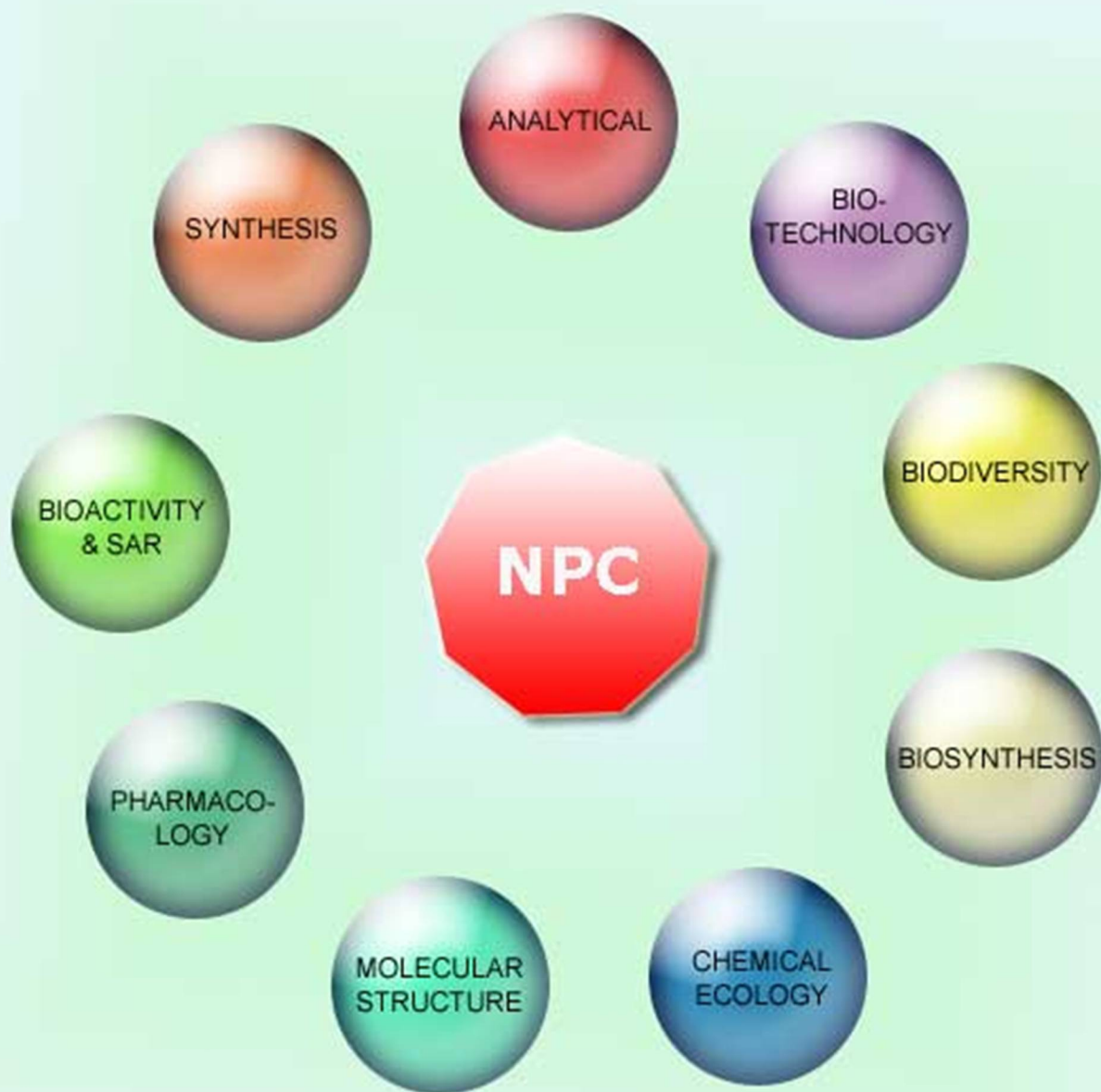


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## Seasonal Variations in the Composition of the Essential Oils of Rosemary (*Rosmarinus officinalis*, Lamiaceae)

