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Seasonal Variations in Essential Oil Composition of Immortelle Cultivated in Serbia

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Abstract: Our previous research has proven that the immortelle (*Helichrysum italicum*) essential oil (EO) grown in Serbia possesses respectable biological potential and desirable composition of volatile compounds with the potential for a wide range of applications in the food, cosmetics, and pharmaceutical industries. Within this study, the impact of seasonal variations (temperature, precipitation, and insolation) during three successive years (2017, 2018, and 2019), on the volatile profile of γ-curcumene + *ar*-curcumene immortelle chemotype was determined. Steam distillation was utilized to extract EO from the plant material, followed by chromatographic mass spectrometric analysis revealing 50 volatile compounds. A multiple linear regression model was developed, and principal component analyses were conducted to deliver detailed information regarding the prediction, component profile, and parallel contents of active compounds of the immortelle EO. Under Serbian agro-ecological conditions, with appropriate harvest method, immortelle can achieve two harvests per year: the first in July, and the second in August. The γ-curcumene + *ar*-curcumene chemotype usually occurs as the dominant chemotype in the region of the ex-Yugoslavia countries. This chemotype probably developed as an adaptation to climatic conditions, and spring and summer precipitation positively influenced curcumene accumulation in plants. Such a phenomenon was especially noticeable in the first harvest.

Keywords: *Helichrysum italicum*; essential oil; principal component analysis

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1. Introduction

Immortelle (*Helichrysum italicum* (Roth) G. Don) is a native plant of the Mediterranean region [1], and it is widely spread in southern Europe (Bosnia and Hercegovina, Croatia, Italy, Portugal, and Spain) [2]. However, due to climate change, many typical Mediterranean plants can now be grown in a continental climate [3]. Because of that, immortelle, as a plant of arid areas [4], can be successfully cultivated in Serbia and Bulgaria [5,6]. As a perennial plant, immortelle is usually cultivated in the same field for five to eight years [7]. For this reason, choosing the appropriate plant genetics, climate, and soil, as well as altitude, are important parameters that may significantly influence the EO content and composition [8–10]. Further, harvest time and distillation method could also significantly affect the plant's EO content and profile [11–13].

Previous research has shown that the EO quantity in immortelle varied from 0.02 to 0.78%, depending on the origin, growing locality, altitude and climate, subspecies, harvest time, etc. In total, cluster analysis (unrooted phylogenetic tree) of different immortelle accessions reported in the literature shows presence of 10 chemotypes including the following: (1,2) neryl acetate (moderate with 19.5–48.0% and high with 50.5–83.4%), (3,4) α-pinene (moderate with 5.6–20.0% and high with 25.2–53.5%), (5) γ-curcumene, (6) juniper camphor, (7) β-selinene, and (8) italidiones, as well as combined chemotypes such as (9) neryl acetate + *ar*-curcumene and (10) γ-curcumene + *ar*-curcumene [5]. However, in the territory of former Yugoslavia (Herzegovina, Dalmatia, and Montenegro), the latter chemotype (γ-curcumene + *ar*-curcumene) is the dominant one [5,14,15]. This chemotype possesses intense curry-like aroma because of curcumene, which gives a typical spicy odor and is commonly known as the "curry plant" [2].

Immortelle has been a widely used plant in traditional medicine since ancient times in regions where it grows spontaneously. It is consumed internally as herbal tea (against fever, respiratory, and digestive disorders) or externally for treating skin inflammation and wounds [16]. Today its popularity has risen because of the commercial application of its EO in cosmetic products [17]. However, the biological activity of immortelle EO strongly depends on its composition resulting from the interaction of its constituents [18]. This plant is considered to have promising pharmacological applications [19], because the immortelle EO has shown anti-inflammatory [5,20–22], anticancer [23,24], antimicrobial [25–27], and antioxidant activity [28–31]. Further, wound healing and skin care application have been well-known and widely applied [32,33]. Recent clinical trials confirmed the safety profile since no adverse effects have been reported [34]. Therefore, this plant is considered safe to be used externally as well as internally. In addition, immortelle has demonstrated insecticidal activity [35,36], positioning this plant species as a suitable candidate for use in biopesticide formulations [37].

Prior studies dealing with EO quantity and quality show that seasonal variation may significantly affect oregano [38], thyme [39], sage [40], rosemary [41], lavandin [42], and other plant EOs. The chemical composition of immortelle EO collected during five different periods of the year—vegetative phase (February and October), pre-flowering (May), flowering (June), and post-flowering (August) showed changes in the amount of volatile compounds [28]. Furthermore, crop age as well as environmental factors during vegetation significantly influenced EO content and composition [43]. Further, the seasonality of flavonoids and phenylpropanoid derivatives has also been noted in this plant [44]. In addition, fertilization could be an important factor in the production of immortelle. Relatively lower yields in organic production may be offset by a higher price of EO on the market, so that the investment is economically profitable [45,46].

