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Identification of volatile compounds of *Clinopodium odorum* (Lamiaceae): A comparison between HS-SPME and classic hydrodistillation

[Identificación de compuestos volátiles de *Clinopodium odorum* (Lamiaceae): Una comparación entre HS-SPME e hidrodestilación clásica]

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Abstract: In the present work an analytical methodology to micro scale based on the use of the HS-SPME/GC-MS to determine volatile compounds present in *Clinopodium odorum* (Griseb.) Harley (Lamiaceae)was optimized and settled differences and similarities with its essential oil. A systematic description of the volatile components of flowers, stems, leaves and combined aerial parts (whole plant) was constructed via GC-MS analyses of HS-SPME adsorbed compounds and of essential oils obtained through hydrodistillation of the same tissues. Pulegone was the main component of both the HS-SPME analysis and essential oil analysis. In addition, piperitenone oxide and piperitone oxide were the other main components in the essential oil whereas in the HS-SPME analysis cis-isopulegone and menthone prevailed. The HS-SPME method can achieve comparable results to those obtained by essential oil analysis, by using very fewer samples, a shorter extraction time and a much simpler procedure.

Keywords: *Clinopodium odorum*, HS-SPME, essential oil, pulegone, piperitenone oxide, piperitone oxide, menthone, cis-isopulegone

Resumen: En el presente trabajo se ha optimizado una metodología analítica a micro-escala basada en HS-SPME/GC-MS para determinar los compuestos volátiles presentes en *Clinopodium odorum* (Griseb.) Harley (Lamiaceae), y se establecieron diferencias y similitudes con su aceite esencial. Se realizó una descripción sistemática de los componentes volátiles de flores, tallos, hojas y partes aéreas combinadas (planta entera) a partir de los análisis por GC-MS a través del sistema HS-SPME y de los aceites esenciales. Pulegona fue el componente principal tanto del análisis por HS-SPME, como del aceite esencial. Además, el óxido de piperitenona y el óxido de piperitona eran los otros componentes principales en el aceite esencial mientras que en el análisis por HS-SPME, prevalecieron cis-isopulegona y mentona. El método de HS-SPME puede lograr resultados comparables a los obtenidos por el análisis de aceite esencial, mediante el uso de muestras de menor tamaño, un tiempo de extracción más corto y un procedimiento más simple.

Palabras clave: *Clinopodium odorum*, HS-SPME, aceite esencial, pulegonae, óxido de piperitenona, óxido de piperitona, mentona, cis-isopulegona

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LIST OF ABBREVIATIONS

C. Odorum - *Clinopodium odorum* (griseb.) harley.; hs – headspace; spme – solid phase microextraction; gc-ms – gas chromatography with mass spectrometry detector; m.a.s.l – meters above sea level; vocs – volatile organic compounds; dvb – divinilbencene; car – carboxene; pdms – polydimethylsiloxane; amu – atomic mass unit; ki – kovats retention index.

INTRODUCTION

The Lamiaceae, or mint family, is the seventh largest flowering plant family with about 7,500 species organised into 236 genera, including herbs, shrubs, trees and even woody climbers. Many are of horticultural or economic importance, including culinary herbs such as basil (*Ocimum*), fragrant oils such as lavender (*Lavandula*), and important timber trees such as teak (*Tectona grandis*). Lamiaceae are also widely used in traditional medicine. The Lamiaceae is therefore a significant component of global plant diversity, particularly in the drylands of the tropics (Kew Royal Botanic Gardens Database, 2012). The essential oils of this family are regarded as the Mother Nature's chemical factory and they are a heterogeneous group of complex mixtures of organic substances (Singh *et al.*, 2011). Within this family, *Clinopodium* L. (Subfam. *Nepetoideae*, Tribe *Mentheae*, Subtribe *Menthinae*) includes about 100 species of aromatic perennial, rarely annual herbs and shrubs, mostly in the New World (both temperate and tropical) and temperate Eurasia, but a few in Africa, tropical Asia and Indomalaysia.