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Seasonal variations in the composition of the essential oils obtained from rosemary plants of the same genotype cultivated in Belgrade were determined by GC and GC/MS. The main constituents were camphor (18.2 – 28.1%), 1,8-cineole (6.4-18.0%),  $\alpha$ -pinene (9.7-13.5%), borneol (4.4-9.5%), camphene (5.1-8.7%),  $\beta$ -pinene (2.1-8.1%),  $\beta$ -phellandrene (4.6-6.5%), myrcene (3.4-5.9%) and bornyl acetate (0.2-7.9%). Cluster analysis showed that 16 samples that had been collected each month during the vegetative cycle can be separated into three main clades with different compositions of essential oils. In the shoots with fruits ('fruits' – Clade I) and shoots with developed leaves ('old shoots' – Clade III) camphor is dominant. In shoots with young and incompletely developed leaves ('young shoot' – Clade II) camphor and 1,8-cineole had almost the same concentration. The fact that the same genotype during the growing seasons can synthesize oils that are so different that they can be classified as different chemotypes confirms the opinion that the chemical composition of essential oils sometimes critically depends on the time of collection. Also, for the definition of chemotypes it is not enough to base this on a chemical analysis of an oil from one phenophase only.

**Keywords:** *Rosmarinus officinalis*, Essential oils, Seasonal variations, Cluster analysis.

*Rosmarinus officinalis* L. (Lamiaceae) is an evergreen sclerophyll shrub growing widely in the Mediterranean Basin. Many studies on the essential oil composition of rosemary have been reported, and many different chemotypes have been observed [1-4].

Investigations of the influence of geographical, climatic and pedological characteristics of habitats [1, 5-9] generally support the opinion that the geographic positioning of the studied plants should be taken as the factor affecting the chemical composition of the essential oils, as well as that the geographical distribution of different chemotypes of essential oils of *R. officinalis* are largely due to the environmental characteristics of the habitats. At the same time, like in many other aromatic plants [10-16], some research clearly indicates that the oil composition of rosemary is strongly influenced by the stages of ontogenesis [17-18]. This paper aims to describe seasonal variations in the composition of the essential oils of rosemary of the same genotype, and to determine whether during the growing seasons, there are significant changes that could be characterized as a transition from one to another chemotype.

**Yield and composition of essential oils.** The oil yields of the tested samples varied between 0.2% and 1.0%. The lowest values were recorded in the fruiting phase and the largest in young shoots (Table 1). Monoterpenoids were the most abundant compounds (92.3-95.3%), especially oxygenated ones (52.1-62.0%). Thirty-three components were identified, representing 100% of the oil. The main constituents were camphor (18.2-28.1%), 1,8-cineole (6.4-18.0%),  $\alpha$ -pinene (9.7-13.5%), borneol (4.4-9.5%), camphene (5.1-8.7%),  $\beta$ -pinene (2.1-8.1%),  $\beta$ -phellandrene (4.6-6.5%), myrcene (3.4-5.9%) and bornyl acetate (0.2-7.9%) (Table 1).

**Cluster analysis.** Cluster analysis performed on the basis of Pearson distances showed a differentiation of three main clades (Figure 1). The first clade corresponds with sample of shoots with fruits (Clade

I – October), the second with samples of young shoots (Clade II – May), and the third with samples of old shoots (Clade III – June-May). In the third clade, a weak differentiation in oil composition of old spring-summer shoots (Clade IIIa– April-June) in relation to autumn-winter shoots (Clade IIIb– August-March) is observed.

Basic differences in the chemical composition of dominant components of the essential oils, expressed as the mean concentration for all samples belonging to the appropriate basic clades, are shown in Figure 2. The most significant differences are reflected in the concentration of camphor and 1,8-cineole. The main constituent in all clades was camphor, but the relationship 'camphor: 1,8-cineole' varied from 4:1 in fruits (clade I), via 2:1 in old shoots (clade III) to almost 1:1 in young shoots (clade II). Significant differences were recorded also in the concentration of bornyl acetate. While in the young shoots this component formed 7.4% of the oils, in the old shoots and shoots with fruits it was present only in concentrations of 1.2 and 2.2%. Other dominant components ( $\alpha$ -pinene, camphene,  $\beta$ -phellandrene and borneol) have more or less similar concentrations in all three clades (Figure 2).