The dissimilarities acquired in the production and collection of some constituents of the EO, particularly the major ones, appear to be associated, but not solely, with modifications in abiotic environmental aspects, such as soil nutrients and climate, i.e., the habitat where plants grew [5,15,47,48]. However, the correlation between genetic and biotic environmental characteristics on the composition of EOs should also be evaluated. Nevertheless, these results are particularly indicative concerning the difference in the accumulation of α-pinene in the immortelle EO [6,49].

The present paper aimed to evaluate the impact of climatic parameters, such as temperature, precipitation, and insolation, on single compounds content in immortelle EO during three-year vegetation period. This investigation analyzed only flowering tops of immortelle, collected during the full blooming period (once during the first year, and twice during the second and third year). The γ-curcumene + *ar*-curcumene chemotype was selected for the study since it is the most widespread chemotype in the western Balkan countries.

2. Materials and Methods

2.1. Plant Material

Immortelle plantation was established on 30.03.2017 in Banatska Topola (45°40′37″ N; 20°28′32″ E) from seedlings produced in the greenhouse (Čapljina, Bosnia and Herzegovina)

(Figure 1a). The plant species was confirmed as *Helichrysum italicum* (Roth) G. Don, by Milica Rat, curator of BUNS Herbarium (University of Novi Sad) where the accession is deposited under Vouch. No. 2-1373. The experiment was conducted in a large-scale field plot on 1 ha under a certified organic production system using black agricultural mulch film (Figure 1b). The distance between rows was 2 m, and 0.3 m between plants in the row, with approximately 16,700 plants per hectare. Figure 1b illustrates the immortelle field utilized in this investigation.

(**a**) (**b**)

Figure 1. Immortelle seedlings production in a greenhouse, ready for transplantation (**a**); immortelle production field (**b**).

The first harvest of immortelle under the agro-ecological conditions of Serbia was performed in the second half of July, and due to regrowth, the second harvest was performed in August. However, during the first field production year, the plants developed slowly, five months after transplanting, the immortelle plants were in the phenophase of full blooming, and the harvest was in the middle of October before the first frost occurred.

2.2. Weather Conditions

Weather conditions during the three growing seasons are given in Figure 2, using data from the nearest meteorological station (~1 km). Figure 2a shows the average daily temperatures, Figure 2b shows the sum of precipitation, and Figure 2c shows the sum of insolation for 2017, 2018, and 2019 years, which were monitored in this investigation (months listed on the *x*-axis of the graph). Using the yearly averages of observed data, multiple linear regression models were computed to indicate the quantity of characteristic volatile compounds in the immortelle EO. In addition, the attained multiple linear regression models demonstrated the effect of the seasonal variations (the average daily temperatures, the sum of precipitation, and the sum of insolation) on the concentration of individual EO compounds.

Average daily temperatures during spring months (March–May) were very favorable for the initial growth of immortelle. During the second growing season, in 2018, the lowest temperatures and insolation as well as the highest precipitation amount in comparison to other years were recorded in March, while higher temperatures characterized April and May compared to other years. The average daily temperatures during the summer months (June–August) ranged between 21.0 °C and 24.5 °C, while the monthly sum of sunny hours was between 281.7 and 361.8 h.

