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Multivariate optimization of a HS-SPME/GC-MS technique for the characterization of volatile compounds present in *Hedeoma multiflorum* Benth

[Optimización Multivariada de una técnica HS-SPME/GC para la caracterización de los compuestos volátiles presentes en *Hedeoma multiflorum* Benth]

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Abstract: The aim of the present work was to optimize the main experimental variables of a procedure using HS-SPME/GC-MS as the analytical methodology to establish the profile of the volatile compounds present in aerial parts of *Hedeoma multiflorum* Benth. The influence of the type of fiber, equilibrium time, extraction time and extraction temperature on the composition of the volatile compounds was determined using response surface methodology (RSM), and the parameters of the models were corroborated by multiple linear regressions. The results showed that the regression models generated adequately explained the data variation and represented the relationships between the parameters and their responses. The optimal analysis conditions from the contour plots were established (DVB/CAR/PDMS fiber, with a 10 min equilibrium time, 10 min extraction time, and 40°C). Under these conditions, 41 volatile components in the whole plant were determined, which represents more than those reported using hydrodistillation.

Keywords: *Hedeoma multiflorum*; HS-SPME/GC-MS; Volatile organic compounds; Response surface methodology.

Resumen: El objetivo del presente trabajo fue optimizar las principales variables experimentales de un procedimiento HS-SPME/GC para establecer el perfil de compuestos volátiles presentes en la parte aérea de *Hedeoma multiflorum* Benth. Se determinó la influencia de las variables tipo de fibra, tiempo de equilibrio, tiempo de extracción y temperatura de extracción sobre la composición de los volátiles, utilizando una met odología de superficie de respuesta (RSM) y los parámetros del modelo se corroboraron por regresión lineal múltiple. Los resultados demostraron que los modelos de regresión generados explican adecuadamente la variación de los datos y representaron significativamente las relaciones reales entre los parámetros y sus respuestas. Las condiciones óptimas de análisis fueron establecidas (DVB/CAR/PDMS, con un tiempo de equilibrio de 10 minutos, un tiempo de extracción de 10 minutos y trabajando a 40°C). Utilizando esta metodología, se determinaron 41 componentes volátiles en planta entera, más que los reportados mediante hidrodestilación.

Palabras clave: *Hedeoma multiflorum*; HS-SPME/GC-MS; Compuestos volátiles; Metodología de superficie de respuesta

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INTRODUCTION

The *Hedeoma* Pers. genus (Lamiaceae) includes about 43 species of annual or perennial herbs distributed throughout America. A species of this genus is used in North America to prepare herbal tea or as a spice (Viveros-Valdez *et al*., 2008). In Argentina, there are three species that grow in different environments: *Hedeoma multiflorum* Benth., *Hedeoma medium* Epling, and *Hedeoma mandonianum* Wedd. (Slanis & Bulacio, 2005), with the first one of these known as "tomillo de las sierras", which is a perennial endemic herb that is grown in dry, calcareous soils and good lighting situations (Barboza *et al.*, 2006). Infusions of this herb are used in folk medicine for digestive and anti-spasmodic purposes (Goleniowski *et al*., 2006). Research studies have revealed that this aromatic plant has *in vitro* antioxidant and cytotoxic properties (Dadé *et al*., 2011). In addition, due its desirable flavor, the flowers, leaves, and stems are blended with other species, particularly *Ilex paraguariensis*, in the manufacturing of "yerba mate" a composite used in a traditional infusion in Argentina and neighboring countries (Dade *et al*., 2009).

Different analytical methods have been developed to determine the compounds that contribute to flavor in plants, such as solvent extraction and hydrodistillation, among others. However, these methods are laborious and time-consuming as they require a lot of samples. Also, due to the long interval time required between sample preparations and obtaining the essential oil, the enzymatic degradation process that occurs during this time period can lead to erroneous conclusions being made about the original characteristics of the original aroma (Mazida *et al*., 2005). In the literature, we have found reports on essential oil analysis from hydrodistillation of the aerial parts of *H. multiflorum*, (Diaz *et al*., 2010; van Baren *et al*., 2010), but none on the composition of the VOCs present in each aerial parts.

