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Variations in essential oil yields and compositions of *Salvia officinalis* (Lamiaceae) at different developmental stages

Branislava S. Lakušić [1*], Mihailo S. Ristić [2], Violeta N. Slavkovska [1], Danilo Lj. Stojanović [1] and Dmitar V. Lakušić [3]

1 Department of Botany, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia
2 Institute for Medicinal Plant Research "Dr Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia
3 Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia

ABSTRACT: Variations in the yield and composition of essential oil of Dalmatian sage (*Salvia officinalis* L., Lamiaceae) in various stages of development were analyzed in two individuals (= genotypes) of different geographic origin. Both plants have been successfully grown ten years under identical environmental conditions in a private garden in Belgrade. The amount and composition of the oils were analyzed during one growing season starting from the young to old overwintered leaves.

The results of the cluster analysis showed that leaf age and origin of the plants has a significant impact on the composition of the essential oils. All oil samples formed two main clades. The first clade are oils collected from young leaves from April to June and belong to α-humulene type. The second clade includes oil obtained mainly of old leaves, which were collected from August to June, and belonging to the camphor and thujone type. In the second clade samples originating from Učka are completely separated from samples originating from Belgrade.

Also, based on these analyzes showed that the same individual (the same genotype) during one growing season significantly change the chemical composition of the essential oils. Thus, it is possible to distinguish three different "phenological types of essential oils": young leaves ("yl-oils"), early old leaves ("early-ol-oils") and a late old leaves type ("late-ol-oils"). Analysis of the seasonal changes in the composition of the essential oils of Dalmatian sage is shown that different components have different dynamics and different directions of change in the concentration during the season.

Keywords: *Salvia officinalis,* essential oil, camphor, thujone, α-humulene, developmental stage

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INTRODUCTION

Dalmatian sage (*Salvia officinalis* L.) economically is one of the most important species of the genus *Salvia* which comprises nearly 1000 species throughout the Old and New Worlds, and represents one of the largest genera in the Lamiaceae family. Dalmatian sage, also known as common sage or garden sage, is a perennial subshrub native to the northern coastal region of the Mediterranean and grows wild in the calcareous regions of northern and central Spain, southern France and the western part of the Balkan Peninsula (Hedge 1972). It is widely cultivated in countries of the continental part of the Balkans, throughout the Mediterranean region, but also in Lithuania, Poland, Slovakia, Hungary, Romania, France, UK, USA, Cuba, New Zeland, etc.

Given that it is used as a herb with beneficial healing properties and its dried leaf (*Salviae officinalis folium*) is

*correspondence: blakusic@pharmacy.bg.ac.rs

an official drug in most pharmacopoeias, the chemical characteristics of Dalmatian sage essential oils have been the subject of many studies. A broad overview of this research was published by Tucker & Maciarello 1990; Perry *et al.* 1999; Ristić *et al.* 1999; Bernotienė *et al.* 2007; Ben Farhat *et al.* 2009; Šatović 2011.

According to Franz (1993) variation in the composition of essential oil within a species appears to be the rule rather than the exception. He suggested that this variation is influenced by three major factors: (1) individual genetic variability; (2) variation among different plant parts and their different stages of development; and (3) modifications due to the environment. The literature data reviewed in many papers indicated that all of these factors would be important for Dalmatian sage oils (Kuštrak *et al.* 1984; Pitarević *et al.* 1984; Putievsky *et al.* 1990; Tucker & Maciarello 1990; Länger *et al* 1993; Boelens & Boelens 1997; Piccaglia *et al.* 1997; Skoula *et al.* 1999; Perry *et al.* 1999; Santos-Gomes & Fernandes-Ferreira 2001; Mockute *et al.* 2003; Zutić *et al.* 2003; Avato *et al.* 2005; Marić *et al.* 2006; Bernotienė *et al.* 2007; Maksimović *et al.* 2007; Ben Farhat *et al.* 2009; Šatović *et al.* 2011; Jug-Dujaković *et al.* 2012). These factors influence the plant's biosynthetic pathways and, consequently, the relative proportion of the main constituents. Because of such variation, sage essential oil composition sometimes does not match the profile defined by standard ISO 9909 (Bruneton 1999).

The aims of our study were: (1) to describe variations in yields and chemical composition of sage essential oil; (2) to determine which of the factors (different stages of leaf development, different months in one vegetational season, or plant origin) had the dominant influence on variation in chemical composition of Dalmatian sage essential oil.

MATERIAL AND METHODS

Plant material. Ten oil samples from one individual and 10 samples from the second individual (= genotype) of Dalmatian sage (*Salvia officinalis*) with different geographic origin (**Serbia**: Beograd – a commercial plant from cultivation **and Croatia**: Učka – a plant from a natural population) were investigated. Both plants were successfully grown ten years under identical environmental conditions in a private garden in Beograd (Serbia) under the same sub-continental climate conditions with cold and snowy winters. All samples were classified into two groups: **"yl"** – "young" incompletely developed leaves and **"ol"** – "old" completely developed leaves. Harvesting was done ten times during the vegetation season 2008-2009, throughout the complete growing cycle, which began with young and incompletely developed leaves, and ended with the old well-developed leaves (Table 1).

Oil isolation. Oils were isolated from fresh material, using always the same distillation apparatus, under the same conditions. The essential oils were isolated by hydrodistillation, according to the standard procedure reported in the Sixth European Pharmacopoeia (European Pharmacopoeia 2007) using a Clevenger type apparatus. Duration of distillation was 2 h. Oil samples were dissolved in ethanol and analyzed by GC/FID and GC/MS.

Analytical gas chromatography (GC/FID). GC/FID analysis of the oils was carried out on a HP-5890 Series II GC [Hewlett-Packard, Waldbronn (Germany)], equipped with split-splitless injector and automatic liquid sampler (ALS), attached to an HP-5 column (25 m · 0.32 mm, 0.52 μm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40 to 260°C (at 4°/min). Solutions of essential oil samples in ethanol (~1%) were consecutively injected by ALS (1 μl, split mode, 1:30). Area percent reports, obtained as a result of standard processing of chromatograms, were used as a basis for quantification.

Gas chromatography/mass spectrometry (GC/MS). The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with an HP-5MS column (30 m · 0.25 mm, 0.25 μm film thickness), using 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 m/z range 40-450. Sample solutions in ethanol (~1 %) were injected by ALS (200 nl, split mode, 1:30). The components of the oil were identified by comparison of their mass spectra with those from Wiley275 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.), compared with those from available literature (Adams 2007), and used as an additional tool to approve MS findings.