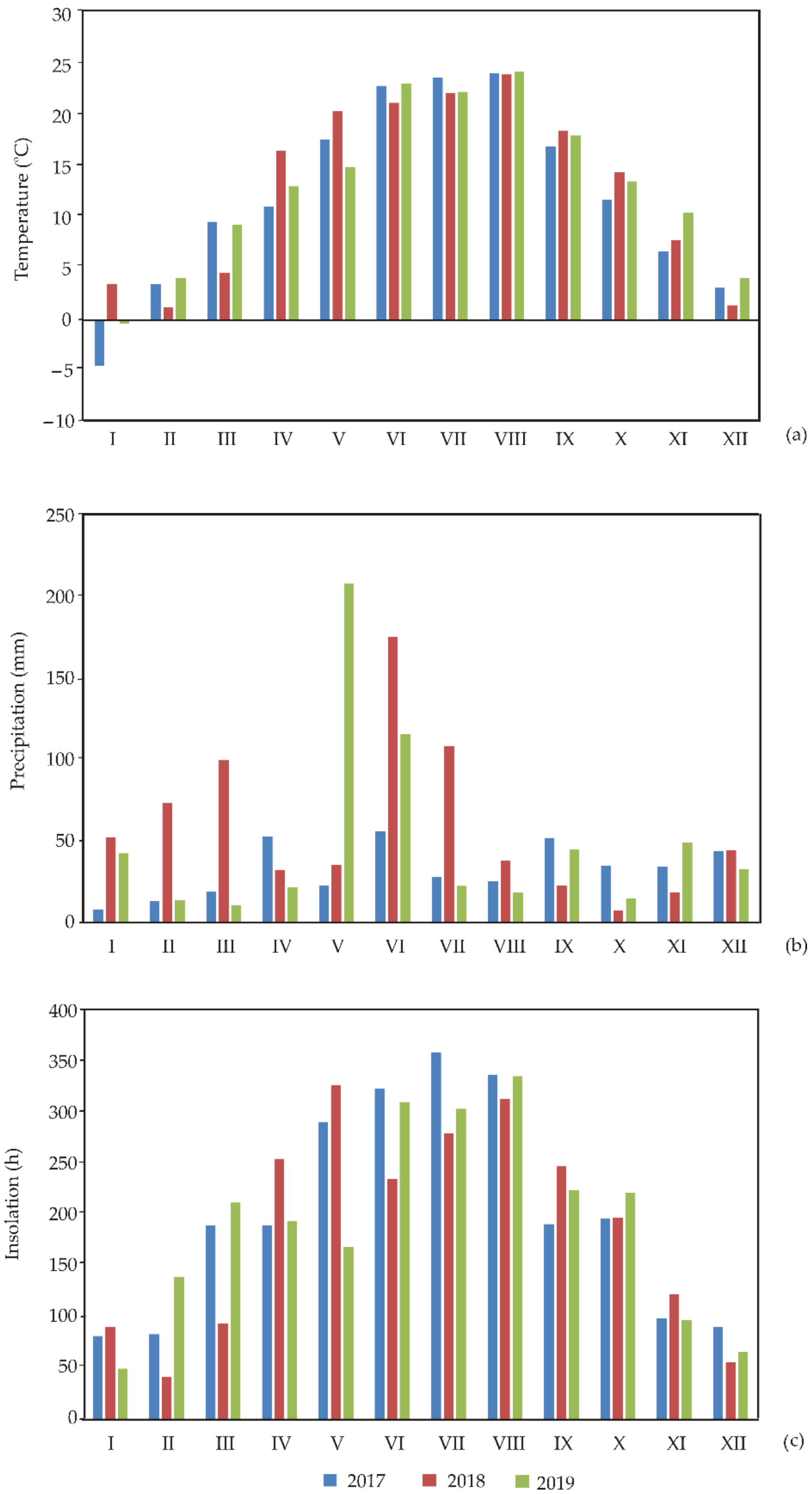

Figure 2. The average daily temperatures (**a**), the sum of precipitation (**b**), and the sum of insolation (**c**) during observed period.

2.3. Soil Condition

The immortelle plantation was established on calcareous chernozem soil type, at an altitude of 78 m. Basic chemical properties of the soil were determined in samples of the 0 to 30 cm layer before crop establishment in the spring of 2017, and again in the spring of 2020 (Table 1).

Table 1. Basic chemical characteristics of the soil.

Year	pH [1]		$CaCO_3$ [2] (%)	Humus [3] (%)	Total Nitrogen [4] (%)	AL-P_2O_5 [5] (mg/100 g Soil)	AL-K_2O [5] (mg/100 g Soil)
	in KCl	in H_2O					
2017	7.33	8.32	3.16	3.76	0.210	28.04	39.59
2020	7.08	8.12	4.51	3.72	0.255	20.54	38.60

[1] pH value of soil was determined potentiometrically in suspension with 1 M KCl and distilled water; [2] $CaCO_3$ content was determined by volumetric method; [3] Organic carbon (humus) was determined by sulfochromic oxidation; [4] Total nitrogen was determined using the Semimikro–Kjeldahl method; [5] Available phosphorus and potassium in soil were determined by extraction with ammonium lactate (AL) solution.

2.4. Essential Oil Extraction

Immediately after harvest, fresh immortelle plant material was distilled in an industrial steam distillation unit. On average, 250 kg of immortelle was placed in the extraction tank (volume 1200 L), closed with a lid, and supplied with steam from a detached steam-generator unit. The tank is connected to the condenser and cooler, and the end distillate was collected in a Florentine tank. After 4 h, the essential oil was decanted and placed in a separation funnel overnight. All samples of EO obtained from one harvest were homogenized in order to obtain one aggregate sample. The EO was dried over anhydrous Na_2SO_4, filtered, and stored in dark glass at 4 °C.