An ideal sample preparation technique for evaluating volatile compounds should be simple, fast, inexpensive and compatible with a range of analytical instrumentations. In this sense, the HS-SPME technique provides many advantages, as it is easily automatized, simple to manage, inexpensive to set up, and does not use any organic solvents (Mendes *et al*., 2012). As the HS-SPME mechanism is based on the equilibrium of analytes among three phases (fiber coating, headspace and sample), the analysis of headspace volatile compounds by HS-SPME is highly affected by the vapor pressure of the volatile compounds in the vial. Related to this, the main variables that influence the vapor pressure and equilibrium of the volatile components in the headspace have been reported to be extraction temperature, headspace equilibrium time and extraction time (Zhang *et al*., 2009; Fatemi *et al*., 2013). Other parameters which affect the extraction process include fiber type, salting, pH, desorption time, temperature and sample amount (Sousa *et al*., 2006; Moreira *et al*., 2016). However, although an increase in extraction temperature increases the rate of extraction, it can decrease the distribution constant and in addition cause a decrease in the sensitivity of the extraction process (King *et al*., 2003). For these reasons, a adequate balance between sensitivity and extraction rate with respect to the extraction temperature can only be achieved through an rigorous optimization of the parameters involved (Balasubramanian & Panigrahi, 2011).

On the other hand, response surface methodology (RSM) allows the effects of many explanatory variables and their interactions on the response variables to be evaluated (Loi *et al*., 2010), with it being widely used in research, in particular for the optimization of the analysis conditions, as it permits the optimal working conditions to be attained from a lower number of determinations than by performing a univariate study (Ma *et al*. 2013). However, the main objective of the present work was to optimize HS-SPME conditions for the analysis of VOCs present in fresh aerial parts of *Hedeoma multiflorum*, with the effects of the variables (fiber type, extraction temperature, and equilibrium and extraction time) being interpreted using a multivariate analysis.

MATERIALS AND METHODS

Standards and materials

Standard solutions of *n*-alkanes C_6-C_{40}, linalool, menthone, pulegone and β-caryophillene were acquired from Sigma-Aldrich (Argentina). The SPME fibers, Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm), Polydimethylsiloxane/Divinylbenzene (PDMS/DVB, 65 μm), Carboxen/Polydimethylsiloxane (CAR/PDMS, 85 μm), Polydimethysiloxane (PDMS, 100 μm) and Polyacrylate (PA, 85 μm) used in this study to extract the volatile compounds from the samples, and also the SPME holder for manual

sampling, were purchased from Supelco (Sigma-Aldrich, Argentina).

Plant samples

Specimens of *H. multiflorum* Benth were collected in the Sierras de Córdoba, Argentina. A whole plant was deposited in the Museo Botánico of the Facultad de Ciencias Exactas, Fisicas y Naturales de la Universidad Nacional de Córdoba (Register Number CORD 7069).

Solid-phase microextraction

To perform this analysis, samples (100±0.1 mg) of fresh aerial parts previously chopped up were placed in glass vials of 20 cm^3, which were sealed with Viton septa and aluminum seals provided by Supelco (Sigma-Aldrich, Argentina). The extraction was performed by placing the vial in a thermostatic water bath with a temperature accuracy of ± 0.2°C (PolyScience 8005). The SPME extractions were carried out from the headspace (HS) of the samples, after optimization, according to the following conditions: DVB/CAR/PDMS (50/30 μm) fiber; equilibrium; extraction time of 10 min; and extraction temperature of 40°C. Following volatile compound microextraction, the fiber was then inserted directly into the GC injector for 5 min. All extractions were performed in triplicate. Significant differences were determined for the results of the analysis using a parametric ANOVA and Tukey´s test (α=0.05) on the aerial parts separately (flowers, leaves and stems).

Gas chromatography

Analyses were performed using a gas chromatograph Buck Scientific Model 910/310 equipped with a flame ionization detector, a manual injection port operating in splitless mode and a ZB-5 capillary column (30 m x 0.25 mm ID x 0.25 μm film).

The identification of the volatile components was performed using a gas chromatograph HP 5890 Series II equipped with a manual injection port operating in a splitless mode and coupled to an HP 5970 Mass Detector, with the column used being an HP-5 capillary column (30 m x 0.25 mm ID x 0.25 μm film). The mass spectrometer was operated at 70 eV, and the spectra were recorded in the range of m/z 50-550 amu in the acquisition mode "scan-full". The data processing system used was the HP-MS ChemStation including database Wiley 275. The GC conditions were: injector: 225ºC; initial temperature: 40ºC (5 min); final temperature: 200ºC (5 min); heating rate: 5°C/min; and interface: 230°C. The carrier gas in GC-FID was N_2 99.99% (5 psi) and in GC-MS was He 99.99% (5 psi).