Statistical analysis. Basic statistics (mean value, minimum and maximum value, standard deviation and standard error of the mean) were performed for each continuous character. The significance of differences betweeen oil samples was tested by analysis of variance (ANOVA) and represented by critical value from an F-test (F) and statistical significance (p). ANOVA was performed on plant stage ("yl" or "ol"), month of harvesting and geographical origin of plants as grouping factors. The Unweighted pair-group average linkage (UPGMA) cluster analysis based on Pearson distances was used to measure the similarities between each measured unit. The regression analyses were performed and R^2 factors were calculated to identify the level of dependency of variation of chemical composition of essential oils in regard to the month of harvesting. Statistical analyses were performed with the package Statistica 5.1 (StatSoft 1996) and scatterplots with trendlines were drawn in the package Excel for Windows 97.

RESULTS AND DISCUSSION

Yields of the Essential Oils. The oil yields varied between 0.2-2.9 % (Table 1). The lowest oil percentages were recorded in the plant from Učka ("ol" - June), and the largest in leaves from Beograd, which were overwintered ("ol" - March). In plants with different geographical origin, we registered significant differences in the oil yields from leaves which were overwintered. Thus, the March "ol" sample from Beograd gave the highest yield of 2.9%, while the April "ol" sample from Učka yielded only 0.5% of oil.

Composition of the Essential Oils. The essential oils contained 49 constituents, 46 of which were identified (Table 1). The 11 main constituents, representing more than 5% of the total oil content in at least a single sample, were: **camphor** (1.9 -32.7%), ***cis*-thujone** (6.7-28.5%), **α-humulene** (3.4-33.3%), **viridiflorol** (2.9-12.4%), **manool** (1.4-14.5%), **camphene** (0.2-8.6%), **1,8-cineole** (1.2-19.4%), **limonene** (0.5-9.1%), **β-pinene** (0.3-13.5%), ***trans*-thujone** (0.7-14.5%) and **α-pinene** (0.4-5.2%). In the oils of young leaves (April – "yl") sesquiterpenoid constituents dominated (50.7 - 57.0%), while the development of leaves increased the amount of monoterpenoids in the oil (55.4-88.4%). Age of leaves had a significant influence on the composition of essential oil.

Compared with literature data (Ivanić *et al.* 1978; Rhyu 1979; Kuštrak *et al.* 1984; Ristić *et al.* 1999; Couladis *et al.* 2002; Šatović 2011; Jug-Dujaković *et al.* 2012), we found that dominant components of our "ol" oils were very similar to essential oils from leaves of Dalmatian sage in the Balkans.

Statistical analyses of the chemical composition of the essential oils for the complete data set of 20 samples is presented in Table 2**.** Differences between oils from leaves in different stages ("yl" and "ol"), in different months and different origin of plants were statistically significant.

Analysis of variance (ANOVA) has shown that the origin of plants had a dominant influence on variation in chemical composition of the Dalmatian sage essential oil. Namely, ANOVA showed that thirty constituents were statistically significant when the origin of plants was selected as the grouping factor. Stage ("yl" vs. "ol") with fifteen constituents, and month of harvesting with only eight constituents giving statistically-significant contributions, showed weaker influences on variation in chemical composition of the essential oils (Table 2). Earlier studies of Dalmatian sage also showed that differences in the composition of essential oils isolated from different plant parts, on different harvesting dates and from different

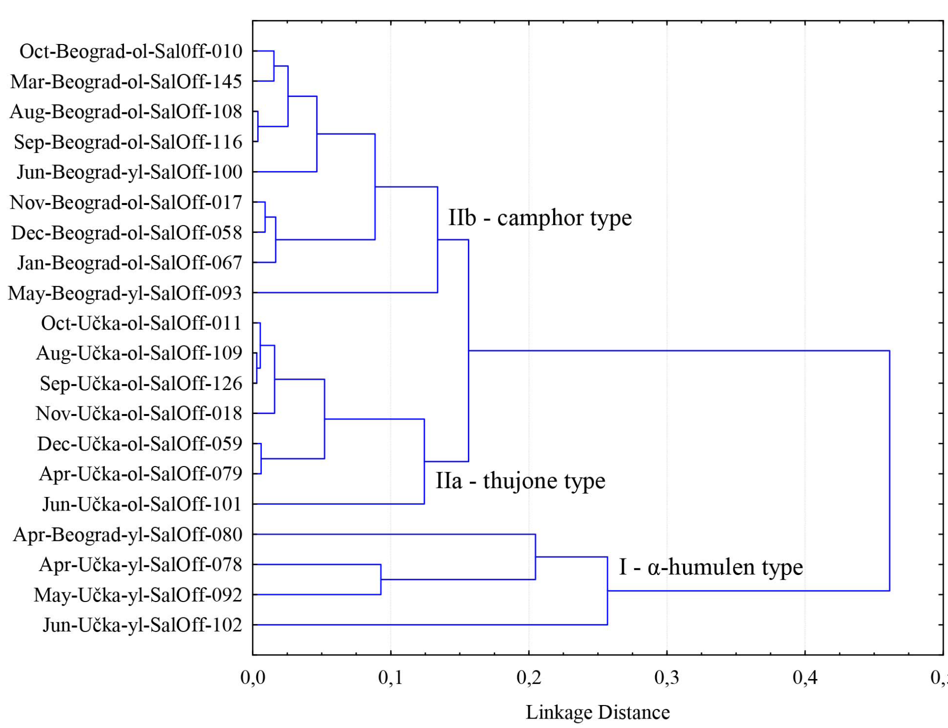

Fig. 1. Cluster analysis of the chemical composition of essential oils of *Salvia officinalis* cultivated in Beograd performed on the basis of Pearson distances calculated for "yl-oils" and "ol-oils" from different months.