2.5. Chemical Analysis of Essential Oils

The EOs samples were dissolved in absolute ethanol due to their good solubility in low concentrations in solvents that are less polar than water and analyzed on an Agilent 6890N (Beijing, China) gas chromatograph equipped with a CTC Analytics CombiPal autosampler (Zwingen, Switzerland), a non-polar HP5-MS column (length: 30 m, inner diameter: 250 µm and film thickness: 0.25 µm), and an Agilent 5975B mass spectrometer (Santa Clara, CA, USA). The injector temperature was 230 °C with an injection volume of 1 µL, a split ratio of 1:50, and a carrier gas (He) flow rate of 1.0 mL/min. The oven temperature program was as follows: 60° C, ramp rate of 3 °C/min, and final temperature of 240 °C held for 2 min. The MS source temperature was set to 230 °C, the single quad temperature was 150 °C, and the transfer line temperature was set to 280 °C. The mass range analyzed by the mass spectrometer was 35.00–500.00 amu. Identification of EO constituents was performed by a computer matching of mass spectra with ADAMS and NIST mass spectral database and by comparison of their Linear Retention Indices (LRI) relative to a series of n-hydrocarbons (C9–C40). Since a mass detector is more sensitive than FID, and components in low concentrations had to be analyzed in order to have valid results, and mass detector data were used for quantitative analysis. The content of a particular component in the immortelle EO was determined as percentage of particular peak area in sum of all peak area.

2.6. Multiple Linear Regression Model

Following temperature, precipitation, and insulation data, a multiple linear regression model was developed to predict the immortelle EO main constituents. Additionally, the valid regression coefficients were defined in the form of $x = b_{Temp} \times \text{Temp} + b_{Ins} \times \text{Ins} + b_{Perc} \times \text{Perc}$, where x is the percentage of the compound in EO (%); b_{Temp}, b_{Ins}, and b_{Perc} present the regression coefficients; Temp is temperature (°C); Ins is insolation; and Perc is precipitation. The regression coefficients utilized in linear regression models were computed by minimizing the sum of squared residuals per model by employing the Solver function in Microsoft Excel ver. 2015, Redmond, Washington, US.

2.7. Statistical Analysis

Principal Component Analysis (PCA) was completed to investigate the immortelle EO profiles. The perspective tendency for a deeper cognition of the EO component profile could be acknowledged by adopting the grouped samples' PCA diagram. Statistical study of the data was accomplished using the Statistica 10 software (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results

The dominant compounds in the immortelle EO during the three years of the investigation were as follows: sesquiterpenes γ-curcumene (12.30–19.60%) and *ar*-curcumene (10.00–15.78%), followed by α-pinene (7.52–19.24%), neryl acetate (5.89–8.71%), and *trans*-caryophyllene (4.69–6.71%); after that, the compounds α-selinene (3.95–5.27%), italicene (3.01–4.55%), α-copaene (2.87–3.84%), limonene (1.18–3.11%), 2-methylcyclohexyl pentanoate (0.00–3.68%), δ-cadiene (1.31–2.00%), *trans*-α-bergamotene (1.19–1.49%), γ-cadiene (1.02–1.51%), neryl propanoate (0.82–1.10%), and caryophyllene oxide (0.18–1.04%) were found (Table 2). In all five harvests, the dominant class was sesquiterpenes with variations between 52.27 and 64.59% (Table 2).

Table 2. Composition of immortelle EO during the three-year period (in %).