Clinopodium odorum (Griseb.) Harley [synonymy: *Satureja odora* (Griseb.) Epling], is a shrub up to 2 meters tall, native from Bolivia and Argentina, that have a pleasant aroma and grows wild in the mountain range of central Argentina. It is found in undisturbed sites of mountain slopes, between 1500 and 2000 m.a.s.l. It is also known as "muña-muña", "salviolora", "peperina" or "piperina" (Barboza *et al.*, 2006). It is used as a flavoring agent in food and like a medicinal plant by local people as an anticatarrhal, antispasmodic, stringent, carminative, diuretic, laxative, stomachic, anti-oxidant, antiacid, stimulant, soporific, vermifuge, menstrual suppressor, or in the treatment of flatulence, colic, altitude sicknesses, headaches and stomachaches, as well as an anti-spasmodic and to help in parturition (Harley and Granda Paucar, 2000, Mahady, 2005, Barboza *et al.*, 2009).

"Muña-muña" is usually found in local health food stores marketed as vegetable crude drugs, which are attributed with digestive properties. As its production comes entirely from wild collection, this species is subject to a high extraction pressure resulting from its use in herbalism, the beverage industry, yerba mate composite (a traditional infusion) and folk medicine (Goleniowski *et al.*, 2006). Despite its collection and trade represents a source of income for local populations, it is necessary to make efforts to prevent its extinction.

Previous investigation of the essential oils obtained by hydrodistillation of aerial parts of *C. odorum* (presented like *S. odora*) has indicated that pulegone is the major constituent (Fester *et al.*, 1951; Fester *et al.*, 1961; Muschietti *et al.*, 1996; Goleniowski *et al.*, 2006; Barboza *et al.*, 2009). However, Zygadlo *et al.* (1993) established that piperitenona was the main component in samples collected in the Pampa de Achala (Córdoba, Argentina) whereas Viturro *et al.* (2007) found that E-isocitral was the largest compound in samples collected in Tafi del Valle (Tucumán. Argentina). This situation reveals the existence of chemotypes within this species (Viturro *et al.*, 2007). Additionally, isoeugenol, isoborneol, cedrol, linalool and carvacrol were also present, albeit at smaller amounts.

Several techniques have been used to characterize the VOCs present in plants, with hydrodistillation being the most common extraction technique employed to obtain essential oils from aromatic plants (Saroglou *et al.*, 2006). However, this methodology is a laborious and time-consuming process that requires large amounts of samples, thus representing a major problem when there is a very limited amount of samples, which is often the classic problem that occurs when trying to establish the profile of the volatile organic compounds present in specimens obtained by "in vitro" propagation. Moreover, in many cases where investigators have extracted essential oils from a plant matrix to characterize the profile of VOCs, little attention has been paid to the possibility that the extraction methods may yield different essential oil profiles, or even worse, a sample degradation, despite it being well known that chemical reactions can occur during the distillation process (Babu and Kaul, 2007). Thus, the final composition of the product may not be representative of the original material, and the observed variations in oil composition may be strongly dependent on the type of distillation method used (Babu *et al.*, 2002; Babu *et al.*, 2004; Babu *et al.*, 2005). This fact makes it particularly important that

researchers explore the various advantages and disadvantages of a given extraction technique before carrying out an analysis.

Solid-phase microextraction (SPME) was introduced by Pawliszyn and co-workers (Arthur and Pawliszyn 1990), with this technique proving increasingly useful in organic analytical chemistry due to it being a rapid and simple procedure of extraction that possesses a great capacity of pre-concentration without the need for any organic solvent and the necessity of only a very small amount of samples (Vas and Vekey, 2004). HS-SPME has been used for the extraction of volatile compounds in various matrices, such as vegetables, fruits, juices, soft drinks or alcoholic beverages (Kataoka *et al.,* 2000), and it has the advantage of minimizing the sample handling and consequently decreasing the loss of volatile compounds. Furthermore, it is a simple and fast modern tool which has been used to characterize the volatile fraction of aromatic and medicinal plants (Smith, 2003; Vázquez *et al.,* 2011), thereby offering a valid alternative to the classic Clevenger essential oil hydrodistillation for the gas chromatographic analysis of volatile constituents.