Table 1. Chemical composition of *Salvia officinalis* essential oils (%)

	Sample-Acronym SalOff-	080	093	100	108	116	010	017	058	067	145	078	079	092	102	101	109	126	011	018	059
	Origin	Beograd, Serbia										Učka, Croatia									
	Month of harvesting	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Mar	Apr	Apr	May	Jun	Jun	Aug	Sep	Oct	Nov	Dec
	Plant part	yl	yl	yl	ol	ol	ol	ol	ol	ol	ol	yl	ol	yl	yl	ol	ol	ol	ol	ol	ol
	Yield (%)	0.8	0.3	0.4	0.6	1.2	0.6	1.0	0.6	1.0	2.9	0.4	0.5	0.4	0.4	0.2	2.1	0.5	0.7	0.8	0.6
No.	Constituents (%)																				
1	*cis*-Salvene	0.1	0.0	0.0	0.1	0.1	0.1	0.2	0.2	0.1	0.3	0.0	0.6	0.0	0.3	0.2	0.3	0.3	0.4	0.6	0.5
2	*trans*-Salvene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1
3	Tricyclene	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1
4	α-Thujene	0.3	0.2	0.1	0.1	0.1	0.2	0.3	0.2	0.2	0.2	0.9	1.7	0.9	0.3	1.2	1.1	1.5	1.5	1.5	1.5
5	**α-Pinene**	3.8	4.1	4.2	4.3	4.5	4.4	4.2	4.1	3.4	**5.2**	0.6	0.5	0.4	1.9	0.7	0.7	0.7	0.7	0.6	0.7
6	**Camphene**	3.6	**5.6**	**6.9**	**8.5**	**8.6**	**8.4**	**7.1**	**6.7**	**5.6**	**8.3**	0.2	2.1	0.9	1.2	2.8	3.6	3.6	3.6	3.2	3.2
7	Sabinene	0.4	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.0	3.0	2.1	2.9	0.4	2.2	0.9	1.7	1.5	1.7	1.5
8	**β-Pinene**	**13.5**	**6.7**	4.4	2.7	2.8	4.1	4.1	5.2	4.4	**5.3**	3.5	0.5	1.0	**6.5**	0.9	0.3	0.4	0.4	0.4	0.4
9	Myrcene	0.6	0.8	0.9	1.1	1.1	1.2	0.8	1.0	0.8	1.0	0.6	0.9	0.8	0.8	1.1	1.2	1.2	1.3	0.7	1.2
10	α-Phellandrene	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.2	0.2	0.2	0.2	0.3	0.1
11	α-Terpinene	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.2	0.5	0.6	0.7	0.1	0.9	1.5	1.2	1.4	1.0	0.9
12	*p*-Cymene	0.1	0.0	0.0	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3	3.6	0.3	0.3	0.5	2.0	2.0	2.6	2.0	3.5
13	**Limonene**	2.2	**5.9**	**7.8**	**8.3**	**9.1**	**7.4**	**5.2**	**5.1**	**5.2**	**7.6**	0.5	1.7	1.0	1.0	1.7	2.6	2.3	2.5	2.2	2.5
14	**1,8-Cineole**	4.2	**6.9**	**6.9**	**5.8**	4.5	**5.1**	4.2	3.7	3.0	**5.8**	**5.8**	1.5	**5.2**	**19.4**	3.9	2.5	2.4	2.0	1.6	1.2
15	*cis*-β-Ocimene	2.0	3.2	1.1	0.2	0.1	0.0	0.0	0.4	0.4	0.2	4.8	0.1	1.7	0.3	0.3	0.0	0.0	0.0	0.0	0.0
16	*trans*- β-Ocimene	0.3	0.6	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0	1.7	0.0	0.5	0.1	0.1	0.0	0.0	0.0	0.0	0.0
17	γ-Terpinene	0.3	0.2	0.2	0.3	0.3	0.4	0.3	0.3	0.2	0.3	1.8	1.9	2.2	0.4	1.8	2.9	2.4	2.5	1.7	1.9
18	*cis*-Sabinene hydrate	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.5	1.5	0.9	0.4	1.0	1.0	1.1	1.2	0.9	0.9
19	Terpinolene	0.1	0.3	0.4	0.6	0.5	0.5	0.2	0.2	0.1	0.2	0.3	0.4	0.6	0.2	0.8	0.9	0.8	0.8	0.5	0.6
20	*trans*-Sabinene hydrate	0.6	0.9	0.9	0.7	0.7	0.6	0.3	0.7	0.6	0.6	1.2	2.1	1.7	0.8	2.0	1.8	2.0	2.1	0.8	0.4
21	***cis*-Thujone**	**6.7**	**7.0**	**8.5**	**13.3**	**15.2**	**20.0**	**19.2**	**21.0**	**19.7**	**15.9**	**10.6**	**28.5**	**15.9**	**16.5**	**15.8**	**25.9**	**27.5**	**28.3**	**28.5**	**28.1**
22	***trans*-Thujone**	0.7	0.9	1.1	1.7	2.0	2.4	2.2	2.4	2.3	1.8	1.4	4.1	2.3	2.4	**14.5**	4.3	4.1	3.9	4.0	4.2
23	α-Campholenal	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.2	0.2	0.2	0.0	0.1
24	*iso*-3-Thujanol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
25	*trans*-Sabinol	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.3	0.1	0.2	0.2	0.0	0.0
26	**Camphor**	**7.0**	**18.0**	**26.4**	**32.7**	**30.6**	**27.4**	**21.6**	**19.2**	**17.7**	**24.2**	1.9	**16.4**	**12.7**	**10.7**	**26.2**	**30.4**	**28.2**	**27.7**	**24.9**	**18.7**
27	*trans*-Pinocamphone	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.0	1.0	0.2	0.4	0.2	0.2	0.2	0.1	0.2	0.0	0.1
28	Borneol	1.3	3.0	3.0	2.0	1.7	1.6	2.2	1.3	1.3	1.7	0.3	0.4	0.9	1.9	0.9	0.6	0.5	0.7	0.9	0.5
29	*cis*-Pinocamphone	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.9	0.0	0.0	0.0
30	Terpinen-4-ol	0.2	0.2	0.2	0.1	0.1	0.5	0.2	0.3	0.3	0.4	0.9	1.7	1.0	0.3	1.1	0.1	0.2	2.2	0.7	1.2
31	α-Terpineol	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.0	0.0
32	Myrtenol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.1
33	Verbenon	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
34	Bornyl acetate	0.1	0.8	1.7	1.4	0.8	0.7	0.5	0.4	0.3	0.2	0.0	0.1	0.2	0.4	0.9	0.4	0.2	0.3	0.3	0.2
35	*trans*-Sabinyl acetate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
36	β-Caryophyllene	4.7	4.3	2.6	1.2	1.2	1.0	2.1	1.7	2.0	1.3	2.5	0.5	2.2	1.2	1.0	0.4	0.4	0.4	0.6	0.5
37	**α-Humulene**	**18.9**	**11.4**	**6.9**	3.4	3.7	3.8	**8.0**	**7.5**	**8.8**	4.9	**33.3**	**6.7**	**26.7**	**16.2**	**7.4**	4.5	4.5	4.5	**6.9**	**6.1**
38	Dehydroaromadendrane	0.2	0.1	0.1	0.1	0.0	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.0
39	γ-Muurolene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0