The volatile compounds were identified by comparing their mass spectra with library data (match ≥90) and by the determination of the respective Kovat retention index (KI), using a homologous series of *n*-alkanes C_6-C_{40}. The retention indices were compared with values reported in the literature for similar chromatographic columns (NIST, 2018; Pherobase, 2018). The percentage of individual peaks was obtained by peak area normalization measured without correction factors. All determinations were performed in triplicate, and the variation coefficient was less than 10%. Significant differences were determined using parametric ANOVA and Tukey´s test (α=0.05).

Optimization of the SPME conditions

The type of fiber coating which had the highest affinity for the volatile compounds from *H. multiflorum* was the first parameter to be evaluated in this study, using a univariate method (Mesquita *et al*., 2017). Five types of fiber coating were exposed to the headspace from the same amount of sample (100.0 mg), temperature (40°C), equilibrium time (10 min) and extraction time (10 min). The comparison made for the five types of fiber coatings was based on the total peak area (extraction efficiency) and the number of detected peaks. Each experiment was performed in triplicate and reproducibility (% RSD) presented an error of less than 10% in all cases. Significant differences were determined using parametric ANOVA and Tukey´s test (α=0.05).

The effect of three SPME independent variables, namely, extraction temperature (x_1, 30-70 °C), equilibrium time (x_2, 10-60 min) and extraction time (x_3, 10-60 min) were evaluated using the response surface methodology (RSM). A total 45 treatments were analyzed for the optimization procedure, based on a three-factor central composite design (CCD). The levels employed in these experiments are listed in Table 1. The response evaluated for all experiments was the total sum of peak areas. The response surface model was used is expressed by the following equation (Bezerra *et al*., 2008):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \varepsilon$$

where Y is the response value predicted by the model; X_i are independent variables (extraction

temperature, equilibrium and extraction time); β_0 is a constant; β_i is the linear coefficient, and ε is the residual associated to the experiments. The statistical experimental design and optimization calculations were performed using Infostat v2018 and Minitab 17 software.

Table No. 1
Variables levels used for screening for central composite design (CCD) optimization of the extraction method by SPME of *H. multiflorum*

Variable	Coded variable		
	-1	0	+1
Extraction temperature, °C	30	50	70
Equilibrium time, min	10	35	60
Exposition time, min	10	35	60

RESULTS AND DISCUSSION

HS-SPME optimization

The comparison among the five types of fiber coatings was based on the total peak area (extraction efficiency) and the number of detected peaks. According to the results obtained, the DVB/CAR/PDMS fiber extracted a significantly greater total peak area (p<0.05) (Figure No. 1). The number of compounds was the same in all cases, with the extraction efficiency of the five tested fibers having the following order (based on the results of the ANOVA test): DVB/CAR/PDMS > PDMS > PA = PDMS/DVB = CAR/PDMS. Thus, DVB/CAR/PDMS was selected for the following optimization assays and for the determination of the volatile profile of *H. multiflorum*.

