A Comparative Study of *Juniperus communis* and *Juniperus virginiana* Extracts

The Influence of method, solvent, and provenience

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The paper presents a comparative study of the Juniperus communis and Juniperus virginiana hydrophobic extracts from the method, solvent, and source influence point of view. Hydrophobic solvents (such as hexane and ethyl acetate) were used for obtaining of J. communis and J. virginiana extracts. Two extraction methods (solvent reflux and sonication) were used for obtaining hydrophobic extracts by using different plant parts (branches, needles, and berries) collected from autochthonous area and other zones from Austria and Syria. Fifteen main compounds were identified and quantified in all hydrophobic extracts by using gas chromatography-mass spectrometry analysis (such as α- and β-pinene, β-phellandrene, caryophyllene, and β-cubebene). The highest amount of β-pinene is identified in the Syrian J. communis branches hexane extract, while β-phellandrene is identified in higher content in the J. virginiana ethyl acetate extract from "Macea" Botanical Garden (Arad, Romania). Principal component analysis of the gas chromatographic data (relative concentration of the main volatile compounds) revealed that the Juniperus species can be classified according to the mono- and sesquiterpene concentrations (limonene, α-pinene, humulene, caryophyllene, cubebene); the provenience of these samples can also be classified by this procedure, but the biologically active compounds concentrations had no significance on the classification according to the extraction method and solvent type.

Keywords: Juniperus communis, Juniperus virginiana, sonication extraction, refluxing extraction, principal component analysis

The genus *Juniperus* (Cupressaceae) contains more than 60 species, and is distributed throughout the forests of the temperate and cold region of the Northern Hemisphere [1-3]; it grows wild in many parts of the world. The genus is divided into three sections: Caryocedrus, Juniperus, and Sabina. Most *Juniperus* species are aromatic and furnish volatile oils with important commercial value. Moreover, plants from this section are tolerant for cold temperature, diseases, and environmental pollution, making them adaptable to a lot of soils and climates [4].

Some *Juniperus* species are present in the Romanian flora: *J. communis* L., *J. sibirica* L. and *J. virginiana*. *J. communis* L., common juniper, is an evergreen shrub and grows in the Apuseni Mountains and Banat's upper hills [5-7].

Juniper contains essential oils, predominantly monoterpenoid hydrocarbons (approximately 70 to 90%) [4-7]. The main components are α-pinene, β-pinene, β-phellandrene, caryophyllene and β-cubebene. According to the literature, analytical data on volatile compounds from *Juniperus communis* needles [8-9], cones (berries) [2,9-10], and branches [9] obtained by hydrodistillation, and by extraction with *n*-pentane [4] have been reported; sonication extraction was used for the isolation of volatile compounds [11] from *J. communis* and *J. virginiana* at room temperature with organic solvents.

The extracts and essential oils of the plant are used in the manufacture of alcoholic and nonalcoholic beverages, frozen desserts, baked goods, meat and meat products [8].

This research was conducted in order to determine the most efficient methods for the extraction of volatile compounds in the two *Juniperus* species harvested from various areas and their analysis by GC-MS. The Austrian (A), Romanian (R) – from different sources, and Syrian (S) berries, needles, and branches were used in the extraction process (refluxing and sonication extraction methods) using two different solvents. The above-mentioned extraction methods have never been investigated for *Juniperus*.

Experimental part

Materials and methods

Plant material (black mature berries, needles, and branches) were harvested from *Juniperus communis* wild-type shrubby trees from hilly areas in Romania (Lipova – LIP, Albac – ALB), Austria – AUS (Stubalte, Steiermark county) and Syria – SYR (Slumfe, Latakia county). The

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samples of *Juniperus virginiana*, cultivated type, were collected from the "Macea" Botanical Garden (MAC) in Romania, and the wild type from Syria in October 2009. The solvents used for refluxing and sonication extractions were ethyl acetate from Chimopar (Bucharest) and hexane from Sigma-Aldrich. All filtered extracts were dried on anhydrous sodium sulfate (Fluka Chemie AG). C_8-C_{20} linear alkane standard mixture (Fluka Chemie AG) was used for determination of Kovats indices.