### Seasonal dynamics of individual components of the essential oils.

At all stages during the season, monoterpenes were dominant in the composition of rosemary essential oils (Table 1). Seasonal changes in the concentration of the dominant components of the essential oils of *R. officinalis* are shown in Figure 3. Camphor and bornyl acetate show weak seasonal changes, while all other dominant components (1,8-cineole,  $\alpha$ -pinene, borneol, camphene and  $\beta$ -phellandrene) have very stable concentrations during the season. Camphor and bornyl acetate have opposite trends in seasonal changes. The lowest percentages of camphor were recorded in young shoots (May – 18.8%). Its concentration in the old shoots

**Table 1:** Yield and chemical composition of the essential oils of *Rosmarinus officinalis* samples.

Yield of oil (%)	Young shoots (Clade II)			Old shoots (Clade III)												Fruits (Clade I)				
				May_a	May_b	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr_a	Apr_b	May_a	May_b	Oct	
				0.5	1.0	0.6	0.6	0.5	0.7	0.4	0.4	0.5	0.5	0.4	0.4	0.5	0.5	0.3	0.4	0.2
Components (%):	KIE	KIL	RRT																	
1	Tricyclene	921.5	921	0.551	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	
2	$\alpha$ -Thujene	927.3	924	0.557	0.5	0.4	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.3	0.2	0.2	0.3	
3	<b><math>\alpha</math>-Pinene</b>	932.7	932	0.572	<b>10.8</b>	<b>9.7</b>	<b>10.6</b>	<b>10.3</b>	<b>10.0</b>	<b>13.5</b>	<b>11.6</b>	<b>11.9</b>	<b>11.4</b>	<b>10.8</b>	<b>11.2</b>	<b>11.1</b>	<b>11.1</b>	<b>11.7</b>	<b>10.5</b>	<b>13.5</b>
4	<b>Camphene</b>	946.9	946	0.600	<b>7.2</b>	<b>6.3</b>	<b>5.8</b>	<b>5.4</b>	<b>5.6</b>	<b>5.5</b>	<b>5.7</b>	<b>5.5</b>	<b>5.4</b>	<b>5.1</b>	<b>5.5</b>	<b>7.0</b>	<b>7.4</b>	<b>6.4</b>	<b>6.6</b>	<b>8.7</b>
5	Thuja-2.4(10)-diene	953.4	953	0.609	0.1	0.1	0.2	0.3	0.2	0.3	0.0	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.2	0.2
6	Sabinene	970.1	969	0.642	0.2	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.1	0.3
7	<b><math>\beta</math>-Pinene</b>	975.5	974	0.651	<b>8.1</b>	<b>5.3</b>	<b>3.9</b>	<b>2.2</b>	<b>2.1</b>	<b>2.5</b>	<b>2.6</b>	<b>2.3</b>	<b>2.5</b>	<b>2.8</b>	<b>5.3</b>	<b>5.5</b>	<b>4.0</b>	<b>4.1</b>	<b>4.7</b>	