No.	Compound	Chemical Group	RI_{lit}	RI_{exp}	2017	2018-1	2018-2	2019-1	2019-2
1	α-pinene	monoterpene	936.1		11.52	7.52	19.24	14.12	14.54
2	α-fenchene	monoterpene	949.4		0.16	0.10	0.39	0.24	0.29
3	β-pinene	monoterpene	977.7		0.30	0.20	0.65	0.35	0.44
4	δ-2-carene	monoterpene	1003.3	1015.7	0.12	0.10	0.24	0.21	0.30
5	*p*-cymene	monoterpene	1024.3	1022.9	0.37	0.19	0.65	0.23	0.12
6	limonene	monoterpene	1029.5	1027.3	1.86	1.18	3.11	2.57	2.73
7	1,8-cineole	monoterpene	1031.8	1029.2	0.35	0.19	0.44	0.33	0.21
8	γ-terpinene	monoterpene	1059.7	1056.5	0.30	0.23	0.54	0.46	0.51
9	terpinolene	monoterpene	1086.9	1087.3	0.13	0.10	0.21	0.20	0.21
10	linalool	monoterpene	1099.0	1100.2	0.87	0.96	0.92	1.06	0.66
11	isoamyl tiglate	ester	1168.0	1152.1	0.97	0.57	0.96	1.38	1.77
12	borneol	monoterpene	1166.2	1163.4	0.11	0.07	0.07	0.12	0.10
13	terpinen-4-ol	monoterpene	1177.1	1175.4	0.32	0.28	0.32	0.31	0.29
14	α-terpineol	monoterpene	1189.7	1189.4	0.32	0.31	0.30	0.35	0.37
15	nerol	monoterpene	1228.9	1227.2	0.26	0.33	0.23	0.43	0.46
16	neryl acetate	monoterpene ester	1362.9	1366.0	7.00	7.43	5.89	6.96	8.71
17	α-ylangene	sesquiterpene	1369.9	1369.1	0.26	0.27	0.25	0.33	0.00
18	α-copaene	sesquiterpene	1376.2	1374.0	2.92	3.26	2.87	3.84	3.34
19	isoitalicene	sesquiterpene	1396.7	1395.9	0.10	0.13	0.10	0.16	0.16
20	italicene	sesquiterpene	1401.8	1401.0	3.10	3.64	3.01	4.55	4.45
21	*cis*-α-bergamotene	sesquiterpene	1414.5	1414.0	1.48	1.59	1.55	1.31	1.50
22	*trans*-caryophyllene	sesquiterpene	1420.1	1417.4	5.65	6.71	4.69	6.20	5.76
23	*trans*-α-bergamotene	sesquiterpene	1434.5	1434.3	1.45	1.37	1.34	1.25	1.19
24	2-methylcyclohexyl pentanoate	ester		1441.0	3.60	3.68	0.00	2.67	0.00
25	α-humulene	sesquiterpene	1453.1	1450.1	0.33	0.39	0.29	0.37	0.37
26	neryl propinoate	monoterpene ester	1452.0	1454.5	0.99	1.07	0.84	0.82	1.10
27	β-acoradiene	sesquiterpene	1465.5	1461.1	0.24	0.27	0.21	0.31	0.30
28	β-patchoulene	sesquiterpene		1473.2	2.15	2.17	1.99	1.90	2.27
29	γ-curcumene	sesquiterpene	1480.3	1481.3	13.64	17.15	12.66	18.08	12.30
30	*ar*-curcumene	sesquiterpene	1482.2	1484.7	15.78	13.41	11.78	10.00	11.80
31	α-selinene	sesquiterpene	1493.4	1494.3	4.97	4.96	4.44	3.95	4.95
32	α-muurolene	sesquiterpene	1498.3	1498.6	0.48	0.53	0.37	0.51	0.51
33	δ-amorphene	sesquiterpene		1505.0	0.09	0.22	0.16	0.35	0.54
34	β-bisabolene	sesquiterpene	1508.4	1507.5	0.18	0.00	0.15	0.00	0.00
35	γ-cadiene	sesquiterpene	1513.1	1511.5	1.02	1.29	1.32	1.12	1.51
36	δ-cadiene	sesquiterpene	1523.2	1521.8	1.45	2.00	1.35	1.58	1.78
37	*cis*-cadina-1,4-diene	sesquiterpene	1531.0	1530.4	0.36	0.46	0.33	0.35	0.44
38	*cis*-α-Bisabolene	sesquiterpene	1540.3	1541.5	0.32	0.39	0.28	0.27	0.31
39	*trans*-nerolidol	sesquiterpene	1560.9	1563.1	0.25	0.36	0.17	0.16	0.16
40	caryophyllene oxide	sesquiterpene	1580.6	1578.8	0.94	1.04	1.01	0.33	0.18
41	guaiol	sesquiterpene	1597.1	1595.1	0.20	0.24	0.16	0.17	0.14

Table 2. *Cont.*

No.	Compound	Chemical Group	RI_{lit}	RI_{exp}	2017	2018-1	2018-2	2019-1	2019-2
42	rosifoliol	sesquiterpene	1600.0	1603.9	0.52	0.61	0.41	0.40	0.32
43	*epi*-1-Cubenol	sesquiterpene	1625.5	1625.5	0.18	0.24	0.13	0.14	0.16
44	γ-eudesmol	sesquiterpene	1630.9	1630.9	0.17	0.22	0.13	0.15	0.11
45	*epi*-α-cadinol	sesquiterpene	1637.8	1638.3	0.20	0.24	0.17	0.14	0.10
46	β-eudesmol	sesquiterpene	1650.1	1646.1	0.13	0.17	0.10	0.11	0.08
47	selin-11-en-4-α-ol	sesquiterpene	1654.9	1650.8	0.37	0.41	0.28	0.26	0.20
48	β-bisabolol	sesquiterpene	1672.0	1667.9	0.25	0.32	0.21	0.17	0.20
49	neryl isovalerate	monoterpene ester		1729.6	0.20	0.28	0.14	0.17	0.15
50	β-costol	sesquiterpene	1756.0	1757.1	0.30	0.25	0.22	0.05	0.00
	Total monoterpenes + monoterpene esters				25.18	20.54	34.18	28.93	31.19
	Total sesquiterpenes				59.68	64.59	52.27	58.68	55.28
	Total esters				4.57	4.25	0.96	4.05	1.77
	Herbage yield (kg/ha)				510	9040	5750	12,940	7260
	Essential oil yield (kg/ha)				0.6	10.6	6.8	17.0	9.5