Extraction methods

Refluxing extraction

The plant material was dried and stored at room temperature. Dried berries, needles, and branches (2 g in each case) were chopped in very small pieces, treated with 15 mL solvent (hexane or ethyl acetate), and refluxed for 30 min. After cooling, the extract was filtered, dried over anhydrous Na_2SO_4, and stored at -4°C in glass containers, until the gas chromatography-mass spectrometry (GC-MS) analysis.

Sonication extraction

In order to obtain volatile compounds from the three anatomical parts (berries, needles, and branches) of two *Juniperus* species, 1 g of dried and grounded plant material was placed in a vial with 6 mL solvent (hexane or ethyl acetate). The vial was covered and then placed in sonication water bath (HK2200, 100W, 50 kHz) for 10 min. After sonication, the extract was filtered, dried over anhydrous Na_2SO_4, and stored at -4°C until GC-MS analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The extracts obtained by refluxing and sonication were analyzed by gas chromatography-mass spectrometry in order to identify the main components. A Hewlett Packard HP 6890 Series gas chromatograph coupled with a Hewlett Packard 5973 mass selective detector (GC-MS) system was used (calibration factor 1.0). A HP-5 MS capillary column was used for the GC system. The temperature program was set up from 50 to 250°C with a 6°C/min rate, using He as carrier gas. The relative percentage concentration of the volatile compounds of two species of juniper was computed from the GC peak areas. The identification of the main compounds was performed by using our previous Kovats indices data obtained for standard compounds [6,12] and/or by matching the experimental mass spectra with those from the NIST/EPA/NIH Mass Spectral Library 2.0.

Principal Component Analysis (PCA)

The statistical multivariate analysis of the GC data was achieved using the PCA analysis of the relative concentration of the main biocompounds identified in the *Juniperus* extracts. This procedure was used in order to identify the importance of some bioactive compounds on the grouping of samples (according to species, plant part and source, as well as extraction method and solvent). We have used an *in house* program with centered data and cross-validation method for validation. Principal component analysis is the basis of the multivariate analysis of the data and presumes an approximation of the data matrix as a product of two reduced matrices, the "object shape" and the "variable shape". The first principal component, PC_1, in the properties space has the maximum variance, the second direction, PC_2, is perpendicular to PC_1, and has the same particularities, and so on. Representation of these PCs characteristics can conduct to information about similarities and possible grouping of the studied objects or properties and the importance of these properties for the model [13,14].

Results and discussion

Composition of Juniperus extracts

The amount of the extracted compounds is expressed as a percentage of the obtained peak area, compared with total area of all peaks (tables 1-3). Of the large number of compounds found in all extracts (over 100) only the most important ones (15) were selected. It should be noted that in all extracts there is a considerable number of monocyclic (limonene, terpinolene and β-phellandrene) and bicyclic (α-pinene, β-pinene, 3-carene, camphene and verbenone)

Table 1
THE MAIN COMPONENTS OBTAINED BY REFLUXING AND SONICATION EXTRACTION FROM *J. COMMUNIS* AND *J. VIRGINIANA* BRANCHES (% OF TOTAL AREA)

