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Agricultural management, season and trace elements effects on volatile oil production from *Melissa officinalis* L. (Lemon balm)

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Abstract The objective of this study was to provide information about organic and mineral fertilization, season and trace elements effects on volatile oil production by the species *Melissa officinalis*. Elemental concentration was determined by instrumental neutron activation analysis and atomic absorption spectrometry. The volatile oil was extracted by hydrodistillation and analyzed by gas chromatography coupled to a mass spectrometer. The elemental content and the main compounds vary according to agricultural management and season. The results indicate that the production of volatile oil main compounds from *M. officinalis* is correlated with the concentrations of Na, Co, Rb, Cd, Cs, La, Sm and Hf.

Keywords *Melissa officinalis* · Trace elements · Agricultural management · Volatile oil · INAA · GC/MS

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Introduction

Melissa officinalis L., Lemon balm, originates from Europe and is now grown all over the world [1]. It is a medicinal and aromatic species popularly used to make tea, which serves as a tranquilizer, promoting sleep and reducing stress and anxiety [2, 3]. These disorders have increased, considerably, in recent years, becoming prevalent diseases affecting a high percentage of the population, [4] which has more and more resorted to herbal medicinal sedative products widely available that can perform the same therapeutic action with fewer side effects, dependence or tolerance than synthetic medicine [5].

The medicinal properties of *M. officinalis* are related to the wide variety of its volatile oil chemical composition, such as terpenoids [6]. These comprise a large group of secondary metabolites, which can vary qualitatively and quantitatively, depending on various factors such as season, plant age, circulating water amount, geographical factors, climate, stress and nutrient availability in the soil [7, 8].

Melissa officinalis essential oil is mainly produced in secretion structures known as glandular trachoma of leaves being geranial and neral (α-citral and β-citral) the major compounds and, also, the ones of great interest for the pharmaceutical industry due to their antioxidative, antimycotic, antiviral and sedative activities [9–11]. Other compounds are, also, found in smaller quantities such as citronellal, citronellol, geraniol and nerol.

Mineral elements are involved in the structure of volatile oil, yet, also, having undesirable effects on their regulation [12]. Chromium (Cr) stress has been found to induce the production of eugenol, a major component of *Ocimum tenuiflorum* essential oil, Holy Basil, (Lamiaceae) [13]. Nevertheless, metal effects on secondary metabolites and the chemical composition concerning the species are largely unknown. Providing information on factors influencing volatile oil production is fundamental to obtain the highest production by the plant, as well as to verify the economic viability of its cultivation [14]. The objective of this study was to afford information about agricultural management, season and the effects of trace elements on volatile oil production by the species *M. officinalis*.

Experimental

Cultive

Container experiments were carried out in the Municipal Gardening School experimental area, in *Ibirapuera* Park, *São Paulo*, Brazil. The experimental design was completely randomized with 4 treatments and with 4 replications. The treatments studied included organic and mineral controls (site soil), organic fertilization (2 t ha^{-1} of poultry manure per season) and mineral fertilization (30 t ha^{-1} of NPK, 6:14:8 per season), in four distinct seasons (spring, summer, autumn, winter). Each of the samples consisted of three individuals. After proper identification, a voucher specimen (No. 15923) was deposited in the Municipal Herbarium of *São Paulo*, SP, Brazil.