8	3-Octanone	991.2	979	0.661	0.3	0.6	2.5	3.0	3.2	3.4	3.8	2.7	3.9	4.1	3.8	2.4	2.3	3.2	2.6	1.3
9	<b>Myrcene</b>	993.1	988	0.670	<b>5.1</b>	<b>5.4</b>	<b>5.1</b>	<b>4.5</b>	<b>4.4</b>	<b>5.5</b>	<b>5.2</b>	<b>5.9</b>	<b>4.8</b>	<b>4.9</b>	<b>4.7</b>	<b>4.7</b>	<b>4.3</b>	<b>4.6</b>	<b>4.3</b>	<b>3.4</b>
10	3-Octanol	1002.4	988	0.677	0.1	0.2	0.6	0.6	0.8	0.9	0.0	0.7	1.1	1.1	1.0	0.7	0.6	0.8	0.7	0.4
11	$\alpha$ -Phellandrene	1005.1	1002	0.699	1.4	1.7	1.7	1.7	1.4	2.0	1.6	2.3	1.4	1.4	1.4	1.4	1.5	1.3	1.3	1.2
12	$\alpha$ -Terpinene	1017.4	1014	0.722	0.3	0.4	0.5	0.7	0.6	0.8	0.7	0.9	0.6	0.6	0.5	0.5	0.4	0.5	0.4	0.3
13	<i>p</i> -Cymene	1025.4	1020	0.736	0.8	0.2	0.5	1.2	1.4	0.9	1.4	1.3	1.5	1.3	1.5	1.4	1.1	1.9	1.4	1.3
14	<b><math>\beta</math>-Phellandrene</b>	1028.9	1025	0.745	<b>4.7</b>	<b>4.9</b>	<b>5.1</b>	<b>4.7</b>	<b>4.6</b>	<b>5.5</b>	<b>5.4</b>	<b>6.0</b>	<b>5.0</b>	<b>5.1</b>	<b>5.2</b>	<b>5.0</b>	<b>5.4</b>	<b>5.1</b>	<b>5.0</b>	<b>6.5</b>
15	<b>1,8-Cineole</b>	1030.9	1026	0.751	<b>15.9</b>	<b>16.2</b>	<b>15.8</b>	<b>18.0</b>	<b>16.2</b>	<b>15.6</b>	<b>15.5</b>	<b>15.7</b>	<b>16.3</b>	<b>15.5</b>	<b>15.8</b>	<b>15.1</b>	<b>12.7</b>	<b>15.4</b>	<b>14.5</b>	<b>6.4</b>
16	$\gamma$ -Terpinene	1059.9	1050	0.799	1.0	1.3	1.1	1.0	0.9	1.1	1.0	1.3	0.8	0.8	0.7	0.9	1.1	0.7	0.9	1.0
17	<i>cis</i> -Sabinene hydrate	1071.1	1065	0.816	0.7	0.9	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.3	0.6	0.2	0.5	0.6
18	Terpinolene	1089.5	1086	0.855	0.6	0.8	0.8	0.8	0.7	0.9	0.8	0.9	0.6	0.7	0.6	0.6	0.6	0.5	0.6	0.6
19	Linalool	1105.9	1095	0.870	0.9	1.1	1.2	0.9	1.1	0.9	1.0	0.8	0.9	1.0	1.0	0.7	0.9	0.8	0.9	1.4
20	Chrysanthenone	1127.2	1124	0.924	0.1	0.2	0.3	0.3	0.5	0.2	0.4	0.2	0.4	0.3	0.4	0.3	0.3	0.3	0.5	0.9
21	<i>trans</i> -Sabinol	1140.6	1137	0.953	0.2	0.3	0.3	0.2	0.2	0.1	0.1	0.0	0.0	0.1	0.2	0.3	0.1	0.3	0.4	0.4
22	<b>Camphor</b>	1144.5	1141	0.964	<b>18.2</b>	<b>19.4</b>	<b>25.2</b>	<b>27.7</b>	<b>28.1</b>	<b>24.0</b>	<b>25.9</b>	<b>24.0</b>	<b>26.5</b>	<b>27.0</b>	<b>26.6</b>	<b>23.4</b>	<b>23.3</b>	<b>24.7</b>	<b>26.0</b>	<b>27.9</b>
23	<b>Borneol</b>	1166.9	1165	1.000	<b>4.4</b>	<b>4.8</b>	<b>5.1</b>	<b>6.8</b>	<b>7.4</b>	<b>5.1</b>	<b>6.4</b>	<b>5.8</b>	<b>6.0</b>	<b>5.8</b>	<b>6.3</b>	<b>6.4</b>	<b>6.3</b>	<b>7.1</b>	<b>7.9</b>	<b>9.5</b>