No.	Compounds	RI	*J. comm.*			*J. virg.*		*J. comm.*			*J. virg.*		*J. comm.*				*J. comm.*			*J.virg.*
			HEXANE refluxing extracts					ETHYL ACETATE refluxing extracts					HEXANE ultrasound extracts				ETHYL ACETATE ultrasonic extracts			
			SYR	LIP	AUS	SYR	MAC	SYR	LIP	AUS	SYR	MAC	ALB	LIP	AUS	MAC	ALB	LIP	AUS	MAC
1.	α-Pinene	935	55.98	21.75	16.02	18.9	21.64	38.72	34.96	51.62	28.52	14.36	8.98	34.81	19.4	32.7	9.39	27.99	23.35	25.64
2.	Camphene	957	0.35	0.06	0.1	0.15	0.12	0.27	0.42	0.3	-	-	0.35	0.29	-	0.19	-	0.23	-	-
3.	β-Phellandrene	977	1.18	19.54	1.16	3.98	12.46	0.17	32.07	0.44	2.69	10.39	25.4	23.29	15.5	6.7	26.77	17.52	0.09	9.32
4.	β-Pinene	986	3.68	2.33	1.29	0.79	0.8	1.55	1.74	1.22	1.09	0.56	1.5	1.34	0.6	0.6	1.17	1.63	0.91	0.59
5.	Ocimene	995	0.07	-	0.09	-	-	0.06	0.18	0.04	0.19	0.12	-	0.38	-	-	0.07	0.06	-	-
6.	3-Carene	1004	0.7	0.3	0.06	4.71	2.22	0.7	-	-	0.25	1.24	0.29	0.21	1.5	-	0.3	0.18	1.79	2.23
7.	Limonene	1022	0.8	3.59	13.31	0.78	0.94	2.17	-	11.75	-	0.66	2.7	4.22	1.7	0.6	2.56	3.07	1.88	0.57
8.	Terpinolene	1075	1.32	0.72	-	0.17	0.34	0.45	-	-	-	0.25	0.3	-	-	0.3	-	0.19	0.16	0.26
9.	cis-Verbenol	1115	0.41	0.65	-	0.38	0.06	0.14	2.16	0.33	0.42	-	0.2	0.58	0.2	-	0.24	1.4	1.01	0.06
10.	cis-Carveal	1203	0.23	-	0.08	-	-	0.03	0.27	0.14	0.38	-	-	-	-	-	-	-	-	-
11.	Verbenone	1218	0.17	0.23	0.13	-	-	0.14	0.82	0.18	-	-	0.1	0.34	0.2	-	0.33	0.4	0.28	-
12.	Copaene	1263	1.53	-	2.27	0.87	-	0.4	0.36	1.02	0.91	-	0.4	-	-	-	-	-	0.22	-
13.	Caryophyllene	1317	0.82	0.75	0.73	0.04	0.06	0.72	0.5	0.62	1.63	3.1	1.5	0.17	0.8	2.8	1.06	0.23	0.8	3.15
14.	α-Humulene	1333	0.44	0.54	0.64	1.36	0.43	0.36	0.17	0.37	1.23	0.35	1.2	0.14	0.8	0.3	0.73	0.25	0.71	0.33
15.	δ-Cubebene	1352	0.84	1.63	4.15	3.48	-	0.84	-	1.63	3.3	-	2.8	0.93	0.9	-	1.22	1.39	0.57	-

Table 2

THE MAIN COMPONENTS OBTAINED BY REFLUXING AND SONICATION EXTRACTION FROM *J. COMMUNIS* AND *J. VIRGINIANA* NEEDLES (% OF TOTAL AREA)