RI_{lit}—Retention indices from literature; RI_{exp}—Retention indices experimental on HP5-MS column; 2017—only one harvest in 2017 (15 October 2017) 2018-1—first harvest in 2018 (19 July 2018); 2018-2—second harvest in 2018 (24 August 2018); 2019-1—first harvest in 2019 (18 July 2019); 2019-2—second harvest in 2019 (10 August 2019)

Higher content of γ-curcumene of 17.15–19.60% was recorded in the first harvest performed in July, and lower content, 12.30–13.64%, was found in the second harvest conducted in August. The same trend was observed with the concentration of α-pinene; its concentration in the EO was 7.52–14.12% in June and July and 11.52–19.24% in August.

Correlation analysis establishes the similarities/dissimilarities in the chemical composition of the immortelle EO (Figure 3). The circles marked in the darker blue tone show a stronger correlation between observed samples, while the lighter color signifies a characteristic distinction between samples. Conversely, the circles marked in red color designate a negative correlation between the samples.

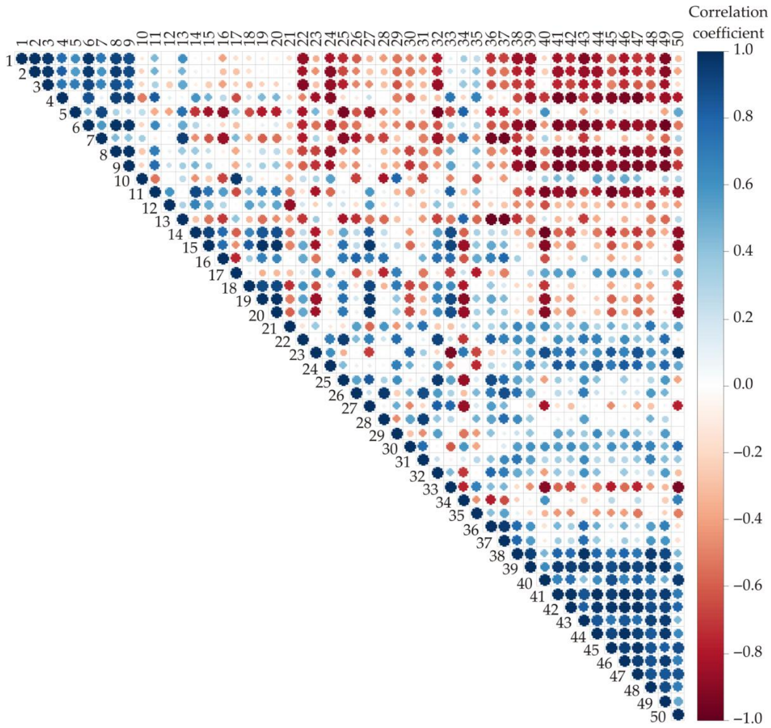

Figure 3. Color correlation diagram of active compounds content in the immortelle EO samples from 2017, 2018, and 2019 (active compounds codes are listed in Table 2).

Thoroughly illustrating the observed data structure, PCA was employed, and the acquired results are shown in Figure 4. The first two PCs illustrated 78.7% of the total variance in the experimental data. The first PC explained 45.72% and the second 32.98% of the total variance between the experimental data (Figure 4).