Elemental concentration determination

Instrumental neutron activation analysis (INAA), a sensitive, fast, nondestructive and multielemental technique, which has been used frequently to evaluate inorganic contents of medicinal plants [15, 16], was applied to determine the elemental concentrations. About 150 mg of fine powder of each sample and certified reference materials (CRMs) were packed in polyethylene bags for irradiation. The samples were irradiated in a neutron flux of approximately 10^{12} cm^{-2} s^{-1} for 8 h, at the IEA-R1 Nuclear Reactor, IPEN, together with the CRMs (Syenite, Table Mountain USGC STM-2 and Estuarine Sediment NIST 1646a) and standard solutions pipeted in paper sheets. Two measurement series were carried out using a Ge detector (Highpure, POP TOP Model) with 20 % efficiency and 1.9 keV resolution for the 1332 keV peak of ^{60}Co and spectra were colleted with EG&G ORTEC counting system. Na, Br, Ba, La and Sm were measured after 7 days cooling time and Sc, Cr, Fe, Co, Zn, Rb, Cs, Ce, Hf, and Th, after 15 days. Spectrum analysis was performed using the VISPECT 2 software [17]. The reference materials Liquen IAEA-336 and tomato leaves NIST 1573a were used to evaluate the effectiveness of the applied methodology.

Graphite furnace atomic absorption spectrometer (GFAAS) was used for the determination of Cd and Pb elements. About 300 mg of each of the powdered plant samples was digested in 22.5 ml of acid solution (HNO_3, HCl, $HClO_4$, H_2O_2 in ratio of 5:15:0.5:2). The corresponding solution was heated until white fumes had appeared. The clear solution was diluted up to 50 ml with distilled water and filtered with Whatman filter paper no. 1. Standard working solutions of the elements of interest were prepared to make the standard calibration curve. Liquen IAEA- reference material 336 was used for quality control of the obtained results in elemental analysis.

Volatile oil extraction

Volatile oil was extracted from *M. officinalis* leaves by hidrodistillation method in a Clevenger apparatus, at the Laboratory of Research Reactor Center (CRPq/IPEN), and analyzed by gas chromatography/mass spectrometry (GC/MS) at the Laboratory of Radiation Technology Center (CTR/IPEN). From the fresh leaves of each plot, a 50 g sample was separated for volatile oil extraction and chemical analysis. The volatile oil was extracted for 4 h [18], stored in amber bottles and maintained in freezer until chemical analysis. The qualitative analysis of volatile oil compounds was performed on a GC/MS (Shimadzu® Co., model QP-5000) using a DB-5 ms (30 m × 0.25 mm × 0.25 μm) capillary column, injection temperature of 220 °C, interface temperature of 230 °C and helium carrier gas (flow: 1.2 mL min^{-1}). Initially, the samples were diluted in hexane (1:10 v v^{-1}), and 1 μL was injected in the column, using splitless mode. Operating conditions were undertaken at oven temperature from 60 to 120 °C, at 3 °C min^{-1} ratio (20 min), 120–300 °C at 15 °C min^{-1}

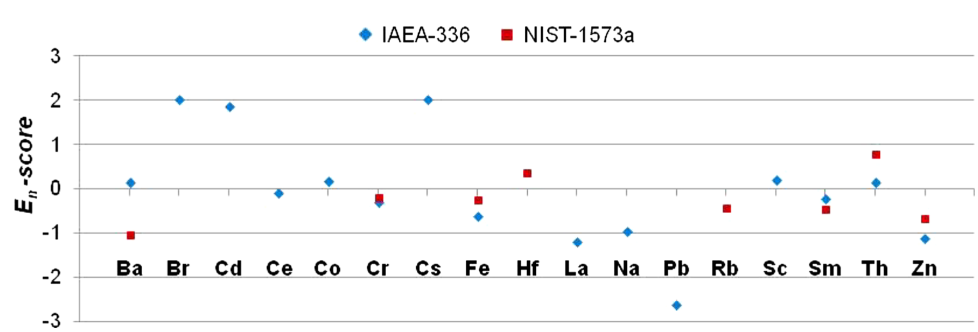

Fig. 1 E_n-score values obtained for the elements determined in IAEA-336 and NIST-1573a reference materials

Table 1 Mean values of element concentration (μg g^{-1}) in leaves of *Melissa officinalis* in four distinct seasons and in the four treatments