No.	Components	KI	*J. communis*						*J. virginiana*				*J. communis*						*J. virginiana*		
			HEXANE refluxing extracts			ETHYL ACETATE refluxing extracts			HEXANE refluxing extracts		EA refluxing extracts		HEXANE ultrasound extracts			ETHYL ACETATE ultrasound extracts			HEXANE ultrasound extracts		EA ultrasound extracts
			SYR	LIP	AUS	SYR	LIP	AUS	SYR	MAC	SYR	MAC	SYR	LIP	AUS	SYR	LIP	AUS	SYR	MAC	SYR
1.	α-Pinene	936	6.29	34.17	20.55	17.37	13.81	29.57	13.63	1.4	7.56	0.6	23.47	35.45	27	20.8	36.36	28.5	25.91	0.39	17.66
2.	Camphene	957	-	0.17	0.11	-	0.08	0.18	0.1	-	0.12	-	0.14	0.18	0.15	-	-	-	2.00	-	0.90
3.	β-Phellandrene	978	0.5	1.15	1.29	0.53	4.28	2.22	0.2	14.25	0.47	7.98	0.5	12.49	6.47	0.3	13.33	5.9	2.81	6.8	3.08
4.	β-Pinene	986	0.52	1.08	0.89	0.79	0.47	1.4	5.67	0.3	0.05	0.22	1.1	1.25	1.12	0.9	1.4	1.2	0.18	0.32	-
5.	Ocimene	1014	0.04	-	0.03	0.08	-	-	0.04	-	0.8	0.05	-	-	0.04	-	-	-	0.32	-	7.13
6.	3-Carene	1052	-	0.25	2.55	-	-	3.39	0.03	0.5	-	-	-	-	0.11	-	0.32	0.2	-	0.03	0.18
7.	Limonene	1023	9.44	2.5	1.82	18.79	2.08	1.65	3.06	1.09	0.19	-	19.8	1.8	1.43	18.8	1.92	1.7	0.74	0.26	0.79
8.	Terpinolene	1075	-	0.21	0.2	-	0.22	0.1	0.47	0.17	0.05	0.52	-	0.33	0.3	-	0.34	0.2	2.93	0.14	6.86
9.	cis-Verbenol	1143	-	0.25	0.11	-	-	0.07	0.06	0.02	0.11	-	-	0.37	0.36	-	0.35	0.3	8.31	-	3.16
10.	cis-Carveol	1169	-	0.02	0.07	0.11	-	0.04	-	-	0.49	-	-	-	0.24	-	-	-	4.39	-	4.53
11.	Verbenone	1219	-	0.15	0.03	-	-	0.03	0.05	-	0.71	-	-	0.29	0.09	0.17	0.35	0.2	2.17	-	1.48
12.	Copaene	1264	2.8	-	0.5	1.5	0.04	0.49	0.31	-	3.7	-	1.9	-	0.46	1.79	-	0.6	-	-	0.73
13.	Caryophyllene	1306	-	0.67	1.63	1.44	0.39	1.51	2.53	1.7	2.34	1.23	-	0.3	1.31	1.2	0.29	1.3	0.35	1.53	-
14.	α-Humulene	1334	2.24	0.53	0.03	1.09	0.25	1.27	1.85	0.18	3.45	0.13	3.3	0.5	1.34	1.1	0.34	1.3	1.03	0.16	0.94
15.	β-Cubebene	1353	16.77	3.51	6.77	0.32	2.14	2.78	6.29	-	-	-	6.9	2.57	4.65	5.97	2.66	4.5	0.32	-	0.40

Table 3

THE MAIN COMPONENTS OBTAINED BY REFLUXING AND SONICATION EXTRACTION FROM *J. COMMUNIS* AND *J. VIRGINIANA* BERRIES (% OF TOTAL AREA)

No.	Components	KI	*J.com.*	*J. virg.*	*J.com.*	*J. virg.*	*J.com.*	*J. virg.*	*J.com.*	*J. virg.*
			HEXANE refluxing extracts		ETHYL ACETATE refluxing extracts		HEXANE ultrasound extracts		ETHYL ACETATE ultrasound extracts	
			ALB	MAC	ALB	MAC	ALB	MAC	ALB	MAC
1.	α-Pinene	937	12.77	2.68	19.73	2.57	6.86	1.60	11.95	-
2.	Camphene	957	0.16	0.05	0.17	-	-	-	0.46	-
3.	β-Phellandrene	979	8.06	35.53	10.14	**46.64**	3.57	0.22	2.52	0.17
4.	β-Pinene	984	1.44	3.95	1.53	0.2	9.02	2.67	17.62	**1.82**
5.	Ocimene	1030	-	-	0.04	0.05	0.08	0.04	0.07	0.06
6.	3-Carene	1005	0.66	0.02	0.61	0.05	0.17	-	0.26	-
7.	Limonene	1023	4.54	1.29	4.75	1.21	0.89	0.81	1.86	0.59
8.	Terpinolene	1075	0.82	0.5	0.68	0.45	0.49	0.23	0.46	-
9.	cis-Verbenol	1143	0.04	-	-	-	0.07	-	-	-
10.	cis-Carveol	1168	0.12	-	-	-	0.04	-	0.02	0.04
11.	Verbenone	1218	0.03	-	0.03	-	-	-	0.02	-
12.	Copaene	1264	0.3	0.06	0.25	-	0.05	-	-	-
13.	Caryophyllene	1306	2.27	2.09	1.83	1.92	**4.82**	2.02	4.14	1.76
14.	α-Humulene	1334	1.95	0.2	1.59	0.14	7.36	7.04	4.93	5.67
15.	β-Cubebene	1354	8.94	2.11	8.22	1.75	**15.21**	1.89	10.46	2.04

monoterpenoids, bicyclic sesquiterpenoids (caryophyllene) and monocyclic sesquiterpenoids (α-humulene).