24	Terpinen-4-ol	1179.4	1174	1.018	1.1	1.0	1.2	1.4	1.3	1.1	1.3	1.2	1.3	1.3	1.4	1.5	1.3	1.4	1.4	1.3
25	$\alpha$ -Terpineol	1193.9	1186	1.040	1.4	1.7	1.9	1.9	2.0	1.8	2.0	1.7	2.1	2.2	2.0	1.6	1.5	1.7	1.7	1.0
26	Myrtenol	1199.9	1194	1.052	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.2	0.2	0.3	0.3	0.2
27	Verbenone	1211.2	1204	1.077	2.0	3.6	4.5	4.1	4.2	5.2	4.5	3.2	4.4	4.9	4.1	4.1	2.8	4.1	2.6	1.3
28	<b>Bornyl acetate</b>	1287.6	1287	1.204	<b>6.9</b>	<b>7.9</b>	<b>3.1</b>	<b>0.4</b>	<b>0.6</b>	<b>0.6</b>	<b>0.5</b>	<b>0.2</b>	<b>0.3</b>	<b>0.2</b>	<b>0.2</b>	<b>2.4</b>	<b>4.9</b>	<b>0.6</b>	<b>2.0</b>	<b>2.2</b>
29	$\alpha$ -Ylangene	1372.7	1373	1.352	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
30	<i>trans</i> -Caryophyllene	1420.4	1417	1.432	1.8	1.3	0.4	0.4	0.3	0.5	0.4	1.4	0.5	0.6	0.5	0.5	0.6	0.4	0.5	0.6
31	$\alpha$ -Humulene	1454.8	1452	1.484	3.5	2.6	0.8	0.7	0.7	1.0	0.9	2.7	0.9	1.1	1.0	0.9	1.2	0.8	1.1	1.2
32	Caryophyllene oxide	1583.8	1582	1.681	0.6	0.5	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.2	0.2	0.4	0.2	0.3	0.4
33	Humulene epoxide II	1610.0	1608	1.717	0.3	0.3	0.2	0.2	0.3	0.2	0.3	0.0	0.3	0.4	0.3	0.2	0.3	0.2	0.3	0.5
<b>Monoterpene compounds</b>				<b>93.3</b>	<b>94.4</b>	<b>95.3</b>	<b>95.1</b>	<b>94.5</b>	<b>93.9</b>	<b>94.4</b>	<b>92.3</b>	<b>93.1</b>	<b>92.5</b>	<b>93.2</b>	<b>95.1</b>	<b>94.6</b>	<b>94.3</b>	<b>94.5</b>	<b>95.6</b>	
Monoterpene hydrocarbons				41.1	36.9	35.8	33.0	32.5	38.9	36.4	39.0	34.4	33.7	34.7	38.8	39.3	37.6	35.8	42.4	
<b>Oxygenated monoterpenes</b>				<b>52.1</b>	<b>57.4</b>	<b>59.5</b>	<b>62.0</b>	<b>62.0</b>	<b>55.0</b>	<b>58.0</b>	<b>53.3</b>	<b>58.7</b>	<b>58.8</b>	<b>58.5</b>	<b>56.3</b>	<b>55.2</b>	<b>56.7</b>	<b>58.7</b>	<b>53.2</b>	
Sesquiterpene compounds				6.3	4.8	1.6	1.3	1.5	1.8	1.8	4.3	1.9	2.3	2.0	1.8	2.5	1.7	2.2	2.7	
Sesquiterpene hydrocarbons				5.4	4.0	1.2	1.0	1.0	1.5	1.3	4.2	1.4	1.7	1.5	1.4	1.8	1.3	1.6	1.8	
Oxygenated sesquiterpenes				0.9	0.8	0.4	0.3	0.5	0.3	0.5	0.1	0.5	0.6	0.5	0.4	0.7	0.4	0.6	0.9	
Other compounds				0.4	0.8	3.1	3.6	4.0	4.3	3.8	3.4	5.0	5.2	4.8	3.1	2.9	4.0	3.3	1.7	
Total				100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	