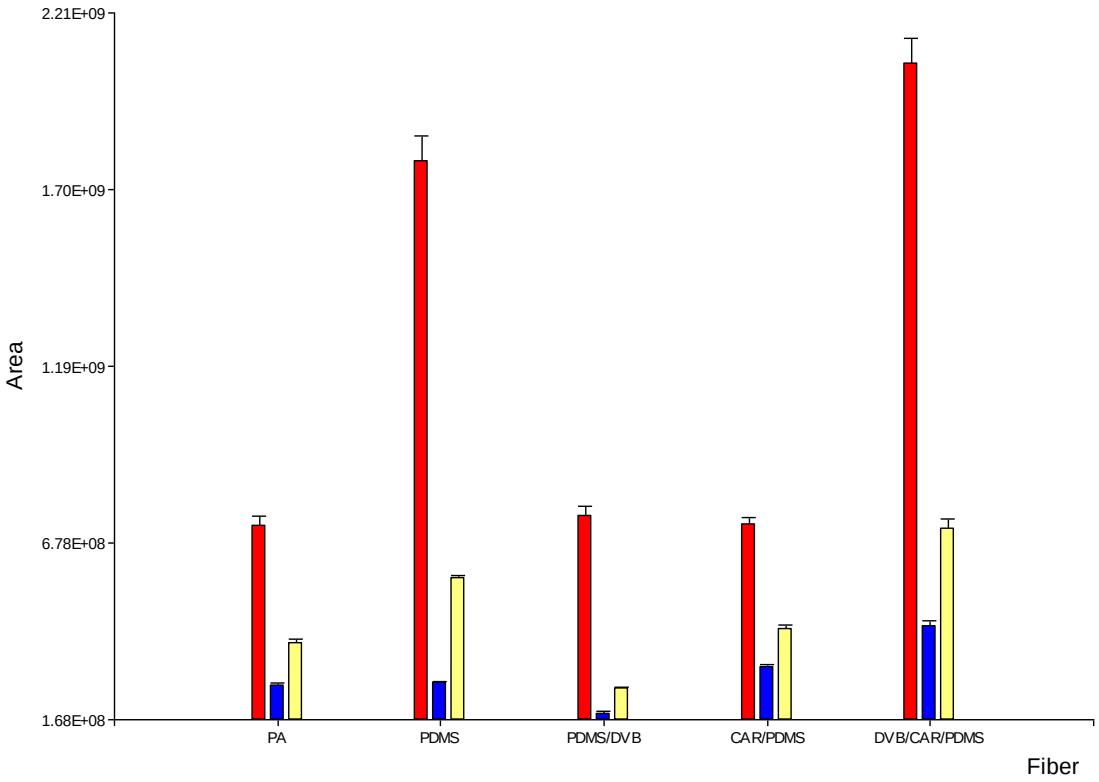


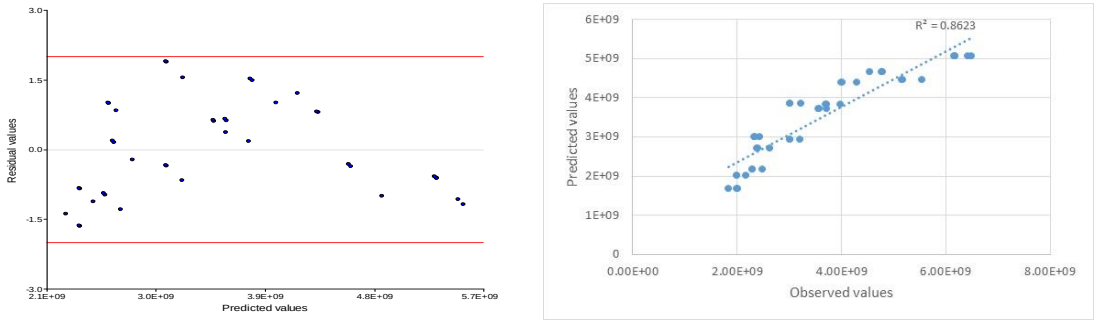
Figure No. 1
Influence of the type of HS-SPME fiber coatings on the extraction efficiency of the VOCs considering total area of the peaks

After selecting the type of fiber coating, the influence of extraction temperature, equilibrium time and extraction time factors in the HS-SPME variables was evaluated through response surface models (RSM). The results of RSM showed that the estimated regression coefficients for the response variables fitted the following equation, with the significance of the factors being confirmed by ANOVA (p<0.05) for all factors:

$$Y = 9.2E09 - 8.3E07\,X_1 - 6.1E07\,X_2 - 6.8E07\,X_3 + 2.6$$

Each response (Y) was assessed as a function of the main, linear and interaction effects of extraction temperature (x_1), equilibrium time (x_2) and extraction time (x_3) (R^2=0.77). In this way, all the response variables could be accurately explained by the response-surface model as a function of the three SPME variables studied, with the results showing that the model was significant (F=48.7 and p<0.05). It is also important to assess the fitted model in order to ensure that it provides a sufficient approximation to the results obtained under the experimental conditions (Anderson-Cook *et al*., 2009). The normality of the data was analyzed using a normal probability plot of the residuals and taking into account the difference between the observed values and those predicted from the regression. It was found that the experimental points were normally distributed around the curve, indicating that the normality assumption was satisfied. A determination coefficient (R^2) of 0.8623 was obtained for this model, which indicated a good fit between the observed and the predicted response values (Cheong *et al*., 2010). The plot of the residual versus the predicted values (Figure No. 2) revealed that the residuals were scattered randomly around zero and did not have outliers, because all of the values were within the accepted range (-3 to +3) for the validation of the model (Roriz *et al*., 2009). Thus, the analysis of variance results were valid, since the model assumptions were satisfied.

For the graphical representation of the functions of this design, graphs which describe the individual and cumulative effects of the variables tested and their effect on the response were used. Figure No. 3 shows the response surface graph in a three-dimensional plane for the regression model fitted to the data.