The GC-MS analyses showed that β-pinene is present in all extracts obtained by refluxing and sonication extraction (tables 1-3). α-Pinene is present in the highest amount (56%) in the Syrian *J. communis* branches hexane extract (table 1, fig. 1). The exception was the *J. virginiana* "Macea" Botanical Garden berries ethyl acetate sonication extract, where only β-pinene (1.8%, table 3) is present.

Two extracts exhibit β-phellandrene as the major component (fig. 2): 46.6% in the *J. virginiana* "Macea" Botanical Garden berries refluxing ethyl acetate extract (table 3) and 26.8% in the *J. communis* Albac branches sonication ethyl acetate extract (table 1). The β-phellandrene content was higher than that reported elsewhere for extracts obtained by hydrodistillation.

Sonication extracts contain higher amounts of limonene (fig. 3), 19.8% in hexane, than the refluxing extracts, 18.8%

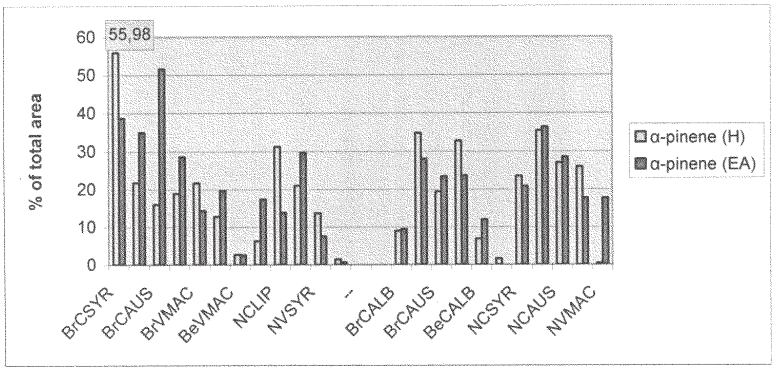

Fig. 1. α-Pinene in *J. communis* (C) and *J. virginiana* (V) extracts obtained by refluxing (left) and sonication (right) extraction (Br-branches, Be-berries, N-needles, H-hexane, EA-ethyl acetate)

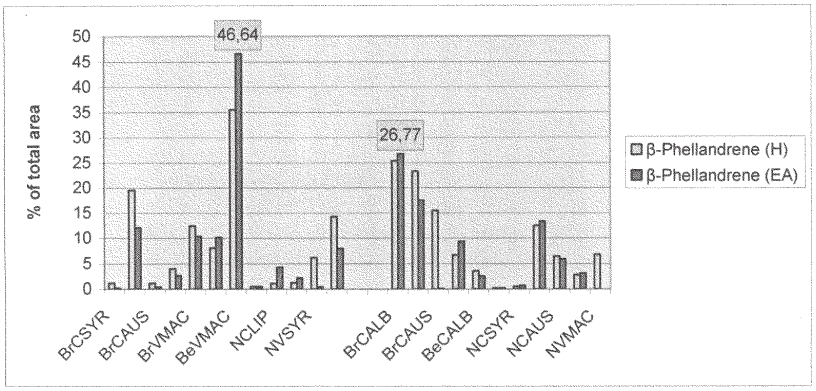

Fig. 2. β-Phellandrene in *J. communis* (C) and *J. virginiana* (V) extracts obtained by refluxing (left) and sonication (right) extraction (Br-branches, Be-berries, N-needles, H-hexane, EA-ethyl acetate)