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	Origin	Beograd, Serbia										Učka, Croatia									
	Month of harvesting	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Mar	Apr	Apr	May	Jun	Jun	Aug	Sep	Oct	Nov	Dec
	Plant part	yl	yl	yl	ol	ol	ol	ol	ol	ol	ol	yl	ol	yl	yl	ol	ol	ol	ol	ol	ol
	Yield (%)	0.8	0.3	0.4	0.6	1.2	0.6	1.0	0.6	1.0	2.9	0.4	0.5	0.4	0.4	0.2	2.1	0.5	0.7	0.8	0.6
No.	Constituents (%)																				
40	Viridiflorene	0.3	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.3	0.1	0.3	0.1	0.0	0.0	0.0	0.1	0.4	0.1
41	δ-Cadinene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
42	Caryophyllene oxide	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.3	0.4	0.1	0.3	0.1	0.2	0.1	0.1	0.1	0.0	0.0	0.2
43	**Viridiflorol**	**11.2**	**10.6**	**9.4**	**6.1**	**6.5**	**5.7**	**8.5**	**10.3**	**12.4**	**9.1**	**10.7**	**8.3**	**6.0**	2.9	4.0	3.3	3.5	3.3	**5.6**	**6.9**
44	Humulene epoxide I	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.2	0.2	0.1	0.5	0.2	0.4	0.1	0.2	0.2	0.2	0.0	0.3
45	Humulene epoxide II	0.8	0.3	0.3	0.4	0.5	0.3	0.4	0.7	1.2	1.4	0.6	3.7	1.0	2.4	0.8	1.1	1.2	1.1	1.6	2.6
46	n.i.=not identified	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.2	0.3	0.2	0.2	0.0	0.5
47	n.i.=not identified	0.0	0.0	0.0	0.3	0.3	0.2	0.3	0.4	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.0	0.2
48	**Manool**	**14.5**	**6.4**	4.1	2.7	2.9	2.1	**5.8**	4.6	**7.5**	1.4	**9.2**	**5.6**	**7.6**	**9.0**	3.5	2.0	2.2	1.7	**5.0**	**6.4**
49	n.i.=not identified	0.7	0.4	0.3	0.2	0.2	0.1	0.3	0.3	0.4	0.1	0.5	0.3	0.5	0.4	0.2	0.1	0.1	0.0	0.5	0.4
	Monoterpenoids	48.5	**66.1**	**76.0**	**85.3**	**84.3**	**86.5**	**73.9**	**74.1**	**67.0**	**80.7**	42.5	**73.8**	**55.4**	**67.2**	**82.7**	**87.7**	**87.2**	**88.4**	**79.2**	**75.7**
	Monoterpene hydrocarbons	27.4	28.0	26.9	27.0	28.0	27.6	23.3	24.7	21.3	29.7	18.7	16.9	14.0	13.8	15.5	18.2	18.4	19.5	16.6	18.7
	Oxygenated monoterpenes	21.0	38.1	49.1	58.3	56.3	58.9	50.6	49.4	45.6	51.0	23.7	56.9	41.4	53.4	67.2	69.4	68.7	68.9	62.6	57.0
	Sesquiterpenoids	**50.7**	33.4	23.7	14.2	15.1	13.2	25.4	25.2	32.5	18.9	**57.0**	25.8	44.1	32.3	16.9	11.7	12.2	11.2	20.4	23.1
	Sesquiterpene hydrocarbons	24.0	16.0	9.7	4.7	5.0	5.0	10.5	9.4	11.0	6.4	36.3	7.4	29.3	17.4	8.4	5.0	5.0	5.0	8.2	6.7
	Oxygenated sesquiterpenes	26.7	17.5	14.0	9.5	10.1	8.2	14.9	15.8	21.5	12.5	20.7	18.4	14.8	14.8	8.6	6.7	7.2	6.2	12.2	16.4
	Notidentified compounds	0.8	0.4	0.3	0.5	0.6	0.3	0.7	0.8	0.5	0.3	0.5	0.4	0.5	0.5	0.4	0.6	0.6	0.4	0.5	1.2
	Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

origins were statistically significant (Piccaglia *et al.* 1997; Perry *et al.* 1999; Ben Farhat *et al.* 2009).

The results of cluster analysis performed on the basis of Pearson distances showed a general differentiation into two main clades (Fig. 1). Samples of the first clade, originated from young leaves ("yl") harvested from April to June (Clade I), belonging to the **α-humulene type**. The second group corresponded to samples obtained mainly from old leaves ("ol"), harvested from August to June (Clade II), belonging to the **camphor** or **thujone type**. In the second clade, samples from Učka (Clade IIa) were separated from the Beograd samples (Clade IIb). In the Učka subgroup (Clade IIa), samples belonged to the **thujone type.** In the Beograd subgroup (Clade IIb), samples belonged to the **camphor type.** The concentration of camphor under colder conditions generally increased, as in the case of rosemary from Beograd (Lakušić *et al.* 2012).

Slight deviations from this general structure were shown for May and June samples of "yl" from Beograd, which were nested within the group of "ol". The fact that May and June samples of "yl" from Učka were placed in the "yl" cluster suggests that during the season, Dalmatian sage from different habitats have different dynamics of synthesis of individual essential oil constituents.

Thus, the same individual (same genotype) during one growing season significantly changed the chemical composition of its essential oil. So, it is possible to distinguish at least three different "phenological types of essential oils": young leaves ("yl-oils"), early old leaves ("early-ol-oils") and a late old leaves type ("late-ol-oils"). The basic chemical specificity of young leaves was reflected in high concentrations of **α-humulene**, **viridiflorol** and **manool**, and relatively low concentrations of **camphor** and ***cis*-thujone**. In early old leaves, concentrations of α-humulene, viridiflorol and manool were significantly reduced, and the concentration of **camphor** and ***cis*-thujone**, gave pronounced increases. Finally, late old leaves (those overwintered) were characterized by decreasing concentrations of camphor and increasing concentrations of α-humulene, viridiflorol and manool (Fig. 2).

Thus, leaves of the same plant, in different stages of development, form different types of oil. It is important to emphasize that in April, May and early June it is possible to isolate two chemically very different essential oils ("yl-oils", "ol-oils") from the same plant.