Season	Treatment	Ba	Br	Cd	Ce	Co	Cr	Cs	Fe	Hf
Autum	Organic control	102 ± 3	56.1 ± 0.2	0.096 ± 0.004	3.42 ± 0.09	0.366 ± 0.008	9.8 ± 0.2	0.26 ± 0.02	4138 ± 31	1.15 ± 0.01
	Organic fertilization	49 ± 2	10.87 ± 0.07	0.059 ± 0.002	0.78 ± 0.04	0.165 ± 0.005	5.1 ± 0.1	0.13 ± 0.01	697 ± 5	0.22 ± 0.01
	Mineral control	86 ± 3	53.6 ± 0.2	0.093 ± 0.001	2.96 ± 0.08	0.272 ± 0.010	12.2 ± 0.2	0.20 ± 0.02	3564 ± 27	1.17 ± 0.01
	Mineral fertilization	46 ± 2	65.8 ± 0.3	0.053 ± 0.001	2.04 ± 0.05	0.357 ± 0.007	6.2 ± 0.1	0.15 ± 0.01	1911 ± 12	0.55 ± 0.01
Winter	Organic control	73 ± 4	54.0 ± 0.3	0.057 ± 0.004	0.49 ± 0.06	0.37 ± 0.02	6.5 ± 0.2	0.033 ± 0.009	785 ± 13	0.72 ± 0.07
	Organic fertilization	79 ± 3	9.3 ± 0.1	0.060 ± 0.002	0.65 ± 0.06	0.92 ± 0.01	83 ± 1	0.08 ± 0.01	644 ± 6	0.07 ± 0.01
	Mineral control	82 ± 4	60.8 ± 0.3	0.032 ± 0.001	0.85 ± 0.08	0.57 ± 0.03	42.9 ± 0.9	0.075 ± 0.02	1104 ± 15	0.79 ± 0.07
	Mineral fertilization	52 ± 2	10.3 ± 0.1	0.123 ± 0.001	0.64 ± 0.04	2.19 ± 0.02	146 ± 2	0.10 ± 0.01	1022 ± 7	0.15 ± 0.01
Spring	Organic control	ND	48.9 ± 0.7	0.048 ± 0.004	1.34 ± 0.12	0.80 ± 0.01	66 ± 1	0.14 ± 0.02	1464 ± 16	0.43 ± 0.01
	Organic fertilization	58 ± 2	13.5 ± 0.5	0.030 ± 0.002	0.53 ± 0.05	0.53 ± 0.01	48 ± 1	0.05 ± 0.01	507 ± 5	0.125 ± 0.008
	Mineral control	ND	47.0 ± 0.7	0.044 ± 0.001	1.13 ± 0.07	1.69 ± 0.02	163 ± 3	0.08 ± 0.02	1654 ± 18	0.25 ± 0.01
	Mineral fertilization	60 ± 2	11.04 ± 0.09	0.035 ± 0.001	0.63 ± 0.07	0.77 ± 0.01	71 ± 1	0.08 ± 0.01	748 ± 7	0.149 ± 0.008
Summer	Organic control	ND	24.2 ± 0.5	0.038 ± 0.001	0.62 ± 0.06	0.52 ± 0.02	43 ± 1	0.18 ± 0.03	777 ± 10	ND
	Organic fertilization	63 ± 3	14.6 ± 0.1	0.027 ± 0.002	0.75 ± 0.08	2.80 ± 0.03	283 ± 5	0.12 ± 0.01	1360 ± 13	0.045 ± 0.009
	Mineral control	86 ± 4	18.6 ± 0.5	0.021 ± 0.001	0.62 ± 0.06	0.29 ± 0.01	24.8 ± 0.7	0.11 ± 0.02	881 ± 11	0.19 ± 0.01
	Mineral fertilization	64 ± 2	14.8 ± 0.1	0.035 ± 0.002	0.54 ± 0.04	0.69 ± 0.01	61.0 ± 0.9	0.09 ± 0.01	475 ± 4	0.061 ± 0.004