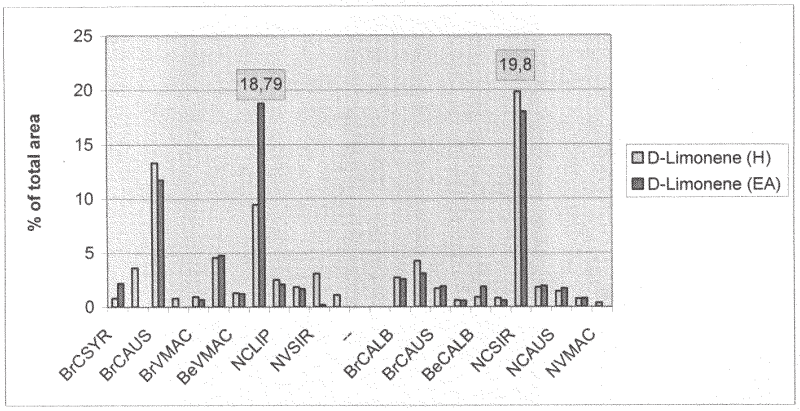

Fig. 3. Limonene in *J. communis* (C) and *J. virginiana* (V) extracts obtained by refluxing (left) and sonication (right) extraction (Br-branches, Be-berries, N-needles, H-hexane, EA-ethyl acetate)

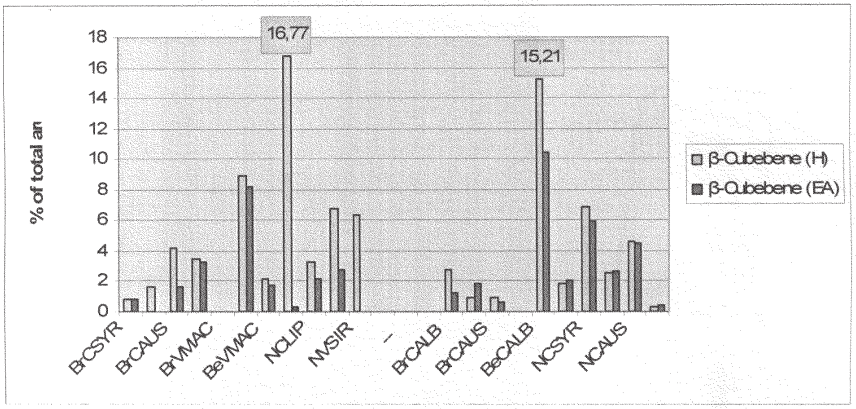

Fig. 4. β-Cubebene in *J. communis* (C) and *J. virginiana* (V) extracts obtained by refluxing (left) and sonication (right) extraction (Br-branches, Be-berries, N-needles, H-hexane, EA-ethyl acetate)

(table 2), in the case of Syrian *J. communis* needles ethyl acetate extracts.

The highest content of caryophyllene is found in the *J. communis* Albac sonication hexane extract (4.8%) (table 3). β-Cubebene (fig. 4) is found in highest amounts in the Syrian *J. communis* needles hexane extract, 16.8% (table 2), for the refluxing extraction, and in the *J. communis* Albac berries hexane extract, 15.2% (Table 3), for the sonication extraction method.

Principal component analysis

Statistical multivariate analysis (PCA) revealed that the relative concentrations of volatile compounds extracted with hydrophobic solvents are important for sample classifications. Thus, PCA analysis using all relative concentrations data obtained for the main compounds conduct to the classification of samples according to species, plant part, solvent, and extraction method. The variance of the data is 88% which is explained by the first three principal components (PC_1 52%, PC_2 28%, and PC_3

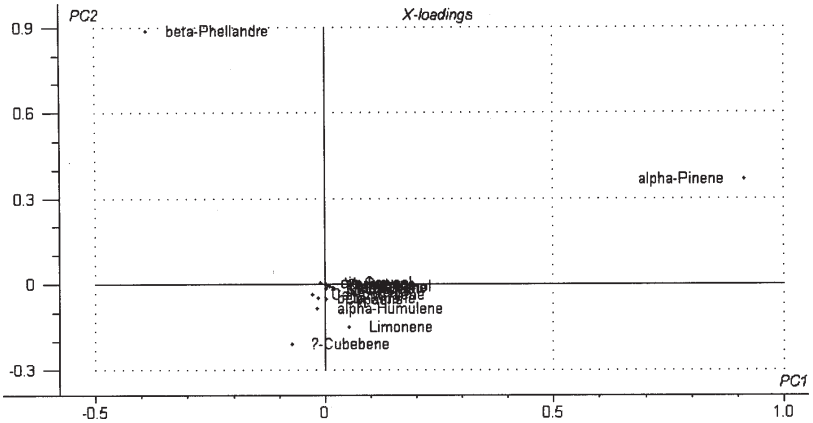

Fig. 5. Loadings plot from the PCA analysis of the GC data of the main compounds from *Juniperus* extracts