To our knowledge, there are no reports in the literature about direct SPME analysis of the volatile constituents in whole plants of *C. odorum* or their aerial parts. For this reason, and in order to develop an analytical micro scale methodology to help identify the VOCs present in *C. odorum*, the present study describes the use of the HS-SPME/GC-MS method for the analysis of volatile compounds in this species to determine the SPME optimal parameters in terms of fiber type, extraction temperature, equilibrium time and extraction time. Also, a comparison between the results obtained by HS-SPME and essential oil analyses for the characteristic GC-MS profiles was performed using both qualitative and semi-quantitative analyses of VOCs from *C. odorum.*

MATERIALS AND METHODS

Plant Samples

Flowering individuals of *C. odorum* were sampled in February 2012 at Sierras Grandes of Córdoba, Argentina (35°32´384 S; 64°33´252 O; altitude: 1663 m.a.s.l.). A specimen was deposited in the Herbarium Marcelino Sayago (Register Number ACCOR 401), Faculty of Agricultural Sciences, Catholic University of Córdoba.

VOCs obtained by HS-SPME

To perform the HS-SPME/GC-MS analysis, samples (100.0 ± 0.1 mg) of fresh aerial parts (previously chopped with a clean cutter) were placed in glass vials of 20 cm^3, which were then sealed with Viton septa and aluminium seals provided by Supelco (Sigma-Aldrich, Argentina). The vials containing these samples were immersed in a thermostatic water bath at 40° C (PolyScience 8005, accuracy 0.2º C), and after 10 min the SPME device was inserted into the sealed vial by manually penetrating the septum and the fiber was exposed to the sample headspace for 10 min. After extraction, the needle on the SPME manual holder was set to its maximum length in the GC injector, and the fiber was directly exposed to the hot injector at 250° C for 5 min in a splitless mode.

Essential oil

Fresh aerial parts (500 g) of plants that had been previously dried (15 days) were hydrodistilled for 3 hours. The aqueous distillate was extracted with chloroform (3 x 20 mL), and the organic layer was separated, dried over anhydrous $MgSO_4$ and filtered. The solvent was evaporated in a rotary evaporator (ambient temperature) to obtain 1.93 mL of essential oil (yield of 0.39% v/w). 1.0 μL of this essential oil was dissolved in 1.0 mL of chloroform and 1.0 μL of this solution was analysed by GC.

Selection of extraction fiber

Five different commercial fibers were evaluated: PDMS 100 μm, PDMS-DVB 65 μm, CAR-PDMS 85 μm, DVB-CAR-PDMS 50/30 μm and PA 85 μm, which were all supplied by Supelco (Sigma-Aldrich of Argentina). Using a manual holder (Supelco), all fibers were conditioned in a GC injector at 225° C for 8 hours before use. The vials containing the samples were immersed in a thermostatic water bath at 40° C (PolyScience 8005, accuracy 0.2º C), and after 10 min the SPME device was inserted into the sealed vial by manually penetrating the septum and the fiber was exposed to the whole plant material headspace for 10 min. After extraction, the needle on the SPME manual holder was set to its maximum length in the GC injector and the fiber was directly exposed to the hot injector at 250° C for 5 min in a splitless mode

HS-SPME optimization

Having established which fiber had the highest affinity for the volatile components (greatest total area and greatest peak area of the main components in the chromatogram), tests aimed at discovering the optimum extraction temperature, equilibrium time

and exposure time were performed. To carry this out, two of the parameters were maintained invariable while the other parameter was altered. The tests to determine the optimum temperature of extraction were performed in the range 30 to 70º C. Those to determine the optimal equilibrium time were carried out in the range 10 to 60 min, and the ones to determine the optimal time of exposure to HS were performed in the range 10 to 60 min.

Gas Chromatography-Mass Spectrometry

The identification of volatile components was performed using a gas chromatograph Hewlett Packard 5890 Series II equipped with a manual injection port operating in a splitless mode and coupled to an Hewlett Packard 5970 Mass Detector. The column used was an HP-5 capillary column (30 m x 0.25 mm ID x 0.25 m film). The working conditions were: injector: 225º C; interface: 230° C; gas carrier: He 99.99%; head pressure: 5 psi; initial ramp: 40º C to 90º C (2º C/min); middle ramp: 90º C to 130º C (10º C/min); final ramp: 130º C to 200º C (5º C/min). 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 the Wiley 275 and NIST databases. The volatile components were identified by comparing their mass spectra with library data (match ≥ 90%) and by the determination of the respective Kovat´s retention indices (KI), with alkane standards provided by Sigma-Aldrich. The Retention indices were compared with those reported in the databases (NIST 2012; Pherobase 2012).