Season	Treatment	La	Na	Pb	Rb	Sc	Sm	Th	Zn	
Autum	Organic control	1.94 ± 0.03	239 ± 4	2.5 ± 0.1	17.2 ± 0.5	1.211 ± 0.005	0.204 ± 0.008	1.95 ± 0.07	58 ± 1	Mean ± error
	Organic fertilization	0.34 ± 0.01	120 ± 3	20.83 ± 0.02	22.7 ± 0.5	0.159 ± 0.001	0.045 ± 0.002	0.30 ± 0.01	31 ± 1	
	Mineral control	1.72 ± 0.03	245 ± 4	3.725 ± 0.004	16.4 ± 0.5	1.123 ± 0.004	0.162 ± 0.007	1.76 ± 0.07	48 ± 2	
	Mineral fertilization	1.70 ± 0.03	266 ± 4	36.92 ± 0.01	19.5 ± 0.5	0.508 ± 0.002	0.230 ± 0.006	0.89 ± 0.03	34 ± 1	
Winter	Organic control	0.40 ± 0.02	45 ± 1	5.8 ± 0.1	14.5 ± 0.8	0.615 ± 0.011	0.037 ± 0.003	0.80 ± 0.05	57 ± 2	Mean ± error
	Organic fertilization	0.21 ± 0.02	23 ± 3	6.11 ± 0.02	19.1 ± 0.5	0.063 ± 0.001	ND	0.06 ± 0.01	64 ± 2	
	Mineral control	0.53 ± 0.03	44 ± 1	1.643 ± 0.004	16.3 ± 0.8	0.116 ± 0.002	0.045 ± 0.003	1.03 ± 0.05	61 ± 2	
	Mineral fertilization	0.37 ± 0.01	45 ± 1	1.87 ± 0.01	16.2 ± 0.5	0.107 ± 0.001	0.047 ± 0.005	0.19 ± 0.01	34.5 ± 0.8	
Spring	Organic control	0.64 ± 0.04	57 ± 13	1.4 ± 0.1	14.4 ± 0.6	0.322 ± 0.002	ND	0.484 ± 0.022	61 ± 2	Mean ± error
	Organic fertilization	0.23 ± 0.01	41 ± 1	11.24 ± 0.02	23.8 ± 0.7	0.057 ± 0.001	0.026 ± 0.002	0.090 ± 0.006	73 ± 2	
	Mineral control	0.51 ± 0.03	55 ± 13	2.449 ± 0.004	10.4 ± 0.5	0.244 ± 0.002	ND	0.390 ± 0.018	56 ± 2	
	Mineral fertilization	0.36 ± 0.01	45 ± 4	2.00 ± 0.01	21.1 ± 0.7	0.106 ± 0.001	0.044 ± 0.002	0.170 ± 0.008	59 ± 1	
Summer	Organic control	0.32 ± 0.03	ND	2.15 ± 0.02	ND	0.159 ± 0.002	0.04 ± 0.01	0.21 ± 0.01	48 ± 2	Mean ± error
	Organic fertilization	0.23 ± 0.01	23 ± 2	4.60 ± 0.02	22.1 ± 0.8	0.034 ± 0.001	0.047 ± 0.007	0.08 ± 0.01	53 ± 1	
	Mineral control	0.50 ± 0.05	ND	2.07 ± 0.00	15.7 ± 0.8	0.192 ± 0.002	ND	0.27 ± 0.02	91 ± 4	
	Mineral fertilization	0.232 ± 0.008	29 ± 2	6.35 ± 0.03	21.2 ± 0.5	0.051 ± 0.001	0.033 ± 0.003	0.088 ± 0.005	41 ± 1	

Values represent mean ± standard error ($n = 4$)