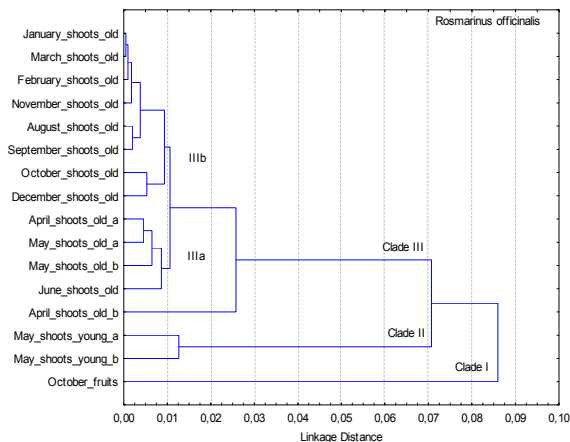
KIE - Kovats (retention) index experimentally determined (AMDIS); KIL - Kovats (retention) index - literature data [25]; RRT - relative retention time to selected constituent (borneol=1.000); a – 2008. Year; b – 2009. Year.

(from June to May) was uniform, and oscillated in the range from 23.3% to 28.1%. On the contrary, bornyl acetate had its largest concentration in young shoots (May – 7.4%), but, during leaf development, its concentration declined rapidly. So, from August to March, bornyl acetate concentration was lower than 1% (Figure 3).

Recent studies of rosemary essential oil composition of indigenous and cultivated plants in the Mediterranean area revealed the existence of 6 monodominant and 6 intermediate chemotypes. The most commonly recorded monodominant chemotypes are **1,8-cineole** [1,5-7,17,19-20] and **camphor** chemotypes [1,5-7,21]. Less common are **verbenone** [19-20,22], and  **$\alpha$ -pinene** [20,23], chemotypes, while in only one sample **linalool** [19] and ***p*-cymene** [24] chemotypes were recorded. Intermediate chemotypes **1,8-cineole/linalool** [19], **1,8-cineole/camphor** and **1,8-cineole/camphor/borneol** [1] were also recorded for only single samples.

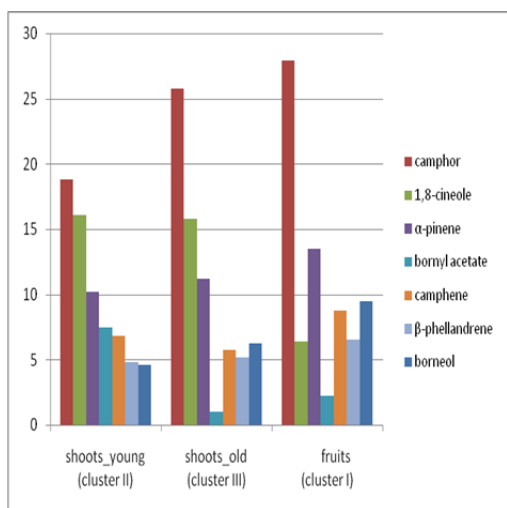
The results of our cluster analysis, performed on the same genotype of rosemary cultivated in Belgrade, have shown that 16 samples collected from each month during the vegetation cycle can be separated into three main clades, with different compositions of essential oils (Figure 1). In the shoots with fruits ('fruits' – Clade I) and shoots with developed leaves ('old shoots' – Clade III) camphor is dominant, and these oils can be classified as **camphor chemotype**. In shoots with young and incompletely developed leaves (young shoots – Clade II) camphor and 1,8-cineole have almost the same concentration, so these oils can be classified as an intermediate **camphor/1,8-cineole chemotype**.

Since the intermediate chemotype is registered only in the young shoots in May (Clade II), and one of the variants of monodominant chemotypes only in October (Clade I), we might conclude that the analyzed genotype of rosemary belongs to a variant of the camphor chemotype in which the relationship 'camphor:1,8-cineole' is 2:1.

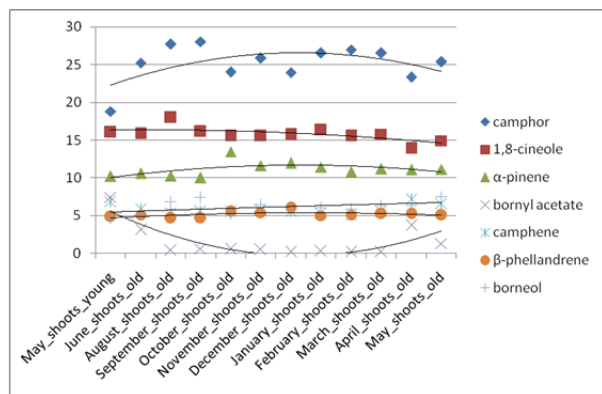


a – 2008; b – 2009.