Dynamics of individual constituents. Analysis of seasonal changes in the composition of essential oils from Dalmatian sage showed that different constituents had different dynamics and different trends of changes in concentration during the season. Almost all differences can be classified into three basic types of change (decreasing, increasing and fluctuating type), which was statistically supported by very high R^2 factors calculated in regression analysis with aim to identify the level of dependency of variation of chemical composition of essential oils in regard to the month of harvesting (Table 3; Fig. 3)

Decreasing-type (Fig. 3A) – highest concentration occurred in spring, in the early stages of development of young leaves (April-May - "yl"). The concentration decreased rapidly during the hot and dry summer months (June-September - "ol"), then moderately fluctuated or rose again during the wet and cold autumn-winter period (October-December - "ol"). This type of seasonal variation was typical for **α-humulene, viridiflorol, manool, β-caryophyllene** and **β-pinene.**

Increasing-type (Fig. 3B) – lowest concentration occurred in the spring, in the first stages of development of young leaves (April - "yl"), then the concentration increased or fluctuated during the summer, autumn and winter period (June- March - "ol"). This type of seasonal variation was typical for **1,8-cineole, camphene, camphor, *cis*-thujone, limonene** and ***trans*-thujone**.

Fluctuating-type (Fig. 3C) – the lowest concentration occurred in the early stages of the development of young leaves (April-May - "yl"), then the concentration rose sharply during the first summer months (May-Jun - "yl"), which led to regular fluctuations in concentration. In the warmest period (August-September) the concentration decreased, then increased again, reaching a second maximum in the autumn (November - "ol"), after which concentration decreased, returning to the winter months. This type of seasonal variation was typical for **borneol** in all samples, **bornyl acetate** for the "Učka" sample and **1,8-cineole** for the "Beograd" sample.

Many studies on season-dependent variations in the essential oil composition of *Salvia officinalis* have shown that the essential oil composition varied significantly over the year (Putievsky *et al.* 1986; Perry *et al.* 1999; Santos-Gomes & Fernandes-Ferreira 2001; Zutić *et al.* 2003; Mirjalili *et al.* 2006; Bernotienė *et al.* 2007, Baranauskiene *et al.* 2011). The asynchronous variations

Table 2. Chemical composition of *Salvia officinalis* essential oils (%) – basic statistics

	DESCRIPTIVE STATISTICS						ANOVA					
							Stage		Month		Origin	
	Valid N	Mean	Minimum	Maximum	Std.Dev.	Standard Error	F	p	F	p	F	p
cis-Salvene	20	0.23	0.02	0.65	0.19	0.04	**7.667**	**0.012**	0.430	0.892	**9.047**	**0.007**
trans-Salvene	20	0.03	0.00	0.10	0.03	0.01	**5.785**	**0.027**	0.547	0.813	**6.182**	**0.022**
Tricyclene	20	0.16	0.00	0.33	0.12	0.03	1.244	0.279	0.503	0.844	**128.061**	**0.000**
α-Thujene	20	0.67	0.13	1.67	0.59	0.13	1.255	0.277	0.202	0.988	**62.272**	**0.000**
α-Pinene	20	2.62	0.38	5.18	1.88	0.41	0.031	0.863	0.273	0.969	**296.300**	**0.000**
Camphene	20	4.91	0.21	9.17	2.81	0.61	4.178	0.055	0.935	0.532	**50.351**	**0.000**
Sabinene	20	0.96	0.00	3.00	0.98	0.21	0.422	0.524	0.455	0.877	**40.429**	**0.000**
β-Pinene	20	3.37	0.30	13.49	3.15	0.69	**7.214**	**0.015**	0.335	0.944	**10.651**	**0.004**
Myrcene	20	0.98	0.62	1.35	0.22	0.05	**15.918**	**0.001**	**6.111**	0.003	0.080	0.780
α-Phellandrene	20	0.09	0.00	0.28	0.07	0.02	3.011	0.099	0.446	0.882	**16.555**	**0.001**
α-Terpinene	20	0.48	0.00	1.46	0.49	0.11	1.883	0.186	0.219	0.985	**40.563**	**0.000**
p-Cymene	20	0.91	0.03	3.63	1.18	0.26	3.716	0.069	0.319	0.951	**14.599**	**0.001**
Limonene	20	4.28	0.47	9.05	2.87	0.63	1.590	0.223	0.630	0.751	**49.813**	**0.000**
1,8-Cineole	20	4.78	1.24	19.39	3.77	0.82	**8.818**	**0.008**	0.864	0.580	0.073	0.790
cis-β-Ocimene	20	0.71	0.00	4.81	1.26	0.28	**26.509**	**0.000**	1.784	0.181	0.003	0.960
trans-β-Ocimene	20	0.17	0.00	1.67	0.38	0.08	**14.224**	**0.001**	1.079	0.445	0.518	0.480
γ-Terpinene	20	1.08	0.22	2.86	0.94	0.21	0.505	0.486	0.178	0.992	**66.615**	**0.000**
cis-Sabinene hydrate	20	0.54	0.11	1.46	0.44	0.10	0.904	0.354	0.140	0.997	**65.227**	**0.000**
Terpinolene	20	0.44	0.07	0.90	0.24	0.05	2.818	0.110	1.446	0.278	**8.286**	**0.010**
trans-Sabinene hydrate	20	1.11	0.32	2.07	0.60	0.13	0.239	0.631	0.299	0.960	**34.470**	**0.000**
cis-Thujone	20	18.63	6.73	28.54	7.17	1.56	**18.154**	**0.000**	0.788	0.634	**7.719**	**0.012**
trans-Thujone	20	3.10	0.74	14.46	2.84	0.62	3.017	0.099	0.341	0.941	**5.832**	**0.026**
α-Campholenal	20	0.04	0.00	0.19	0.07	0.01	3.465	0.078	0.464	0.870	**5.974**	**0.024**
iso-3-Thujanol	20	0.02	0.00	0.13	0.03	0.01	1.147	0.298	1.116	0.425	0.289	0.597
trans-Sabinol	20	0.06	0.00	0.28	0.08	0.02	0.646	0.432	0.650	0.736	**11.766**	**0.003**
Camphor	20	21.42	1.87	32.71	8.26	1.80	**16.314**	**0.001**	**4.378**	**0.012**	0.750	0.397
trans-Pinocamphone	20	0.16	0.00	0.98	0.21	0.05	1.872	0.187	0.495	0.850	**4.543**	**0.046**
Borneol	20	1.36	0.35	3.04	0.78	0.17	2.304	0.146	0.562	0.802	**22.300**	**0.000**
cis-Pinocamphone	20	0.21	0.00	1.94	0.56	0.12	1.172	0.293	1.980	0.142	1.718	0.206
Terpinen-4-ol	20	0.58	0.07	2.16	0.57	0.12	0.329	0.573	0.566	0.799	**11.328**	**0.003**
α-Terpineol	20	0.06	0.00	0.18	0.06	0.01	2.584	0.124	**3.763**	**0.021**	0.818	0.377
Myrtenol	20	0.03	0.00	0.13	0.05	0.01	0.344	0.564	1.053	0.460	**5.661**	**0.028**
Verbenon	20	0.01	0.00	0.17	0.04	0.01	0.588	0.453	1.190	0.386	1.391	0.253
Bornyl acetate	20	0.51	0.00	1.73	0.44	0.09	0.032	0.859	1.332	0.322	**4.677**	**0.044**
trans-Sabinyl acetate	20	0.00	0.00	0.07	0.01	0.00	0.388	0.541	1.106	0.430	1.106	0.306
β-Caryophyllene	20	1.58	0.36	4.70	1.21	0.26	**21.018**	**0.000**	1.037	0.469	**6.425**	**0.020**
α-Humulene	20	9.45	3.43	33.30	7.96	1.74	**27.455**	**0.000**	1.715	0.197	1.547	0.229
Dehydroaromadendrane	20	0.09	0.00	0.31	0.08	0.02	0.206	0.655	**5.990**	**0.004**	1.147	0.298
γ-Muurolene	20	0.01	0.00	0.06	0.02	0.00	0.831	0.373	0.990	0.497	2.476	0.132
Viridiflorene	20	0.11	0.00	0.39	0.11	0.02	**4.449**	**0.048**	2.783	0.056	0.735	0.402
δ-Cadinene	20	0.01	0.00	0.06	0.02	0.00	0.810	0.379	0.996	0.494	2.407	0.137