RESULTS AND DISCUSSION

Selection of extraction fiber

Although changes in the extraction conditions can strongly affect the total peak area, the number of chromatographic peaks is not influenced. Therefore in this study, the total peak area was used as a parameter (Mejías *et al.*, 2002; Diaz *et al.*, 2002) to optimize the SPME extraction conditions. Additionally, the effect of these conditions on the peak area of the main components was studied.

Figure Nº 1
Effect of fiber type on the HS-SPME procedure for the volatile components of *C. odorum*

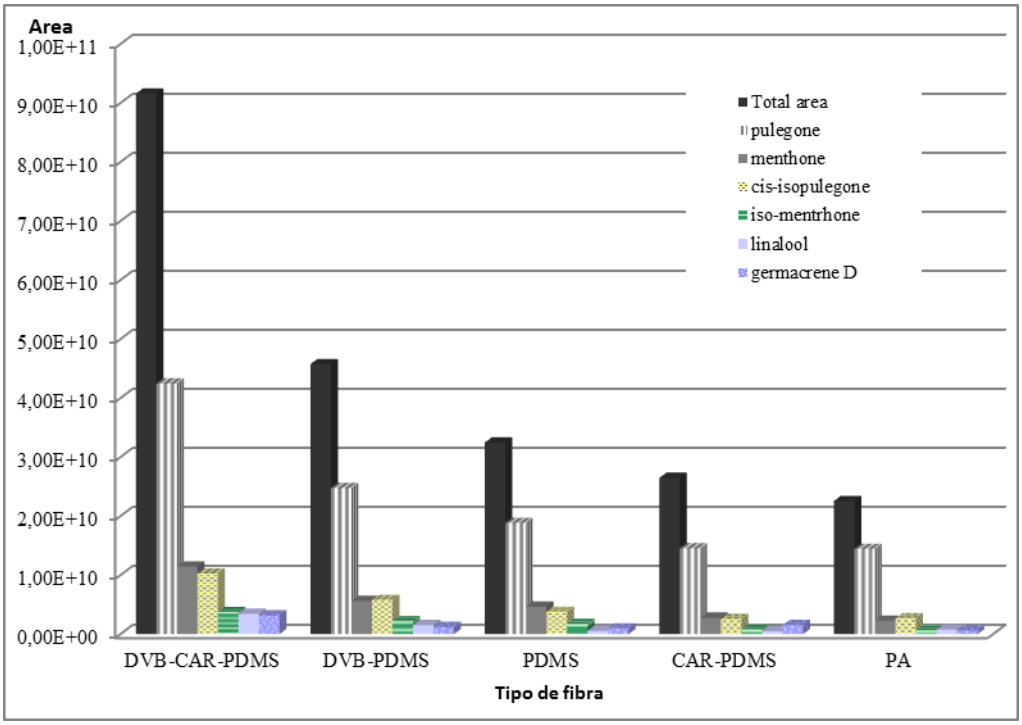


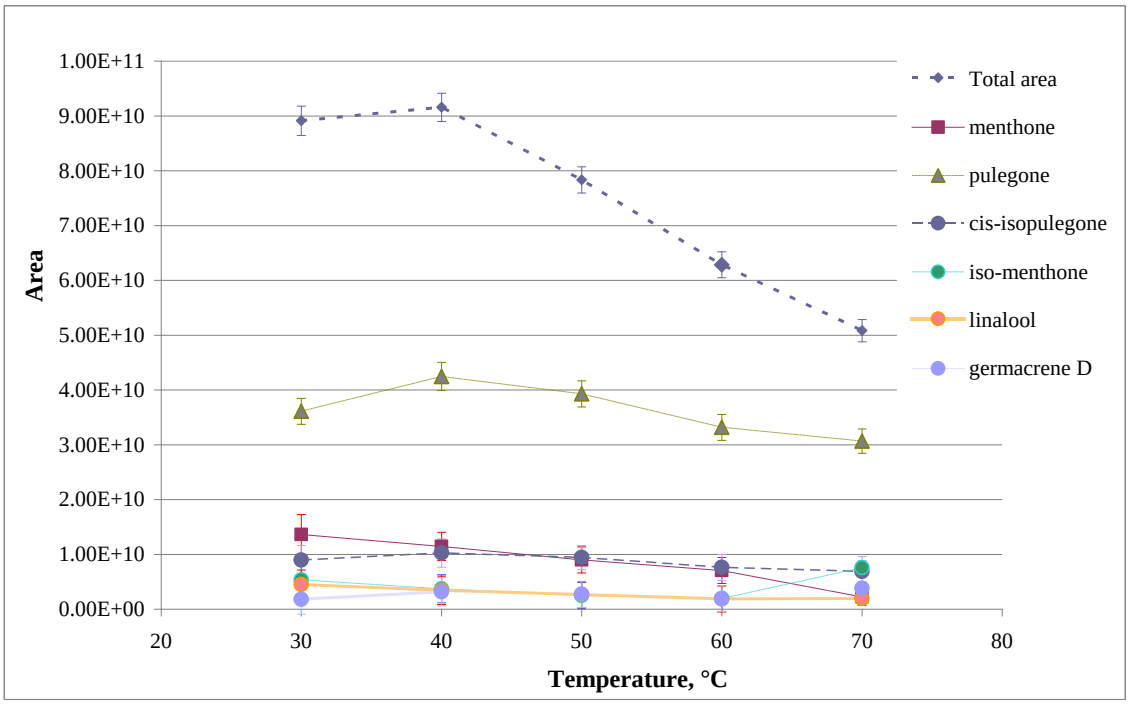
Figure Nº 1 shows the effect of fiber type on the total peak area of the chromatogram and on the peak area of the main components. As can be seen, the order of the extraction efficiencies of the volatile components as determined by the total area of the chromatograms was: DVB-CAR-PDMS > DVB-

PDMS > PDMS > CAR-PDMS > PA, with the fibers of DVB-CAR-PDMS showing a 50-70% higher extraction efficiency than the other fibers tested. In the case of the principal VOCs, the same effect was observed. DVB-CAR-PDMS was very efficient for extracting the volatile compounds present in *C. odorum*, and for this reason this fiber was adopted for the subsequent studies.