ND not determined

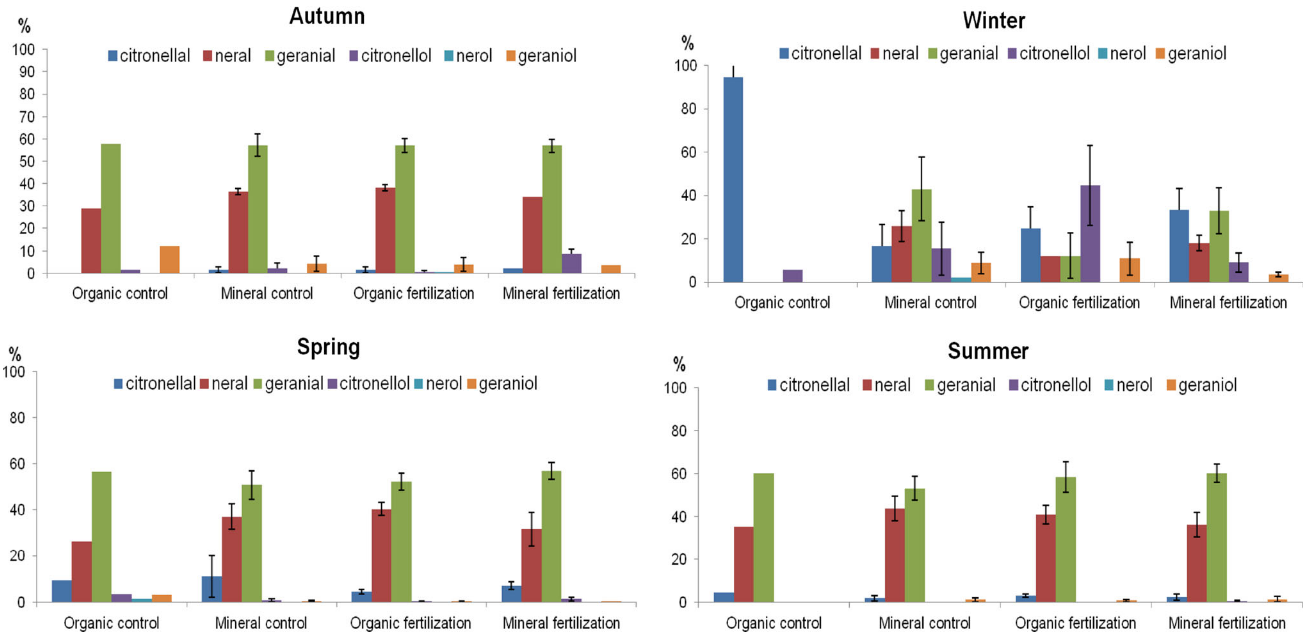

Fig. 2 Major compound percentages of *Melissa officinalis* volatile oil, distilled from fresh leaves harvested at distinct seasons

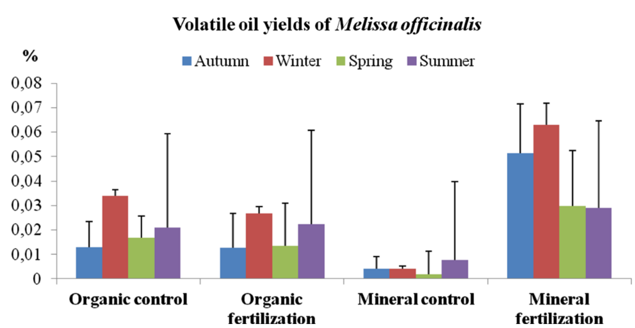

Fig. 3 Volatile oil yield of *Melissa officinalis* for different types of fertilization

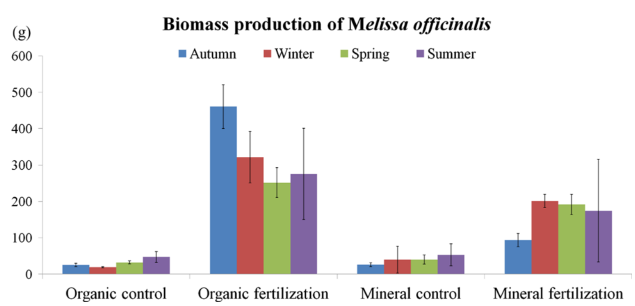

Fig. 4 Biomass of *Melissa officinalis* for the different types of fertilization

ratio (12 min). The major compounds neral, geranial and citronellal were identified by using reference standards from Sigma Aldrich. The citronellol and geraniol constituents were identified by the comparative analysis of the acquired mass spectra with NIST62 database library coupled to GC/MS spectrometer, literature McLafferty & Stauffer, Adams [19, 20], and Kováts retention index [21].