	DESCRIPTIVE STATISTICS						ANOVA					
							Stage		Month		Origin	
	Valid N	Mean	Minimum	Maximum	Std.Dev.	Standard Error	F	p	F	p	F	p
Caryophyllene oxide	20	0.15	0.00	0.42	0.10	0.02	0.291	0.596	**3.847**	**0.020**	2.423	0.136
Viridiflorol	20	7.13	2.92	12.43	2.92	0.64	1.831	0.192	2.208	0.108	**8.573**	**0.009**
Humulene epoxide I	20	0.13	0.00	0.46	0.14	0.03	0.020	0.888	0.445	0.883	**10.085**	**0.005**
Humulene epoxide II	20	1.09	0.29	3.71	0.87	0.19	0.376	0.547	0.337	0.943	**9.536**	**0.006**
n.i.=not identified - 1	20	0.10	0.00	0.50	0.13	0.03	2.019	0.172	1.265	0.351	**5.348**	**0.032**
n.i.=not identified - 2	20	0.13	0.00	0.36	0.13	0.03	**11.792**	**0.003**	**4.345**	**0.013**	2.957	0.102
Manool	20	5.03	1.42	14.49	3.29	0.72	**16.199**	**0.001**	**3.091**	**0.041**	0.054	0.818
n.i.=not identified - 3	20	0.29	0.00	0.72	0.18	0.04	**13.777**	**0.001**	**4.220**	**0.014**	0.341	0.566

