from several localities in Bratislava and Arboretum Mlyňany (Tab. 1). Each tree was evaluated according to the international biomonitoring scale of trees from the aspect of morphological sings of their damage (degrees of the damage needles and thinning of crowns). The degrees of damage are shown according to Abrahamsen [1].

The quantitative terpenoid compounds content is considerably variable,

Table 1

List of the *Pinus* samples from various localities
Zestawienie próbek rodzaju *Pinus* pochodzących z różnych stanowisk

Sample Próbka	Name of tree Nazwa drzewa	Locality Stanowisko	Evaluation of the state of health		
			Needle [a] Tree [b] N° of Age [c] Ocena stanu zdrowotnego		
			Igły [a]	Drzewo [b]	Wick [c]
1	*Pinus nigra*, (1 y.)	Arboretum Mlyňany	0	0	5
2	*Pinus nigra*, (2 y.)	Arboretum Mlyňany	0	0	5
3	*Pinus nigra*, (1 y.)	Bratislava Lamač	0	0	4-5
4	*Pinus nigra*, (2 y.)	Bratislava Petrzalka	0	0	4
5	*Pinus nigra*, (2 y.)	Bratislava Biskupice	0	0	5
6	*Pinus nigra*, (2 y.)	Bratislava Roznavska	0	0	3-4
7	*Pinus nigra*, (2 y.)	Bratislava Koliba	0	0	4-5
8	*Pinus nigra*, (2 y.)	Bratislava Fr. Zupku	0	0	3-4
9	*Pinus nigra*, (2 y.)	Bratislava PRIOR	0	1	3-4
10	*Pinus silv.*, (1 y.)	Arboretum Mlyňany	0	0	3
11	*Pinus silv.*, (2 y.)	Arboretum Mlyňany	0	0	3
12	*Pinus silv.*, (2 y.)	Bratislava Lamač	1	0	3
13	*Pinus silv.*, (1 y.)	Bratislava Petrzalka	0	1	3
14	*Pinus silv.*, (1 y.)	Bratislava Biskupice	0	0	3-4
15	*Pinus silv.*, (1 y.)	Bratislava Roznavska	1	2	4-5
16	*Pinus silv.*, (1 y.)	Bratislava Fr. Zupku	1	0	3

[a] Needles undamaged up to damaged for 100% (range: from 0 to 4)
   Igły nieuszkodzone do uszkodzonych w 100% (zakres: od 0 do 4)
[b] Tree thick crown up to thinned for 60% (range: from 0 to 3)
   Korona w pełni wykształcona do pocienionej w 60% (zakres: od 0 do 3)
[c] Number of the age of needles growing on the tree
   Wiek igieł rosnących na drzewie

which depends on climatic conditions, locality, time and the place of sampling, as well as on increment sectors. These factors were standardized by the sampling effected in the same daytime period, in the same height and, as far as it is possible, on the sunny side of the crown. The influence of increment sectors was eliminated by taking the needles from the last increment sector but one. A choice was made of the uncut trees of about the same age. The material was sampled during the vegetation period (summer) at about 10.00 a.m. and immediately carried into the laboratory for its treatment.

100 g of fresh needles were picked and essential oils were obtained by hydrodestillation and analyzed by GS-MS Hewlett-Packard 5970B mass-spectrometer linked to an HP 5890A gas chromatograph equipped with an HP ULTRA-1 capillary column (25 m × 0.2 mm I.D., 0.33 μm film thickness).

The injection 0.5 μl of sample was made in the splitless mode using the on-line dried helium as a carrier gas at the rate of 25 ml/min. The oven temperature was maintained at 40°C for 1 min and programmed at 7.5°C/min to 260°C and maintained for 10 min. The injector and transfer line temperatures were set at 270°C and 275°C, respectively. The mass spectrometer was operated in the SCAN mode, mass scan range of 33-300 amu, electron energy of 70 eV.

The identification of mixture components was performed automatically, by a PBM (Probability Based Matching) library search system, using the Wiley mass spectral library.

A software package EXCERPT [14] for exploratory data analysis was used for chemometric interpreting the investigated data.