Statistics

Statistical significance was determined by analysis of variance (ANOVA). In addition, the Tukey's test ($P \leq 0.05$) was used to find significant differences among the means. Pearson correlation coefficients were calculated with all data. Hierarchical cluster analysis (HCA) and Factor analysis were used for grouping accessions according to their similarity. The proximity of the groups in the obtained dendrogram reflects the similarity of determined parameters [22]. The analyses were performed with STATISTICA software.

Results and discussion

To assess the accuracy and precision of the methodologies, two certified reference materials IAEA-336 and NIST-1573a were analyzed. The E_n-score values [23], calculated for the elements determined in the reference materials, are shown in Fig. 1. For most elements, the E_n-score values were $|Z| < 1$, which means that the obtained results are in the 95 % confidence interval of the certified values. For Br, Cs and Pb, the E_n-score was high due to the low concentration of these elements in the samples.

The elemental concentration was determined on *M. officinalis* leaves and the results are given in Table 1.

Table 2 Pearson correlation coefficient obtained for trace elements, volatile oil constituents, yield and biomass of *Melissa officinalis*

	Citronellal	Neral	Geranial	Citronellol	Geraniol	Yield	Biomass
Ba	0.05	−0.11	−0.09	0.20	0.21	−0.07	−0.26
Br	−0.06	−0.07	0.17	−0.10	0.22	−0.04	−0.61*
Cd	0.12	−0.41*	−0.23	0.24	0.55*	−0.21	0.02
Ce	−0.25	−0.04	0.23	−0.15	0.32	0.04	−0.41*
Co	0.43*	−0.18	−0.24	0.03	−0.21	0.09	0.19
Cr	0.13	−0.00	−0.11	0.12	−0.24	0.10	0.20
Cs	−0.29	−0.01	0.39*	−0.23	0.29	0.15	−0.19
Fe	−0.10	−0.03	0.14	−0.14	0.29	0.04	−0.38*
Hf	−0.12	−0.03	0.09	−0.08	0.63*	−0.05	−0.51*
La	−0.08	−0.11	0.10	0.22	0.68*	−0.09	−0.44*
Na	−0.23	0.02	0.13	0.32	0.73*	0.04	−0.16
Pb	−0.22	0.11	0.16	0.01	−0.14	−0.11	0.01
Rb	−0.25	0.46*	0.03	0.11	−0.01	0.21	0.48*
Sc	−0.19	0.00	0.19	−0.15	0.37	0.02	−0.48*
Sm	−0.23	−0.09	0.15	0.39	0.71*	−0.13	−0.40*
Th	−0.15	−0.03	0.19	−0.17	0.38	−0.03	−0.49*
Zn	−0.02	0.27	−0.06	−0.05	−0.15	0.26	−0.05
Citronellal	1.00	−0.81*	−0.78*	0.36	0.28	−0.24	−0.04
Neral	−0.81*	1.00	0.69*	−0.71*	−0.43*	0.46*	0.18
Geranial	−0.78*	0.69*	1.00	−0.83*	−0.55*	0.19	−0.20
Citronellol	0.36	−0.71*	−0.83*	1.00	0.70*	−0.41*	0.28
Geraniol	0.28	−0.43*	−0.55*	0.70*	1.00	−0.36	−0.01
Yield	−0.24	0.46*	0.19	−0.41*	−0.36	1.00	−0.03
Biomass	−0.04	0.18	−0.20	0.28	−0.01	−0.03	1.00