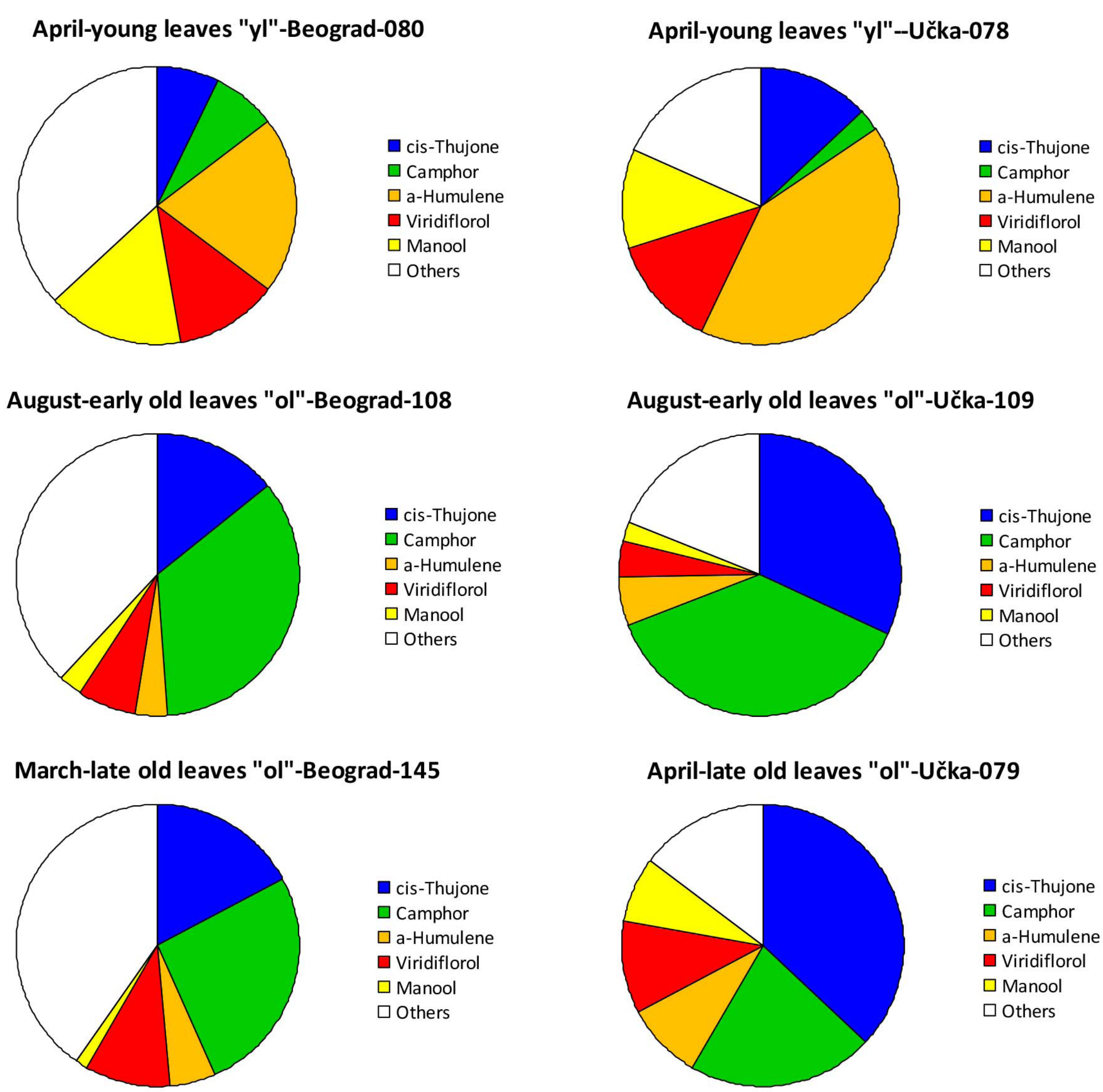

Fig. 2. Composition of main constituents of six oils of *Salvia officinalis* isolated from Beograd and Učka accession harvested in different months: April - young leaves, August - early old leaves, March - late old leaves (overwintered).

Table 3. Chemical composition of *Salvia officinalis* essential oils (%) - different month accessions (only dominant components are shown). A) decreasing-type, B) increasing-type, C) fluctuating-type.

	α-Pinene	Camphene	β-Pinene	Limonene	1,8-Cineole	*cis*-Thujone	*trans*-Thujone	Camphor	Borneol	Bornyl acetate	β-Caryophyllene	α-Humulene	Viridiflorol	Manool
Distribution type	C	B	A	B	B, C	B	B	B	C	B, C	A	A	A	A
R^2	0,37-0,92	0,91-0,98	0,69-0,98	0,92-0,97	0.34-0.82	0,94-0,97	0,89-0,96	0,87-0,94	0,83-0,97	0.96-0.99	0,96-0,99	0,97-0,98	0,93-0,97	0,82-0,95
April-Beograd	3.82	3.60	13.49	2.20	4.18	6.73	0.74	6.98	1.33	0.10	4.70	18.85	11.18	14.49
May-Beograd	4.10	5.56	6.68	5.85	6.90	7.04	0.90	18.02	3.04	0.76	4.34	11.42	10.60	6.39
Jun-Beograd	4.20	6.93	4.38	7.84	6.90	8.52	1.11	26.39	2.97	1.73	2.61	6.91	9.42	4.13
August-Beograd	4.27	8.51	2.71	8.30	5.80	13.34	1.71	32.71	1.96	1.37	1.20	3.43	6.13	2.68
September-Beograd	4.49	8.55	2.78	9.05	4.54	15.16	2.02	30.64	1.65	0.85	1.25	3.71	6.52	2.90
October-Beograd	4.40	8.41	4.07	7.42	5.15	20.05	2.40	27.38	1.62	0.71	1.04	3.84	5.72	2.10
November-Beograd	4.17	7.05	4.14	5.25	4.17	19.21	2.16	21.57	2.16	0.48	2.10	8.02	8.46	5.84
December-Beograd	4.13	6.73	5.23	5.15	3.73	20.96	2.42	19.18	1.27	0.38	1.73	7.46	10.28	4.62
January-Beograd	3.44	5.64	4.39	5.22	3.02	19.65	2.29	17.66	1.32	0.31	2.02	8.81	12.43	7.46
March-Beograd	5.18	8.33	5.33	7.61	5.80	15.87	1.80	24.21	1.73	0.18	1.33	4.89	9.07	1.42
April-Učka-yl	0.60	0.21	3.50	0.47	5.77	10.62	1.41	1.87	0.35	0.00	2.53	33.30	10.69	9.25
May-Učka	0.38	0.90	0.99	0.98	5.20	15.91	2.30	12.66	0.93	0.18	2.19	26.68	6.01	7.58
Jun-Učka	1.90	1.20	6.50	0.97	19.39	16.54	2.39	10.67	1.93	0.45	1.19	16.18	2.92	8.96
August-Učka	0.70	3.63	0.30	2.55	2.46	25.91	4.29	30.44	0.58	0.41	0.37	4.52	3.30	1.98
September-Učka	0.68	3.65	0.45	2.33	2.38	27.48	4.07	28.17	0.54	0.25	0.36	4.49	3.52	2.22
October-Učka	0.67	3.64	0.41	2.46	2.01	28.32	3.88	27.70	0.68	0.26	0.38	4.53	3.32	1.67
November-Učka	0.65	3.19	0.43	2.23	1.62	28.46	3.96	24.88	0.89	0.31	0.60	6.88	5.61	4.96
December-Učka	0.71	3.25	0.36	2.48	1.24	28.12	4.18	18.75	0.54	0.24	0.49	6.10	6.89	6.36
April-Učka-ol	0.51	2.05	0.54	1.69	1.51	28.54	4.11	16.43	0.41	0.09	0.50	6.74	8.34	5.60

in concentration of individual constituents appears to be the rule rather than the exception. This means that a component from plants from different geographical areas and different habitats, may have very different trends of variation during the season.

***Variation in the quality of essential oil in relation to the standard ISO 9909*.** Given that Dalmatian sage essential oil belongs to the group of the most important essential oils, variations in its composition may have a very important practical significance. Too high, or too low a concentration of individual components may cause some plants to lose their medicinal properties, or even to become toxic. Consequently, ISO 9909 (1999) standard for medicinal uses regulates the amounts of the following constituents in the oil: ***cis*-thujone** (18.0–43.0%), **camphor** (4.5–24.5%), 1**,8-cineole** (5.5–13.0%), ***trans*-thujone** (3.0–8.5%), **α-humulene** (≤ 12.0%), **α-pinene** (1.0–6.5%), **camphene** (1.5–7.0%), **limonene** (0.5–3.0%), **bornyl acetate** (≤ 2.5%) and **linalool + linalyl acetate** (≤ 1.0%) (Santos-Gomes & Fernandes-Ferreira 2001; Mockutė *et al.* 2003).

Comparing our essential oils with chromatographic profiles defined by ISO 9909, we found that only 3 samples met the appropriate requirements (SalOff-018 November, SalOff-059 December and SalOff-079 from overwintered leaves in April) (Table 1). The majority of the summer samples did not meet ISO 9909. For example, from June to November plants from Učka gave oils that had too high amounts of camphor, a-humulene and *trans*-thujone and too low concentrations of *cis*-thujone and α-pinene. Oil quality that met ISO 9909 in these plants was achieved only in November, December and only from overwintered leaves in April.