(a) (b)

Figure No. 2
(a) Plot of the residual *versus* the predicted values for the validation of the Response Surface Methodology (RSM) model; (b) Plot of observed values *versus* predicted ones from the RSM equation

Regression coefficients and graphs show that the high level of these response tended to increase with decreasing extraction temperature, equilibrium and extraction time. The effect of the extraction time revealed that, although a longer extraction time favored the occupation of more sites on the fiber by analyte molecules, a prolonged time when all sites are occupied can cause desorption (Ma *et al*., 2013). With respect to the effect of temperature, in general, heating provides energy for analyte molecules to overcome the energy barriers that tie them to the matrix (Alexandrou *et al*., 1992). This will enhance the mass transfer process, increase the vapour pressure of the analytes (Ho *et al*., 2006), and thereby facilitate the release of analytes into the headspace (Zhang *et al*., 2009). However, in this case, the

concentration of total area decreases with increasing extraction temperature (Figure No. 2). Although high temperature is good for the release of analytes from their matrix, it can adversely affect the adsorption of analytes by the coating due to decrease in the partition coefficients. Therefore less quantity is extracted when the temperature increases (Ma *et al*., 2013). In our study, the final optimum conditions predicted to result in the most desirable equilibrium headspace concentrations for *H. multiflorum* volatile compounds were found to be 10 min equilibrium time, 10 min extraction time and 40°C extraction temperature using a DVB/CAR/PDMS fiber.

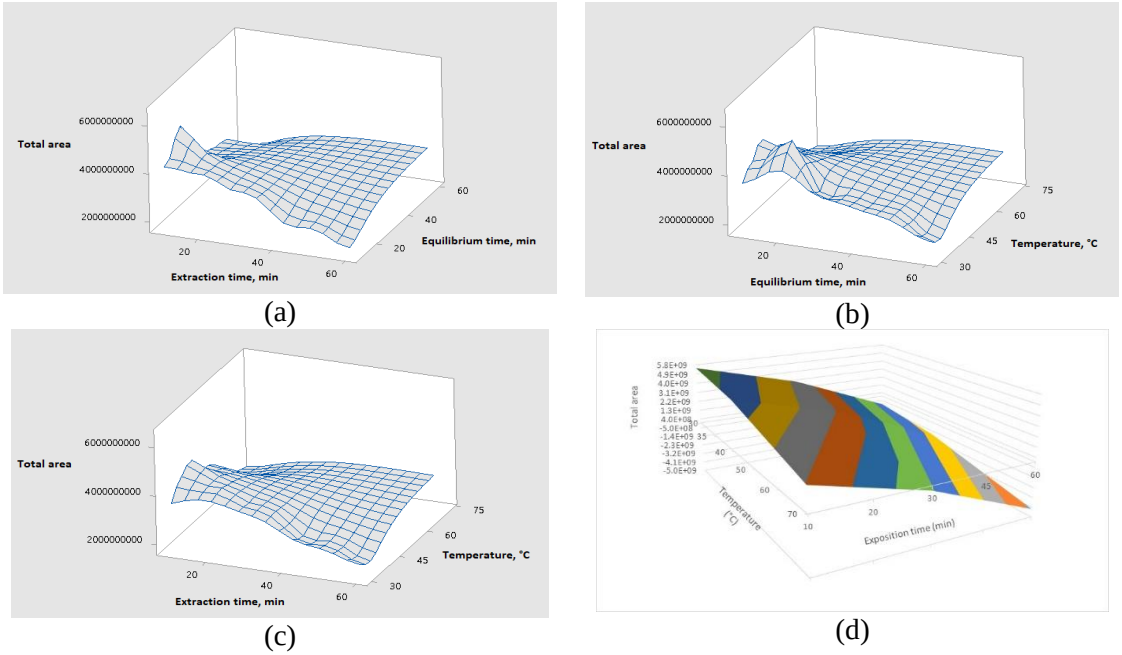


Figure No. 3
Response surface plots of significant interaction effects on total area of (p<0.05): (a) equilibrium and extraction time; (b) equilibrium time and temperature; (c) temperature and extraction time; (d) 3D surface plot for total area from Central Composite Design.

Characterization of volatile compounds from Hedeoma mutiflorum

The HS-SPME/GC-MS optimized method was applied to the analysis of the volatile compound profiles from *H. multiflorum* aerial parts (whole plant, and flowers, leaves and stems separately). Table No. 2 shows all identified volatile compounds, their retention index (RI) and the relative composition average of each compound in the samples. A total of 47 volatile compounds were identified in the whole plant, with pulegone (47.8%), *cis*-isopulegone (14.5%) and menthone (10.8%) being found at the greatest proportions.