* Significant at p level ≤ 0.05

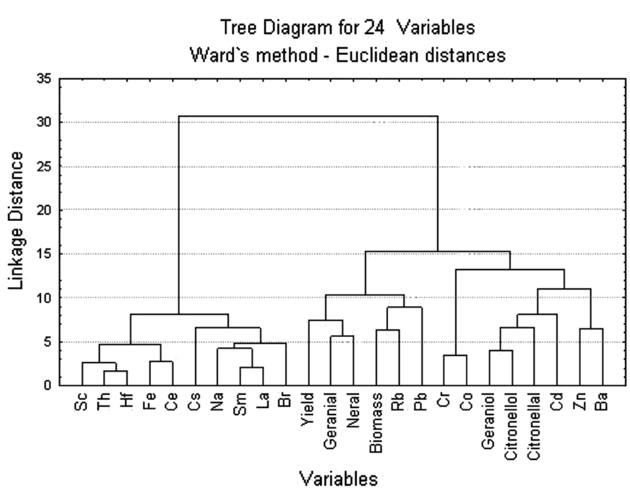

Fig. 5 Cluster analysis dendrogram of trace elements, volatile oil constituents, yield and biomass

Among the elements determined in *M. officinalis* leaves, the lowest variation coefficient was observed for Ba, Cs, Rb and Zn (ranging from 22 to 49 %) and the highest variation coefficient was observed for Co, Cr, Pb, Sc and Th (ranging from 113 to 203 %). For the rare earth elements (REE) Ce, La and Sm, the highest concentration was observed in autumn. For the potentially toxic elements Cd and Pb, similar concentrations among the distinct seasons were observed. Except in autumn, in which Pb presented a higher variation coefficient (147 %) and in the winter, in which Cd presented a higher variation coefficient (94 %). Brazilian legislation determines the maximum tolerance level (MTL) of 0.6 mg kg^{-1} for lead and 0.4 mg kg^{-1} for cadmium, in herbal products [24]. The overall results indicated that this limit was exceeded for Pb in all the samples, while the Cd concentrations were below the MTL. The results for trace elements showed that Cs, Hf and Th presented similar concentrations for the four distinct seasons and different fertilization. The elements Co, Cr and Zn presented the lowest concentrations, and Fe the highest, in autumn. As to elements Zn and Co, the results showed significant difference depending on the treatment.

The components of interest of this study were selected based on the fact that, in commercial essential oil, it is recommended that neral, geranial, and citronellal must be present as major chemical compounds and nerol, preferentially, geraniol and citronellol should be absent [8]. The

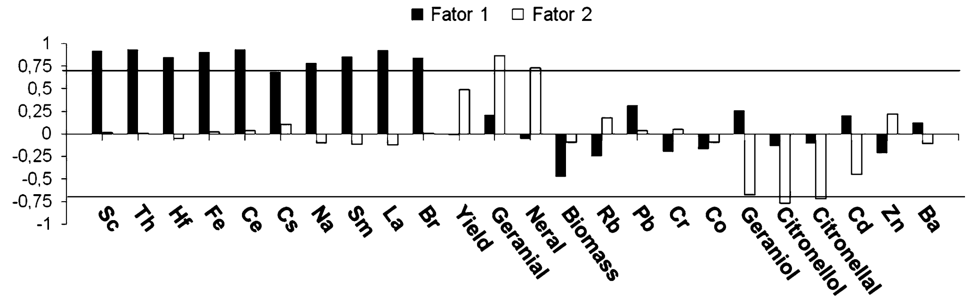

Fig. 6 Factor loadings that contribute for 50 % of the explained variance

obtained percentages of *M. officinalis* volatile oil constituents are given in Fig. 2.