Additionally, our analysis of published data (Ivanić *et al.* 1978; Kuštrak *et al.* 1984; Ristić *et al.* 1999; Couladis

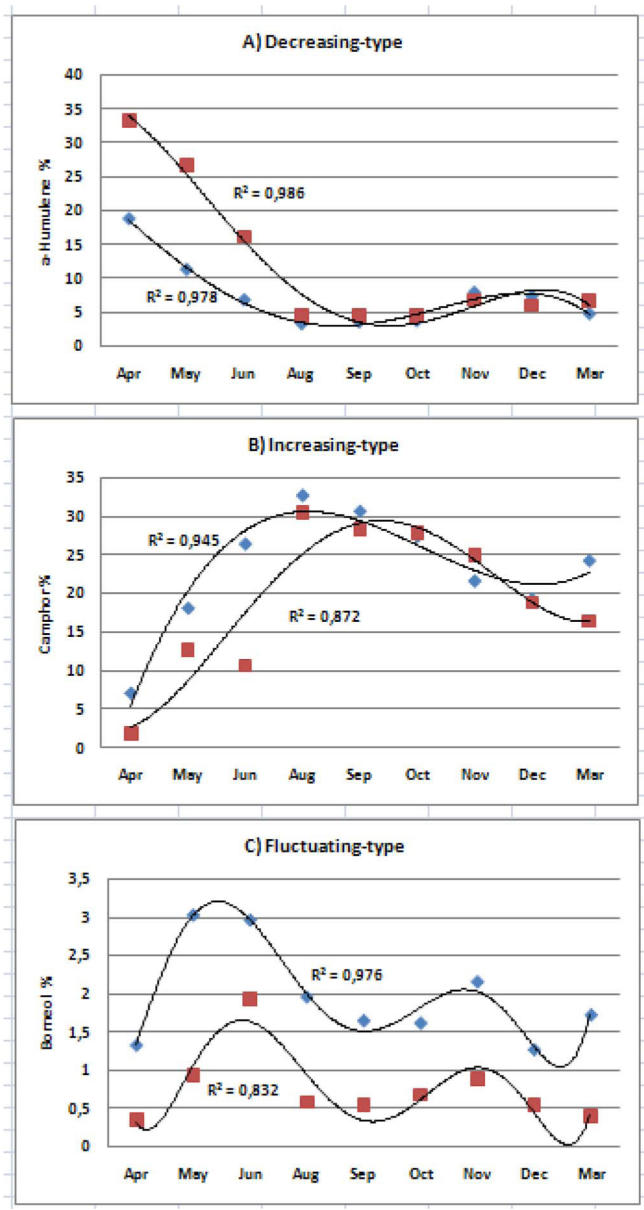

Fig. 3. Basic types of changes in the concentration of constituents of essential oils from *Salvia officinalis* during season (◆ - Beograd, ■ - Učka): A) decreasing-type, B) increasing-type, C) fluctuating-type. Growing cycle, presented the on X-axis, began with young and incompletely developed leaves harvested in April. For details see Table 3.

et al. 2002; Šatović 2011; Jug-Duјaković *et al.* 2012) on 81 oils from wild and cultivated plants throughout the Balkan Peninsula, has shown that even 75.6% of all the oils did not match ISO 9909 standard. It is particularly important to note that in the period May-August, when the sage is usually harvested for industrial purposes, only 20.1% of the samples gave satisfactory oil quality. In contrast, plants harvested in the period December-April, gave satisfactory oil quality in 38.5% of cases.

CONCLUSIONS

There were large variations in the essential oil yields ranging from 0.2-2.9%. The chemical composition of essential oils from the same individuals (same genotypes) during one growing season changed significantly. In the essential oils of young leaves (April – "yl"), sesquiterpenoids were dominant (50.7 - 57.0%), while the development of leaves increased the amount of monoterpenoids (55.4-88.4%). Leaves of the same plant in different stages of development, formed different types of oils. It is important to emphasize that in April, May and early June it is possible to isolate chemically very different essential oils from the same plant.

On the basis of all the analyses, it could be concluded that yield and composition of the oil depends, primarily on the ontogenetic phase of leaf development (young and old leaves), but also on the origin of the plant. Oils tested in the same phenophases differed mainly because of different plant origins. Samples from Beograd ("ol") belonged to the camphor type and those from Učka to the thujone one.

Because of large variations in the concentrations of individual constituents, the majority of essential oil samples of Dalmatian sage did not meet the ISO 9909 standard.

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Botanica SERBICA

REZIME

Variranje prinosa i sastava etarskih ulja dalmatinske žalfije (*Salvia officinalis* Lamiaceae) u različitim fazama razvoja

Branislava S. Lakušić, Mihailo S. Ristić, Violeta N. Slavkovska,
Danilo Lj. Stojanović, Dmitar V. Lakušić

Variranje prinosa i sastava etarskog ulja dalmatinske žalfije (*Salvia officinalis* L., Lamiaceae) u različitim fazama razvoja analizirano je na dve individue (= genotipa) različitog geografskog porekla. Obe biljke su uspešno gajene deset godina pod identičnom ekološkim uslovima u privatnoj bašti u Beogradu. Količina i sastav ulja su analizirani tokom jedne vegetacione sezone počevši od mladih pa sve do starih prezimelih listova.

Rezultati klaster analize su pokazali da starost listova kao i poreklo biljke ima veoma značajan uticaj na sastav etarskog ulja. Svi uzorci ulja su formirali dve osnovne klade. Prvu kladu čine ulja mladih listova sakupljanih od aprila do juna i pripadaju α-humulen tipu. Druga klada obuhvata ulja dobijena uglavnom od starih listova, koji su sakupljani od avgusta do juna, i koji pripadaju kamfor ili tujon tipu. U drugoj kladi uzorci poreklom sa Učke su potpuno odvojeni od uzoraka poreklom iz Beograda.

Takođe, na osnovu ovih analiza je utvrđeno da ista individua (isti genotip) u toku jedne vegetacione sezone značajno menja hemijski sastav etarskog ulja. Tako je moguće razlikovati tri različita „fenološka tipa etarskih ulja": ulja mladih listova, ulja rane faze starih listova i ulja kasne faze starih listova. Analiza sezonskih promena u sastavu etarskih ulja Dalmatinske žalfije je pokazala da različite komponente imaju različitu dinamiku i različite pravce promena u koncentraciji tokom sezone.

Ključne reči: *Salvia officinalis,* etarska ulja, kamfor, tujon, α-humulen