The analysis of aerial parts separately revealed some differences, with the most important ones being that: 39 compounds were found in leaves and flowers, and 35 in stems; flowers had a lower menthone proportion (7.2%) than leaves (9.8%) or stems (10.8%); leaves had less isomenthone (2.0%) than flowers (3.8%) or stems (3.4%); and stems had a higher proportion of pulegone (56.9%) than leaves (52.1%) or flowers (45.1%). Some other less important significant differences were also found.

Table No. 2
Identified volatile compounds from *H. multiflorum* using HS-SPME

Chemical group	Compound[1]	Whole plant %[2]	Flowers %[2,3]	Leaves %[2,3]	Stems %[2,3]	RI_E[4]	RI_R[5]
Oxygenated monoterpenes	pulegone*	47.8	45.1	52.1	56.9	1250	1249
	cis-isopulegone	14.5	13.4	15.0	12.4	1184	1181
	menthone*	10.8	7.2[b]	9.8[a]	10.8[a]	1156	1153
	isomenthyl acetate	6.0	2.8[a]	0.9[b]	1.2[b]	1280	1280
	isomenthone	3.0	3.8[a]	2.0[b]	3.4[a]	1170	1166
	neoisomenthol	1.5	0.5	ND	ND	1195	1192
	linalool*	0.4	0.4[a]	0.1[b]	0.2[b]	1102	1103
	piperitenone	0.2	0.6[a]	0.5[a]	0.2[b]	1343	1339
	piperitone	0.2	0.8[a]	0.2[b]	0.8[a]	1265	1264
	α-terpineol	0.2	0.2	ND	ND	1199	1196
	menthyl acetate	0.1	0.5[a]	0.1[b]	0.2[b]	1307	1310
	piperitenone oxide	0.1	0.3[a]	0.3[a]	0.1[b]	1373	1369
	myrtenyl acetate	0.1	0.2	0.2	0.1	1323	1322
	cis-limonene oxyde	0.1	ND	0.1	ND	1142	1140
Total %		85.2	75.7	81.2	86.3		
Monoterpenes	limonene	0.9	3.5[a]	2.6[b]	1.9[c]	1030	1033
	3-p-menthene	0.4	3.3[a]	0.1[b]	ND	992	993
	α-pinene	0.3	ND	0.1	ND	933	948
	β-myrcene	0.1	0.2	0.1	0.1	983	983
	β-pinene	0.1	ND	0.1	ND	976	980
Total %		1.6	7.0	3.0	2.0		
Sesquiterpenes	bicyclogermacrene	2.7	4.5[a]	4.4[a]	2.6[b]	1499	1498
	β-elemene	1.5	0.2	0.3	0.3	1382	1391
	germacrene D	1.3	0.5[b]	1.4[a]	0.8[b]	1490	1485
	γ-cadinene	1.0	0.3	0.2	0.1	1507	1511
	bicycloelemene	0.9	1.7[a]	1.7[a]	0.9[b]	1333	1334
	aromadendrene	0.9	1.5	1.4	1.3	1448	1446
	α-cadinene	0.7	0.2	0.1	0.1	1540	1539
	α-amorphene	0.4	1.3[a]	0.5[b]	0.4[b]	1484	1481
	δ-cadinene	0.4	1.0	0.7	0.7	1523	1525
	β-caryophyllene*	0.4	0.6	0.6	1.0	1427	1430
	germacrene A	0.4	0.1[b]	0.4[a]	0.4[a]	1486	1484
	allo-aromadendrene	0.3	0.6	0.5	0.4	1455	1450
	β-cubebene	0.3	0.4	0.4	0.3	1437	1432
	α-bourbonene	0.3	ND	ND	ND	1391	1384
	α-copaene	0.2	1.6	1.3	1.1	1398	1400
	α-guaiene	0.2	0.4	0.3	0.2	1438	1438
	α-cubebene	0.2	0.3	0.2	0.1	1349	1345
	β-bourbonene	0.2	0.2	0.2	ND	1402	1407
	δ-selinene	0.2	ND	0.3	0.2	1493	1493
	γ-muurolene	0.1	0.7[a]	0.2[b]	0.1[b]	1469	1467
	ar-curcumene	0.1	0.5	ND	ND	1479	1472
	α-calacorene	0.1	0.1	0.1	0.03	1547	1546
	isoledene	0.1	ND	ND	ND	1368	1373
	α-ylangene	0.1	ND	ND	ND	1388	1387
	α-selinene	0.1	ND	0.1	0.1	1491	1485
	cadina-1,4-diene	0.1	ND	0.1	0.1	1539	1539
	β-cadinene	ND	0.5	0.4	0.3	1517	1520
Total %		13.1	17.0	15.8	11.6		
Oxygenated sesquiterpenes	cedrol	0.04	0.2	ND	0.06	1620	1618
	farnesol	0.05	0.07	ND	ND	1746	1742
Total, %		0.1	0.2	-	0.1		