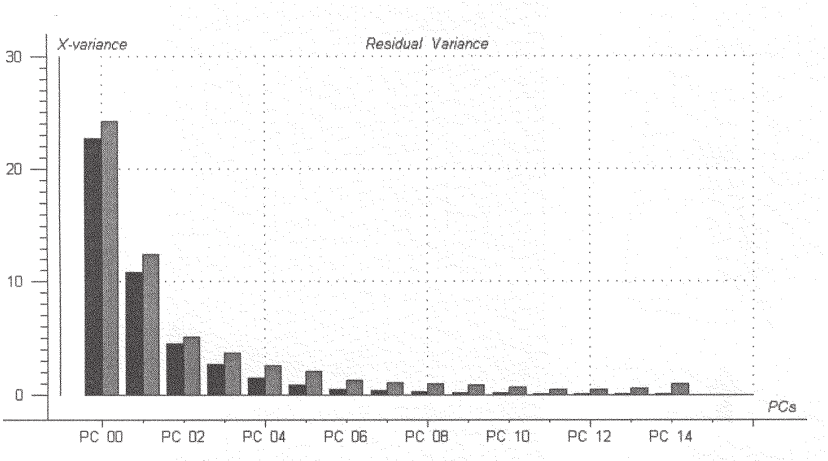

Fig. 6. Residual variance of PCs from the PCA analysis of the GC data of the main compounds from *Juniperus* extracts

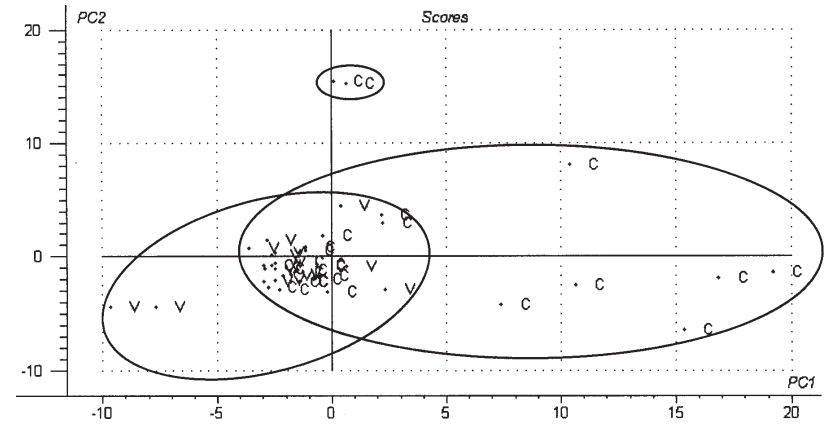

Fig. 7. Scores plot from the PCA analysis of the GC data of the compounds (without α-pinene and β-phellandrene) from *Juniperus* extracts (*Juniperus* species dependent variable: C – *communis*, V – *virginiana*)

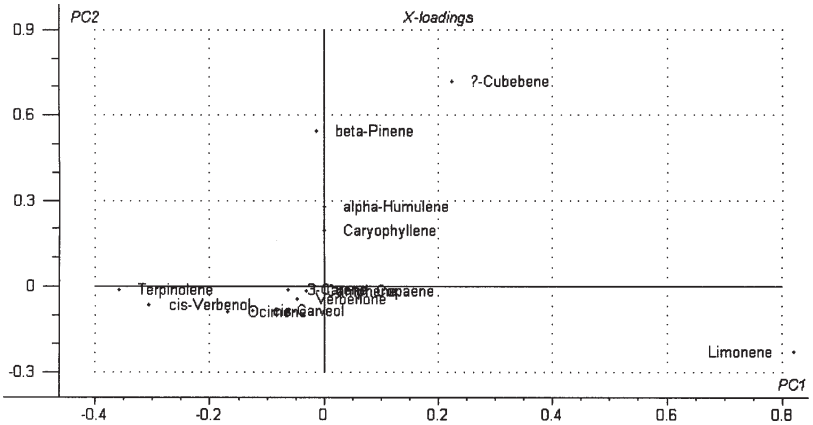

Fig. 8. Loadings plot from the PCA analysis of the GC data of the compounds (without α-pinene and β-phellandrene) from *Juniperus* extracts (species dependent variable)