Selection of extraction temperature

Figure 2 shows the effect of extraction temperature on the total and main components areas observed in the chromatograms, where it can be seen that with increasing temperature there was a slight increase in the total area of the chromatogram peaks until 40° C. Subsequently, the total area began to decrease sharply. Also, the extraction temperature affected each component separately: as extraction temperature increased so did the peak areas of pulegone, *cis*-isopulegone and germacrene-D until 40° C, before subsequently decreasing. However, menthone, *iso*-menthone and linalool always decreased with increasing temperature.

Figure Nº 2
Effect of temperature on the HS-SPME procedure for volatile components of *C. odorum*



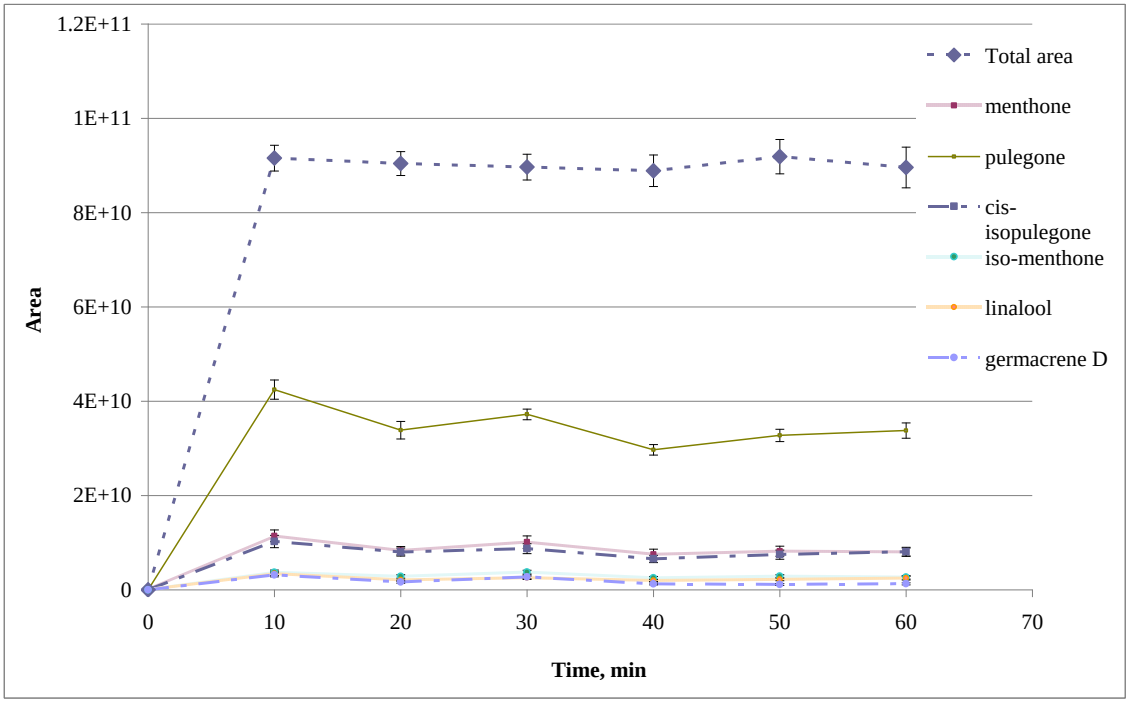
In general, the extraction temperature has a significant influence on the HS-SPME because it affects the distribution coefficients of the volatile components from the sample matrix and HS, as well as between HS and the fiber. As shown in Figure Nº 2, temperature had a different effect on each individual compound.