RESULTS AND DISCUSSION

The indentification of the mixture components performed by the computer library search system was successful in most cases; the Wiley mass spectral library is rich for terpenoid compounds, Tab. 2. The reliability of the PBM library search (correct identification on the first or second position in the hitlist) was about 85%.

Fig. 1 illustrates the typical chromatograms of terpenoid compounds for *P. nigra* and *P. silvestris* species. The evaluation of such rich mixtures is too elaborate and the comparison of plenty of chromatograms would be much more demanding (9 *P. nigra* and 7 *P. silvestris* species, Tab. 1) especially when some of them were apparently quite different from other ones. In this work the chemometric approach was used for evaluating the data by a multivariate statistical analysis. The relative amounts of terpenoids (normalized to the maximal chromatographic peak in the samples) serve as features for the exploratory data analysis. T-test of the data differences between *P. nigra* and *P. silvestris* and species at a 5% significance level gave several significant results (Tricyclene, Camphene, γ-Terpinene, etc.). The most intensive 23 peak of mono- and di-terpenoid compounds were selected (Tab. 2). The data points (species) scattered in this multidimensional space were projected onto a plane which was spanned by the two principal components with the highest amount of information, thus visualizing the grouping of similar species.

The principal component analysis plot shows an evident separation of *P. nigra* and *P. silvestris* species, Fig. 2a, b despite different origin of the samples. The most relevant features (compounds) for the data structure presented results from a loading-loading plot of the first two principal components, Fig. 3: sesquiterpene C15H24 (15), Dodecanoid acid, ethyl ester (18); 1-Limonene (6), α-Pinene (1), β-Myrcene (4); β-Pinen (2), Δ-3-Karen (5); Spathulenol (19), sesquiterpenes C15H24 (20), (21), (22); etc. The position of the feature on the plot in Fig. 3 reflects the influence of the feature to positions of the objects in Fig. 2. This relationship is useful to find out which properties are

Table 2

Composition of the hydrocarbon fractions of the essential oil of *Pinus nigra* Arnold and *Pinus silvestris* L. species

Skład frakcji węglowodorowych olejków eterycznych gatunków *Pinus nigra* Arnold and *Pinus silvestris* L.

N° Nr	RT Czas retencji (min)	Feature Cecha	Compound Związek	PBM [1] Qual %	Compound present Występowanie składnika	
					Pinus silvestris	Pinus nigra
1	8.55	•	Tricyclene	95	+	+
2	9.03	1•	α-Pinen	93	+	+
3	9.24	2•	Camphene	98	+	+
4	9.65	•	Sabinene	93	+	+
5	9.79	3•	β-Pinen	96	+	+
6	10.00	4•	β-Myrcene	95	+	+
7	10.11		3-Hexen-1-ol, actate, (Z)	64	+	+
8	10.32		1-Phellandrene	43	+	−
9	10.36	•	α-Terpinene	97	−	+
10	10.64	5•	Δ-3-Karen	97	+	−
11	10.93	6•	1-Limonene	81	+	+
12	11.25	•	1, 3, 6-Octatriene, 3, 7-dime	98	+	+
13	11.53	•	γ-Terpinene	96	+	+
14	12.22	7•	α-Terpinolene	97	+	+
15	13.24		trans-Pinocarveol	42	+	
16	13.81	•	endo-Borneol	90	+	+
17	14.06		3-Cyclohexen-1-ol, 4-methy	97	+	+
18	14.29	8	1-α-Terpineol	91	+	+
19	14.43		Myrtenol	91	+	+
20	15.55		2-Undecanone	90	+	
21	15.93		2-Octenyl acetate	59	+	−
22	16.29	9	Endobornyl acetate	96	+	+
23	16.54		2-Nonanol	17	+	+
24	16.73	10	Myrtenyl acetate	50	−	+
25	16.87		Bornylene	83	−	+
26	17.48	11	1-P-Menthen-8-yl acetate	83	+	+
27	17.82		α-Cubebene	99	+	+
28	18.38	12	α-Copaene	99	+	+
29	18.60	13	Elemene or Caralene	58	+	+
30	19.37	14	trans-Caryophyllene	96	+	+
31	19.60		Naphthalene, 1, 2, 3, 4, 4a, 5,	94	+	+
32	19.87		α-Humulene	96	+	+
33	19.96		Epi-Bicyclosesquiphellandr	95	+	+
34	20.26		sesquiterpene C15H24	−	+	?
35	20.39	15	sesquiterpene C15H24	−	+	?
36	20.51		sesquiterpene C15H24	−	+	?
37	20.67		α-Muurolene	95	+	+
38	20.92	16	γ-Cadinene	98	+	+
39	21.24	17	Δ-Cadinene	97	+	+
40	21.39		sesquiterpene C15H24	−	+	+
41	21.72		Dodecanoic acid	95	+	+
42	21.46	18	Dodecanoic acid, ethyl est.	95	−	+
43	21.92	19	Spathulenol	86	+	−
44	22.20		Veridiflorol	64	+	−
45	22.50	20	sesquiterpene C15H24	95	+	−
46	22.72	21	sesquiterpene C15H24	59	+	+
47	23.09	22	sesquiterpene C15H24	−	+	+
48	23.35	23	sesquiterpene C15H24	−	+	+