[1] Identified using NIST mass spectral database (match over 85%) and retention index (RI) agrees with literature values (Machiels & Istasse, 2003); [2] Quantified by GC-FID; [3] Different letters indicate significant differences of the ANOVA with Tukey test ($p < 0.05$); [4] Relative retention index calculated in relation to the retention time of *n*-alkane (C_6–C_{40}) series; [5] Literature relative retention index; * Compounds also identified by comparison with Sigma-Aldrich standards; ND: Not detected.

Figure No. 4 shows the chromatogram from *H. multiflorum* whole plant as an example over 12-32 min and also the expanded minor peak images.

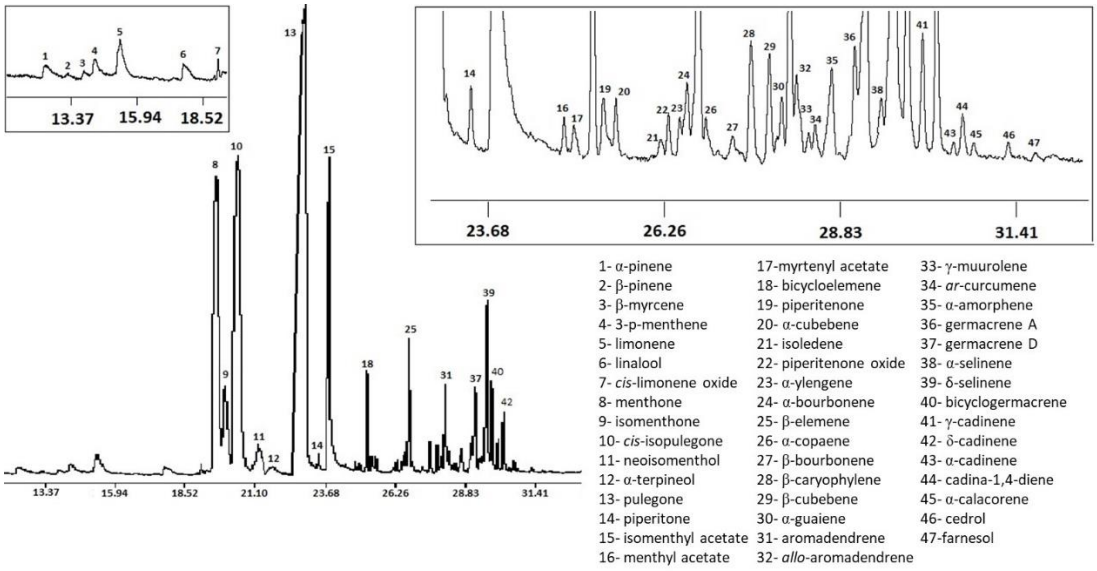


Figure No. 4
Chromatogram from *H. multoflorum* whole plant over 12-32 min by HS-SPME using DVB/CAR/PDMS fiber. The figures on the tops show enlarged minor peaks

The volatile compounds from *H. multiflorum* whole plant were terpenoids, with a high percentage of oxygenated monoterpenes (85.2%) and sequiterpenes (13.1%) being found. At a lower amount, monoterpenes (1.6%) and oxygenated sesquiterpenes (0.1%) were also observed. Based on the chemical composition of volatiles, it can be stated that the main biosynthetic pathways were methylerythritol phosphate (MEP) from pituvate (monoterpenes), and mevalonic acid (MVA) from acetyl CoA (sesquiterpenes), which are shown in Figure No. 5 (Dudareva *et al*., 2013). The chemical structures of the main volatile compounds from *H. multiflorum* and their biosynthetic routes are shown in Figure No. 6 (Croteau *et al*., 2005).

Most of the identified volatile compounds matched those reported for the essential oil of Argentine species of *H. multiflorum* (Koroch *et al*., 1999; van Baren *et al*., 2010). However, in our study, a greater number of volatiles was identified (47 *vs*. 11). These results are in agreement with other investigations, where larger amounts of volatiles were obtained by HS-SPME in comparison with hydrodistillation for a large variety of vegetables (Stashenko *et al*., 2004). These differences were related to the loss of volatiles due to the high temperatures required in the hydrodistillation process, compared to the low temperatures used in HS-SPME (Mohammadhosseini & Nekoei, 2014; Zanousi *et al*., 2016).