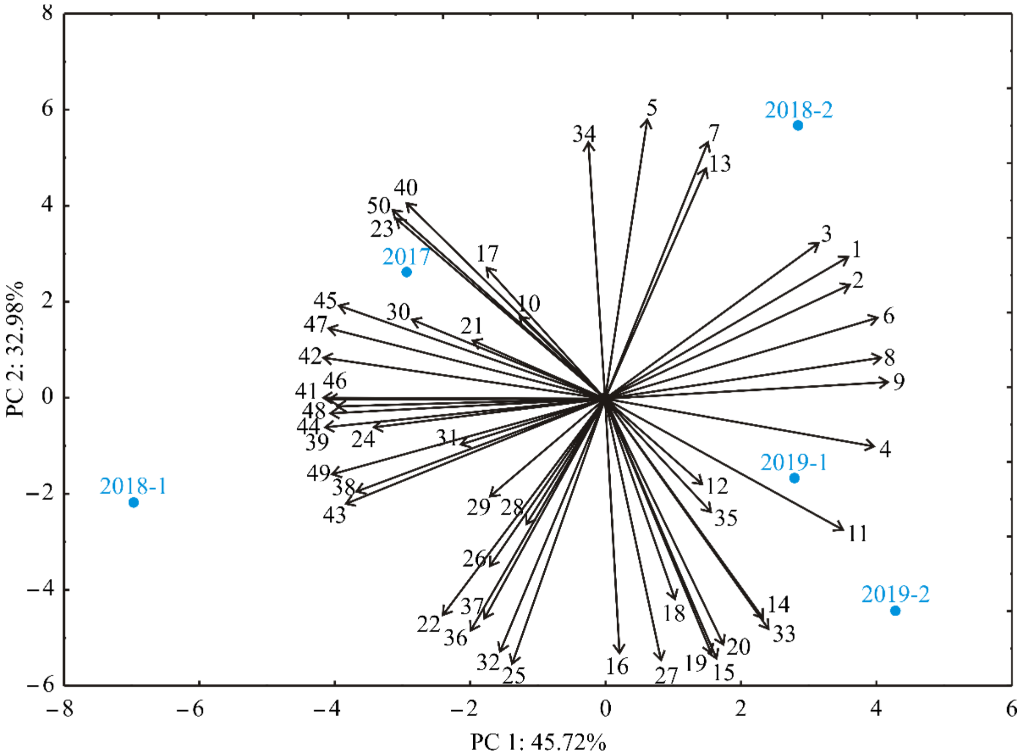

Figure 4. The PCA biplot diagram describing the relations between immortelle EO compounds (marked with black color) of samples from 2017, 2018, and 2019 (marked with blue color) (the compound codes and samples codes are noted in Table 2).

According to the PCA analysis, a greater positive influence on the first principal component (PC1) was observed for the content of the following: *α*-pinene (3.2%, based on correlations), *α*-fenchene (3.2%), *β*-pinene (2.4%), *δ*-2-carene (3.9%), limonene (4.0%), *γ*-terpinene (4.1%), terpinolene (4.3%), and isoamyl tiglate (3.1%). The most prominent negative influence on PC1 calculation was observed for the concentrations of the following: *trans*-*α*-bergamotene (2.3%, according to correlation), *β*-costol (2.4%), *cis*-*α*-bisabilene (3.3%), *trans*-nerolidol (4.2%), caryophyllene oxide (2.1%), guaiol (4.2%), rosifoliol (4.2%), *epi*-1-cubenol (3.6%), *γ*-eudesmol (4.0%), *epi*-*α*-cadinol (3.8%), *β*-eudesmol (4.1%), selin-11-en-4-*α*-ol (4.1%), *β*-bisabolol (3.9%), and neryl isovalerate (4.0%).

The strongest positive influence for the second principal component (PC2) calculation was noticed for the content of *trans*-*α*-bergamotene (2.4%, based on correlation), *β*-costol (2.6%), caryophyllene oxide (2.8%), *β*-bisabolene (4.8%), *p*-cymene (5.7%), 1,8-cineole (4.9%), and terpinen-4-ol 3.9%. The most powerful negative influence for PC2 calculation was noticed for the concentration of *α*-terpineol (3.6%, according to correlation), nerol (5.0%), neryl acetate (4.8%), *α*-copaene (2.9%), isoitalicene (4.7%), italicene (4.4%), *trans*-caryophyllene (3.4%), *α*-humulene (5.2%), *β*-acoradiene (5.1%), *α*-muurolene (4.7%), *δ*-cadiene (4.0%), and *cis*-cadina-1,4-diene (3.6%).

The partition within samples is noticeable from the PCA plot, where the samples from immortelle EO composition during 2019-1 and 2019-2 are grouped on the bottom right, 2018-1 and 2017 on the left, and 2018-2 on the top left side of the diagram. The vector's direction defining the variable in factor space implies an increasing tendency in these variables, while the vector size is proportionate to the square of the correlation values between the fitting value for the variable and the variable itself. The correlation between different compounds' content can be observed in Figure 4 the small angles between connected variables signify a high degree of their correlations.

4. Discussion

The immortelle EO has been widely studied. However, almost all studies have been focused on biological activities and the chemical composition of the plant material [5,9,14,18,20,23–31,33,35,36]. In addition, there have been studies aimed at finding a link between chemical composition and biological activities and explain their relationship [18,50]. For example, analysis of two different immortelle samples showed that samples that were rich in neryl acetate and γ-curcumene (33.87% and 8.84%, respectively) showed more prominent antimicrobial activity, as well as stronger antioxidant potential, in comparison to samples rich in α-pinene and γ-curcumene (20.84% and 16.53%, respectively) [51]. Similar results were reported with samples rich in neryl acetate, α-pinene, and γ-curcumene (15.6%, 11.5%, and 11.3%, respectively) [52]. The efficiency of immortelle EO as an antimicrobial agent is probably the result of a synergistic action between its major as well as minor constituents [50].