• Compounds measured by the headspace analysis

Związki oznaczone specjalną techniką chromatografii gazowej (ang.: headspace technique)

[1] PBM (Probability Based Matching) similarity coefficient

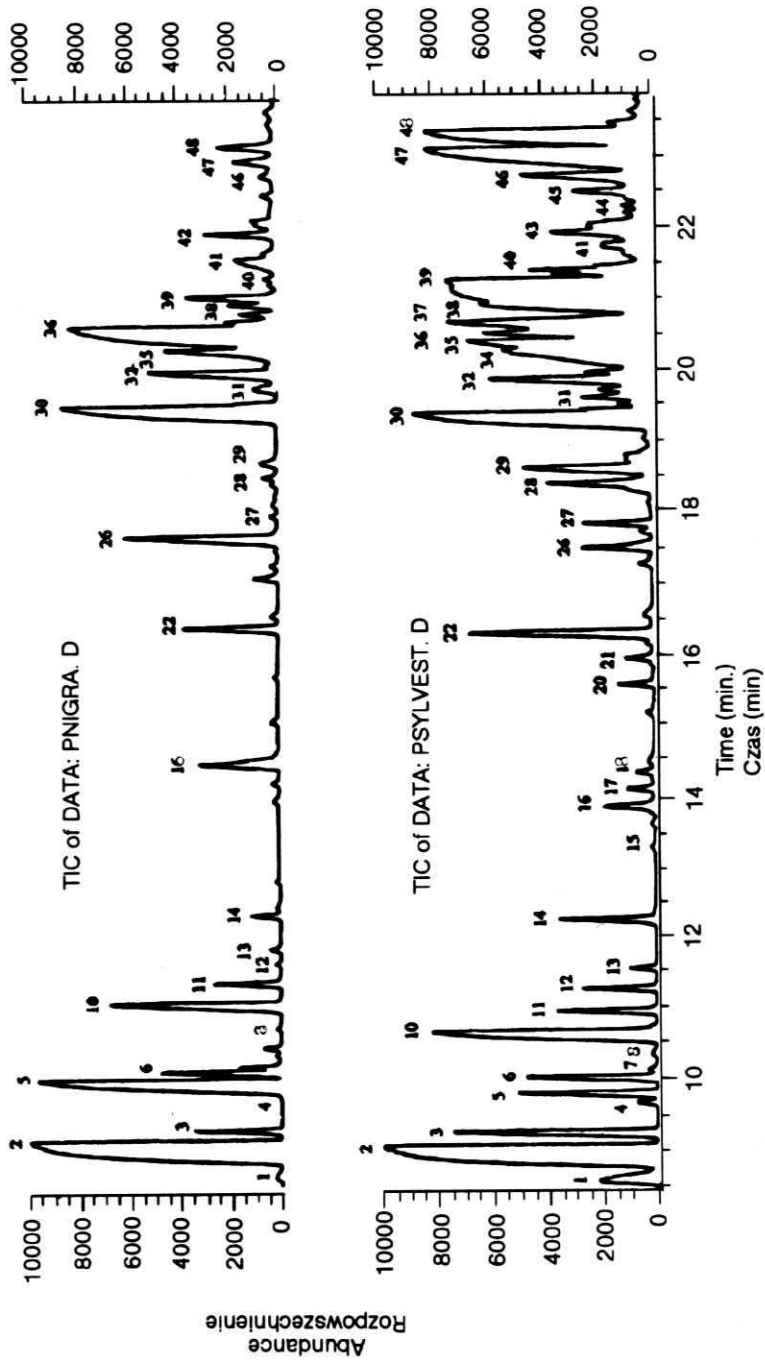System indentyfikacji komputerowej, prawdopodobieństwa %

Fig. 1. Gas chromatograms of the hydrocarbon fractions of the essential oil of *Pinus nigra* Arnold. and *Pinus silvestris* L. The numbers of mixture components refer to Table 2.