This pattern of chemical composition found is in accordance with previous studies conducted on the essential oils of this species (Koroch *et al*., 1999; van Baren *et al*., 2010). In all cases, pulegone was the main component, although van Baren (2010) found that there is a great seasonal variability in the percentage composition of volatiles, especially pulegone, menthone and isomenthone, which are in such equilibrium that when pulegone increases they diminish menthone and isomenthone and vice versa. Therefore, the differences in the percentage composition found may be due to edaphoclimatic factors.

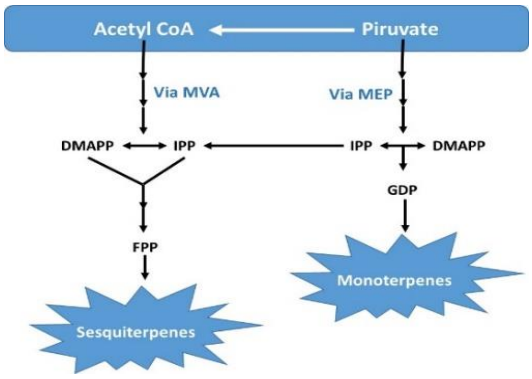


Figure No. 5
Biosynthetic routes of mono- and sesquiterpenes. Abbreviations: MVA, mevalonic acid; MEP, methyleritritol phosphate; IPP, isopentenylpyrophosphate; DMAPP, dimethylallylpyrophosphate; GDP, geranyldiphosphate; FPP, farnesylpyrophosphate

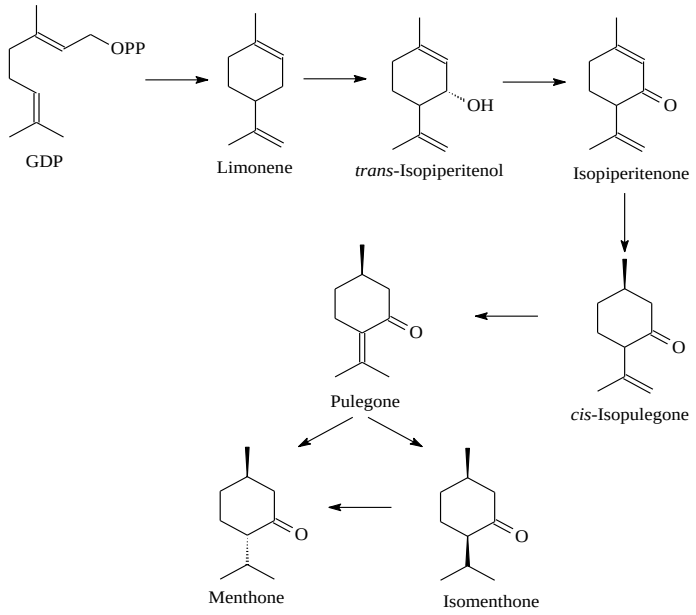


Figure No. 6
Pathway of *cis*-isopulegone, pulegone, menthone and isomenthone biosynthesis according to Croteau *et al*. (2005). Abbreviations: GDP, geranyldiphosphate; OPP denotes the diphosphate moiety

CONCLUSIONS

The optimum HS-SPME conditions for the extraction of volatile compounds in *H. multiflorum*, which significantly contributed to the multivariate regression models using a comprehensive experimental design, were determined in order to study the effect of the SPME variables; namely, equilibrium time, extraction time and extraction temperature. The response-surface analysis showed significant relationships ($p<0.05$) between the SPME variables and the component headspace concentrations, with regression equations of relatively high R^2 values (>0.7). The type of fiber coating which had the highest affinity for the volatile compounds was evaluated using a univariate method. Extraction using DVB/CAR/PDMS fiber; a 10 min equilibrium time; a 10 min extraction time; and an extraction temperature of 40°C was predicted to result in the most favorable equilibrium headspace concentrations for all volatile compounds in this study. The HS-SPME optimized method, coupled with GC analysis, was able to identify 47 VOCs in the whole plant, which represented more than that reported using hydrodistillation. In addition, due to the small amount of sample needed, the VOC composition in the aerial parts could be determined separately (flowers, leaves and stems). The methodology reported here can be used for quality control analyses of commercial samples of *H. multiflorum*.

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