The increase in pulegone area with temperature showed that, until 40º C, the total area was mostly due to this compound. From the results obtained in this study, it was decided to perform the subsequent experiences at 40º C.

Selection of equilibrium time

Figure Nº 3 shows the effect of equilibrium time of HS on the total and main component peak areas of the chromatogram. As can be seen in this figure, the increase of equilibrium time of HS had no effect on the total area or the area of the main components. At all times tested, the measured parameters remained constant. Thus, there was no significant effect of equilibrium time of HS on the SPME process, and for this reason we chose to work up to 10 min.

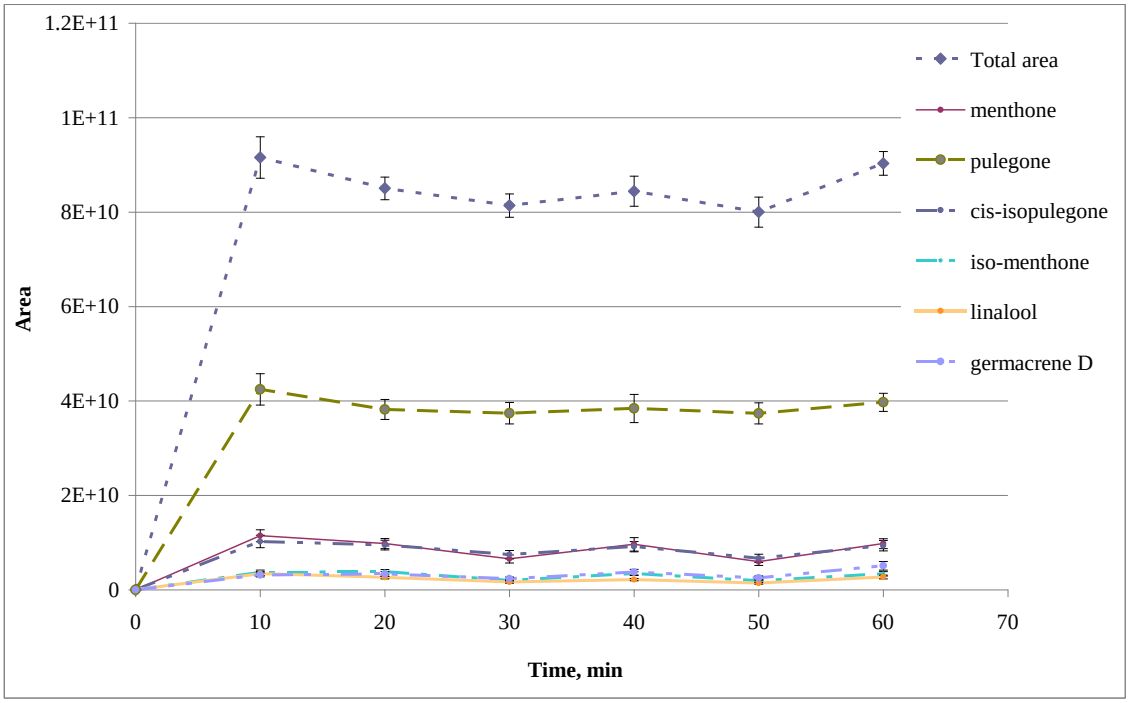
Figure Nº 3
Effect of equilibrium time of HS-SPME procedure for volatile components of *C. odorum*



Selection of extraction time

Figure Nº 4 shows the effect of exposure time of fiber to HS. It can be observed that as exposure time increased, there no was effect produced on the total area of the peaks in the chromatogram or on those peaks corresponding to principal components.