The major concentrations of compounds did not present significant difference for the distinct agricultural managements in autumn, spring or summer seasons. In the autumn, the major components of the volatile oil ranged from 28.8 to 38.1 % for neral and from 56.9 to 57.8 % for geranial, while the minor components ranged from 1.4 to 2.1 % for citronellal, from 0.5 to 8.6 % for citronellol and from 3.6 to 11.9 %, for geraniol. In the spring, neral ranged from 26.3 to 40.3 %; geranial, from 50.8 to 57 %; citronellal, from 4.4 to 11 %; citronellol, from 0.3 to 1.3 % and geraniol from 0.2 to 3.1 %. In the summer, neral ranged from 35.2 to 43.7 %; geranial, from 53.2 to 60.3 %; citronellal, from 1.9 to 4.5 %; citronellol, from 0.4 to 0.6 % and geraniol, from 0.8 to 1.5 %. Conversely, in the winter, the volatile oil percentages presented a decrease for the major components: neral (12 to 25.9 %) and geranial (12.2 to 43 %); and a significant increase for the minor components: citronellal (16.6 to 94.4 %) and geraniol (3.7 to 10.8 %).

There are many studies related to the medicinal effect of *M. officinalis* volatile oil and the factors influencing its production, such as drying temperature, harvest time, seasonality, light intensity, leaves production, fertilization effect. Nevertheless, the trace elements effect on volatile oil production in this species are not known [25, 26].

The yields of *M. officinalis* volatile oil and the biomass production are given in Figs. 3 and 4, respectively.

Pearson correlation coefficients (PCC) and hierarchical cluster analysis (HCA) were applied to examine the relationship and similarities between trace elements present in leaves, chemical constituents of volatile oil, yield and biomass of *M. officinalis*.

In Table 2, presenting the PCC results, it can be observed that neral was negatively correlated with Cd and positively correlated with Rb. Geranial concentration was positively correlated with the concentration of Cs. Citronellal concentration was positively correlated with the concentration of Co. Geraniol concentration was positively correlated with Cd, Hf, La, Na and Sm.

The dendrogram, obtained by HCA, presented in Fig. 5, reveals two main groups: group I includes the monotherpenes: neral, geranial, citronellal, citronellol and geraniol; alkaline earth metal, Ba; alkaline metal, Rb and the transition elements, Cr, Co and Zn. This group, also, includes the toxic Cd and Pb. Group II includes the rare earth elements La, Ce and Sm, alkaline metals Na and Cs and the transition metals Fe, Hf, Th and Sc.

Factor analysis was applied to the elemental concentrations in *M. officinalis* leaves, volatile oil constituents, yield and biomass. The results showed the occurrence of two main factors with eigenvalues greater than one, accounting for 49.5 % of the total variance. The main contribution (34.8 % of variance) comes from variables included in Factor I: Br, Ce, Fe, Hf, La, Na, Sc, Sm and Th. Factor II (14.7 % of variance) is composed of geranial, citronellol and geraniol (Fig. 6).

Conclusions

The results obtained in the present work indicated that there was significant difference in the production of the major compounds of *M. officinalis* volatile oil in winter, in all the tested agricultural managements, while in autumn, spring and summer seasons this difference was not observed. Biomass production was higher in fertilization forms than in the non-fertilized controls. Since there were no variations in the biomass production and essential oil constituents' yields of *M. officinalis* between the organic and the mineral fertilization forms, the former proves to be more advantageous due to its lower cost, easily bio-degradable process and the fact that it does not cause environmental pollution. Considering the metabolites of higher interest, the results indicated that neral was positively correlated with Rb and negatively correlated with Cd concentrations. Geranial and citronellal were positively correlated with Cs and Co concentrations, respectively. Geraniol was positively correlated with Cd, Hf, La, Na and Sm. The biomass has presented inverse correlation with Br, Ce, Fe, Hf, La, Sc, Sm, Th and Zn, and positive correlation with Rb. Therefore, this study should be continued in order to explain some of the reported results. In future research, Mg, Cl, K, Ca, Ti, V, Mn, Ni, Cu, Mo and Pd concentrations should, also, be considered.

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