Rys. 1. Chromatogramy gazowe frakcji węglowodorowych olejków eterycznych *Pinus nigra* Arnold. i *Pinus silvestris* L. Numery składników mieszanin według tabeli 2
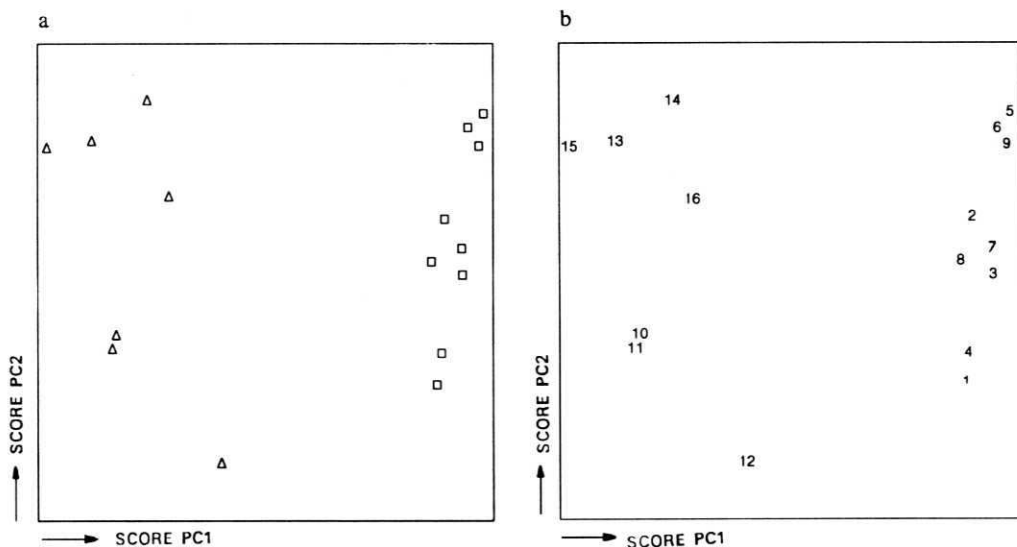
Fig. 2. Principal component analysis of the data of 9 *Pinus nigra* and 7 *Pinus silvestris* species showing the separation of the species. Features: 23 mono- and di-terpenoid compounds (see Tab. 2). Axes: the first and second principal component, 52% and 20% of total variance, respectively. a – species marked by symbols: □ – *Pinus nigra* Arnold., △ – *Pinus silvestris* L. b – species marked by numbers (see Tab. 1)

Rys. 2. Analiza głównych składników 9 drzew *Pinus nigra* i 7 *Pinus silvestris* ukazująca rozróżnienie gatunków. Cechy: 23 związki mono- i diterpenowe (patrz tab. 2). Osie: pierwszy i drugi główny składnik, 52% i 20% wariancji całkowitej, odpowiednio. a – oznaczenie gatunków: □ – *Pinus nigra* Arnold., △ – *Pinus silvestris* L., b – próbki oznaczone numerami (patrz Tab. 1)

responsible for a separation of the classes of species. Several of the properties are highly correlated Δ-Cadinene (17), sesquiterpenes C15H24 (22), (23), Spathulenol (19), sesquiterpene C15H24 (21); Camphene (2), α-Terpinolene (7), etc. (they are closed together in the plot). From Fig. 2 and Fig. 3 it is also evident that species are remarkably extended (due to the features: β-Myrcene (4), α-Pinen (1), 1-Limonen (6) etc.).

To confirm the separation of the samples by PCA, shown in Fig. 2, the cluster analysis was used. Fig. 4 shows the dendrogram resulting from the hierarchical cluster analysis by agglomerative method.