In addition, biochemical and genetic diversity has also been widely addressed [5,53]. For example, EO samples from seven locations in Italy, characterized by different environmental conditions, mainly by soil types, during three different periods (January, May, and October) showed a difference in the composition of the volatile compounds [48]. Further, in a previous study, the same genetic material as in this study was used (introduced from Hercegovina), and the dominant compounds were γ-curcumene (13.6%), β-selinene (12.2%), and α-pinene (11.8%) [5]. Similarly, samples from Montenegro also contained γ-curcumene (14.1%), β-selinene (11.3%), and α-pinene (10.4%) as major EO compounds [54]. These observed differences in the EO composition may result from the various microclimatic conditions and growing technologies, as well as the extraction techniques (hydrodistillation in Clevenger apparatus vs. semi-commercial steam distillation).

Immortelle is known for its affinity to grow in poor to moderately fertile soils [30,47,53]. In this study, this requirement was fulfilled by organic growth of immortelle with no soil fertilization between growing seasons. For that reason, the impact of soil fertility on immortelle EO composition was not analyzed in this study.

Immortelle, due to its morphological and anatomical features, tolerates strong insolation and water deficit well. However, in the case of plantation cultivation, irrigation is needed at transplanting and right after transplanting. Later, when the plants develop their root system, they can tolerate water stress [55]. In this study, precipitation in April 2017 (52.9 mm) had a positive influence on the root development of young plants. Further, moderate temperatures during April and May were favorable for immortelle development, so the extremely hot June, July, and August accompanied by water deficit did not have a negative effect on plants. Moderately warm and rainy weather in September and October favored the formation of immortelle aboveground biomass, and the first harvest was done in the middle of October (510 kg/ha). After the harvest, the plants went into winter dormancy. November and December had average temperatures for Serbia (6.8 °C and 3.2 °C, respectively) as well as average precipitation (34.6 mm and 43.8 mm, respectively), which was positive for the plants. The year 2018 was extremely warm with an average amount of precipitation (mainly distributed in June and July). In first harvest, the fresh biomass yield was 9040 kg/ha, while in the second it was for 37% lower (5750 kg/ha). The 2019 cropping season was similar the 2018 season, but precipitation occurred mainly in May and June. Abundant rainfall during May and June had positive effect on first harvest (12,940 kg/ha), while the second was for 44% lower than the first (7260 kg/ha).

Bearing in mind that drying significantly increases the postharvest processing costs, as well as that the agricultural producers of immortelle in the region practice distillation of fresh plant material, we decided to apply this knowledge. The water content in the fresh immortelle plant material for all five harvests in average was 43.48%. The results showed that in the case of supercritical extraction with carbon dioxide, the use of wetted immortelle flowers (moisture content 28.4%) leads to increased solubility of the solute compared to dry plant material (moisture content 10.5%) [56]. In addition, investigation with *H. odoratissimum* demonstrated that EO components are lost during drying, with significant changes in the share of the main EO components [57].

Adapting the immortelle to some regions' climatic conditions is part of the plants' conservation strategy [58]. Further, variations among the plants are mainly associated with temperature oscillations. In addition, the relationship between precipitation and chemical compounds of the EO shows that curcumene was positively influenced by spring and summer precipitation [8]. It could be a good explanation of why this chemotype is dominant in the region of ex-Yugoslavia countries, which have a long-term average characterized by a significant amount of precipitation during spring and summer. Probably, precipitation after the first harvest influences the regrowth of the immortelle plants and their second flowering period. In addition, growing technology, i.e., cutting high, also influences regrowth and second flowering. Cutting only the inflorescences leads to a quick appearance of new buds.

In addition, cutting only the flowering tops during harvest, without the lower parts of the stems and leaves, influences the preservation of lateral buds, which subsequently stimulates fast regenerative growth and the formation of new/additional flowering tops. This phenomenon is known for some other aromatic crops such as lemon verbena [59], Spanish sage [60], oregano [61], lemon balm [62], and many others. Agro-ecological conditions in Serbia seem to be promising for growing immortelle as a perennial crop and providing two harvests per year when only the flowering tops are harvested.

5. Conclusions

Under the agro-ecological conditions of Serbia, immortelle can provide two harvests per year if the first cut is relatively high, just under the inflorescences, to stimulate fast plant regrowth. The first harvest is usually in July, and the second harvest could be in August. The natural population from the region of ex-Yugoslavia belongs to γ-curcumene + *ar*-curcumene chemotype. Apparently, this chemotype developed as an adaptation to the specific climatic conditions, and spring and summer precipitation would improve the synthesis and accumulation of curcumene in the immortelle plants. The importance of detecting seasonal variations in the immortelle EO profile is important because of the standardization of raw materials.

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