**Figure 1:** Cluster analysis of the chemical composition of essential oils of *Rosmarinus officinalis*.



**Figure 2:** Dominant components of essential oils of basic clades of *Rosmarinus officinalis*.



**Figure 3:** Seasonal changes in the concentration of the dominant components of essential oils of *Rosmarinus officinalis*

Both chemotypes observed in the same individual have already been recorded by us in previous studies [1]. The **camphor type** is relatively frequent, while the **1,8-cineole/camphor chemotype** is rare, and has been found only in the oils of rosemary from the island of Zakynthos in Greece [1]. The fact that the same genotype during the growing seasons can synthesize oils that are so different

that they can be classified as different chemotypes confirms the opinion of those authors who believe that the chemical composition of essential oils sometimes critically depends on the time of picking of the plant material. At the same time, our results suggest that, for the definition of a chemotype, it is not enough to make a chemical analysis of the oil from one phenophase only. To define a chemotype it is necessary to analyze the seasonal variations of composition of essential oil.

**Experimental**

**Plant material:** The seasonal composition of the essential oils of cultivated rosemary from Belgrade (Serbia) was investigated. Samples were collected each month from April 2008 to May 2009. The analyses were performed on shoots with young and incompletely developed leaves ('young shoots'), shoots with developed leaves ('old shoots'), as well as shoots with fruits ('fruits'). To avoid the influence of genetic and environmental influences, all analyzes were made on the same genotype, a 15 years old individual, which is successfully grown in a private garden.

**Oil isolation:** Oils were isolated from dried plant material using always the same distillation apparatus, and working under the same conditions. The essential oils were isolated by hydrodistillation for 2 h, according to the standard procedure reported in the Sixth European Pharmacopoeia, using a Clevenger type apparatus. Oil samples were dissolved in ethanol and analyzed by GC/FID and GC/MS. Chemical analyses (GC/FID and GC/MS) used to identify and quantify essential oil constituents were accomplished using sharply defined and always the same analytical conditions.

**Analytical gas chromatography (GC/FID):** GC/FID analysis of the oils was carried out on a HP-5890 Series II GC apparatus [Hewlett-Packard, Waldbronn (Germany)], equipped with a split-splitless injector and an automatic liquid sampler (ALS), attached to a HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to a flame ionization detector (FID). The carrier gas flow rate (H<sub>2</sub>) was 1 mL/min, split ratio 1:30, injector temperature 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-260°C (at a rate of 4°C/min). Solutions of essential oil samples in ethanol (~1%) were consecutively injected by ALS (1 μL, split mode). Area percent reports, obtained as a result of standard processing of chromatograms, were used as the basis for the quantification purposes.

**Gas chromatography/mass spectrometry (GC/MS):** The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with a HP-5MS (30 m × 0.25 mm, 0.25 μm film thickness) column, using a HP G 1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Instead of hydrogen, helium was used as carrier gas. The transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in the range m/z 40-450. Sample solutions in ethanol (~1 %) were injected by ALS (200 nL, split mode). The components of the oil were identified by comparison of their mass spectra with those from Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1.), and compared with those from available literature [25] and used as an additional tool to support the MS findings.

**Statistical analysis:** Statistical analysis was performed in two steps: in the first, multivariate analysis was done in order to identify the structure of variability and to measure the distances between groups. These analyses were performed on complete data sets. The UPGMA

(unweighted pair-group average linkage) clustering method based on Pearson distances was used to measure the similarities between each measured unit. In the second step, the seasonal dynamics of individual components of the essential oils were examined. These analyses were performed on shoots with leaves – ‘young shoots’ and ‘old shoots’.

Statistical analyses were performed with the package Statistica 5.1 (STATSOFT 1996) and scatterplots with trendlines using the package

Excel for Windows 97. The emergence of new young shoots in May is designated as the beginning of the season in which we analyzed the trends of changes in the composition of essential oils.

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