Fig. 5 shows the PCA plot of data formed by only several compounds, which can be measured by a headspace technique (13 compounds, marked by asterix, up to the retention time 13.81 min, see Tab. 2). Again, we can see a separation of *P. nigra* and *P. silvestris* species. Sample 12 (very low chromatographic signal and therefore compounds No. 1-16 were not detectecd) was omitted in this case. The loading-loading plot of the first two principal components shows, Fig. 6, that the most important
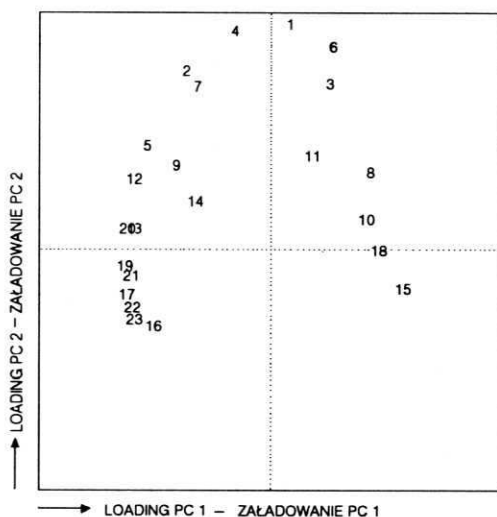
Fig. 3. Loading-loading plot of the first two principal components of the 9 *Pinus nigra* and 7 *Pinus silvestris* species. Numbers correspond to features. See also Fig. 2

Rys. 3. Diagram rozproszenia pierwszych dwóch głównych składników 9 drzew *Pinus nigra* i 7 drzew *Pinus silvestris*. Liczby odpowiadają cechom. Patrz również rys. 2
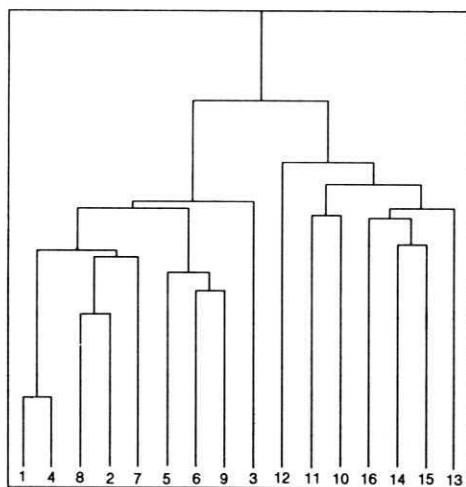

Fig. 4. Minimal spanning tree resulting from the hierarchial cluster analysis corresponding to the data shown in Fig. 2. The height is an indication of the similarity of the samples numbered as in Tab. 1

Rys. 4. Dendrogram przedstawiający wyniki analizy zbioru usystematyzowanego metodą aglomeracyjną odpowiadający danym zawartym na rys. 2. Wysokość jest miarą podobieństwa próbe ponumerowanych według tabeli 1
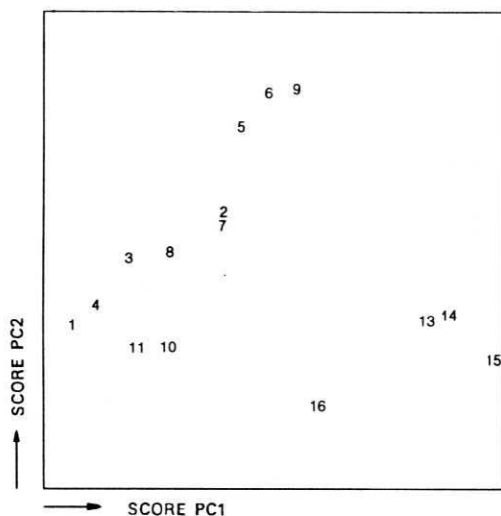
Fig. 5. Principal component analysis of the data of 9 *Pinus nigra* and 7 *Pinus silvestris* species showing the separation of the species by means of compounds, which can be measured by a headspace technique. Features: the 13 compounds till retention time 13.81 min (see Tab. 2). Axes: the first and second principal component 52% and 25% of total variance, respectively. Species marked by numbers (see Tab. 1)