8%); the relative concentration of the α-pinene is the most important independent variable for the PC_1 classification and β-phellandrene is important for the PC_2 (figs. 5 and 6).

PCA analysis of GC data by using species variable as classification variable revealed relatively grouping of the samples in two groups: *J. communis* (C) and *J. virginiana* (V); some of the samples are presented in both classes. These samples are better classified according to the species if the GC data for relative concentrations of volatile compounds (except α-pinene and β-phellandrene) are

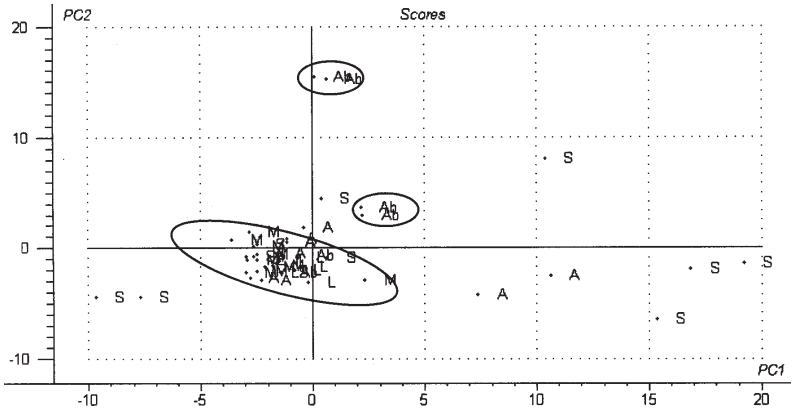

Fig. 9. Scores plot from the PCA analysis of the GC data of the compounds (without α-pinene and β-phellandrene) from *Juniperus* extracts (source dependent variable: M – Macea, Ab – Albac, L – Lipova, S – Syria, A – Austria)

used. Most of samples are grouped in the center of the scores plot (fig. 7), but some of the *J. communis* samples (code C) are grouped in the right side of this plot; few *J. virginiana* samples (V) are grouped in the left side. Limonene, β-pinene, β-cubebene concentrations are the most important variables for this classification (fig. 8), the variance being 39% explained by PC_1 and 29% by PC_2.

Attempts to classify these samples according to plant part, extraction method, and solvent used for extraction did not conduct to significant results; although, the sonication extraction is more grouped than the refluxing extraction. On the other hand, the source of *Juniperus* samples is well grouped if the reduced GC data set (without α-pinene and β-phellandrene) is used; autochthonous samples are clearly grouped in the center and in the upper side of the scores plot (fig. 9), while the Syrian and Austrian samples are distributed along the PC_1 axis.

Conclusion

The following conclusions can be drawn on the gas chromatographic and statistical multivariate analyses of *Juniperus* extracts: (1) the main compounds identified in all *Juniperus* samples are hydrocarbonated terpenes: monoterpenes (such as pinenes, phellandrene, and limonene) and sesquiterpenes (cubebene); (2) most of the *Juniperus communis* and *Juniperus virginiana* samples are good classified by statistical multivariate analysis using the relative concentrations of the above mentioned terpenes; pinene and limonene were most concentrated in the *J. communis* samples; (3) autochthonous (especially "Macea" Botanical Garden, cultivated) *Juniperus* samples are good classified by using the concentration of other volatile compounds than monoterpenes, *i.e.* sesquiterpenes such as cubebene, humulene, caryophyllene, and copaene; (4) seems that the extraction method and the hydrophobicity of the solvent used for extraction has no significant influence on the volatile compound composition.

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