Rys. 5. Analiza głównych składników 9 drzew *Pinus nigra* i 7 drzew *Pinus silvestris* ukazująca rozróżnienie gatunków na podstawie związków chemicznych, które można oznaczyć specjalną techniką chromatografii gazowej. Cechy: pierwsze 13 związków do czasu retencji 13,81 min (patrz tab. 2). Osie: pierwszy i drugi składnik główny, 52% i 25% całkowitej wariancji, odpowiednio. Próbki oznaczone numerami (patrz tab. 1)

features for separation of these 2 species in this case are: α-Terpinen (7), β-Pinen (5), Δ-3-Karen (8), Sabinene (4), Tricyclene (1), Camphene (3) and γ-Terpinen (11). Thus the results of this simple and fast computer technique could serve for the successful chemosystematic study.

It is obvious, that the separation of classes (as shown in Fig. 2 and Fig. 5) is possible and therefore the classification of unknown species (by PCA, DCA or pattern recognition methods) could be performed.

The results achieved in the sphere of the study of volatile products under investigation have contributed to a closer knowledge of the occurrence and composition of ethereal oils in softwood species and have enabled to compare their differences. The main distinctive features are the content of Camphene, β-Pinen, Δ-3-Karen, Spathulenol, several sesquiterpenes, Dodecanoid acid ethyl ester.

The computer processing of GC/MS data (particularly library searching) offers the possibility to obtain relatively fast a great amount of data which can be interpreted by multivariate statistical analysis methods. A more detailed analysis of the terpenoid compounds content has shown that it can serve (even
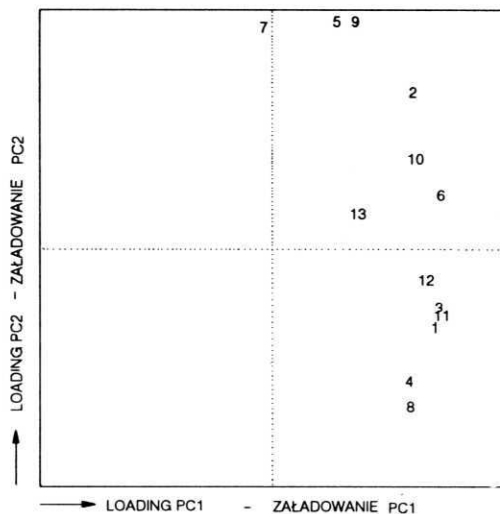
Fig. 6. Loading-loading plot of the first two principal components of 9 *Pinus nigra* and 7 *Pinus silvestris* species. Numbers correspond to 13 selected compounds by asterisk in Tab. 2. See also Fig. 5

Rys. 6. Diagram rozproszenia pierwszych dwóch podstawowych składników 9 drzew *Pinus nigra* i 7 drzew *Pinus silvestris*. Numery odpowiadają 13 wybranym związkom oznaczonym gwiazdką w tabeli 2. Patrz również rys. 5

after the remarkable reduction of the number of variables or when the headspace technique is necessary) in the diagnosis of species. The next work will lead to determining the damage degree of species.

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CHEMOMETRIA W CHEMOTAKSONOMII I MONITOROWANIU ZAMIERANIA LASÓW

II. STUDIA CHEMOSYSTEMATYCZNE GATUNKU *PINUS SILVESTRIS* L. I *PINUS NIGRA* ARNOLD

Streszczenie

Wielozmienne dane dla sosny zwyczajnej (*Pinus silvestris* L.) i sosny czarnej (*Pinus nigra* Arnold.) dotyczące GC/MS profili terpenowych zostały poddane analizie wstępnej. Podejście do zagadnienia z punktu widzenia techniki komputerowej wskazuje na możliwość rozróżnienia gatunków na podstawie składu olejków eterycznych. Najbardziej wyraźną cechę stanowią zawartości kamfenu, α-pinenu, δ-3-karenu, spatulenolu, różnych seskwiterpenów, estru etylowego kwasu dodekainowego. Jest oczywiste, że bardziej staranna analiza zawartości związków terpenowych jest wskazana, również po znacznej redukcji liczby zmiennych w przypadku zastosowania techniki w diagnozie gatunku drewna i dla oceny jego odporności lub reakcji na zmienione warunki środowiska.

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