Figure Nº 4
Effect of exposure time on the fiber on HS-SPME procedure of volatile components of *C. odorum*



It is noteworthy that increases in the extraction time no produced a significant effect on the exposure time of fiber to HS and on the equilibrium time of HS after 10 min. Moreover, after 10 min the exposure time of fiber to HS a slight decrease of the total area can be observed. Therefore, for the HS-SPME parameters for the extraction of volatile compounds from fresh aerial parts of *C. odorum*, 40° C was adopted as the extraction temperature, 10 min for the equilibrium time and 10 min as the extraction time.

HS-SPME of leaves

As can be seen to below in Table Nº 1, the existence of 66 different components in the volatile fraction of leaves of *C. odorum* were established, of which 62 were successfully identified (93.94%). Thus, a positive identification was achieved in 99.69% of the total area observed in the chromatogram.

Table Nº 1
Volatile compounds observed in *Clinopodium odorum* (Griseb) Harley

Peak	Rt [a] (min)	Compound [b]	KI [c] (Ex)	KI [d] (Lit)	%				
					L.[e]	S.[f]	F.[g]	W.P.[h]	E.O.[i]
1	16.40	α-pinene	952	952	0.07	-	1.39	0.15	1.20
2	17.20	β-pinene	964	969	0.12	-	0.73	0.26	0.75
3	17.84	β-myrcene	985	983	0.12	-	0.28	0.30	0.45
4	19.38	sabinene	1007	1001	0.78	-	0.23	0.33	-
5	20.17	δ-3-carene	1017	1016	0.16	-	-	0.34	-
6	20.38	limonene	1020	1033	2.73	16.37	4.02	1.16	2.68
7	20.57	eucalyptol	1022	1023	-	-	9.96	-	2.43
8	22.05	*trans*-β-ocimene	1042	1044	0.26	-	0.65	0.52	0.59
9	22.64	γ-terpinene	1050	1050	0.21	-	-	-	0.15
10	26.02	linalool*	1095	1092	1.36	-	2.01	1.40	1.92
11	27.08	1-octenyl-3-acetate	1111	1111	4.89	-	-	0.22	0.52
12	28.42	*cis*-limonene oxyde	1132	1140	9.95	-	-	0.05	0.14
13	29.70	isomenthone	1153	1153	0.47	1.79	0.40	2.18	0.16
14	30.56	menthone	1166	1166	2.82	7.99	2.78	9.15	4.23
15	31.21	*cis*-isopulegone	1177	1173	3.66	4.16	1.59	12.10	1.88
16	31.96	*trans*-isopulegone	1188	1188	0.26	-	1.56	0.18	0.22
17	32.36	α-terpineol	1195	1196	0.19	1.65	3.20	0.44	1.72
18	32.39	dodecane	1201	1200	-	-	0.88	-	0.50
19	32.61	*cis*-α-terpineol	1204	1209	-	-	1.16	-	-
20	33.12	β-fenchyl acetate	1223	1222	-	-	2.23	-	-
21	33.49	α-fenchyl acetate	1225	1226	-	-	0.72	-	0.36
22	34.23	pulegone*	1249	1249	35.46	35.10	16.06	51.76	40.96
23	34.51	piperitone oxide	1258	1259	5.84	2.05	7.23	1.32	13.93
24	34.72	carvone oxide	1264	1263	11.44	-	6.18	-	0.27
25	34.83	diosphenol	1268	1273	0.31	-	1.41	0.43	0.12
26	34.95	isobornyl acetate	1272	1275	0.30	-	0.95	-	0.31
27	35.31	*trans*-anethol	1283	1283	2.35	-	-	0.20	0.44
28	35.40	*trans*-sabinyl acetate	1286	1284	1.04	2.38	4.13	0.09	0.38
29	35.52	*cis*-sabinyl acetate	1290	1291	0.24	-	1.26	0.19	0.20
30	35.72	thymol	1296	1295	0.27	-	0.63	0.31	0.68
31	35.82	carvacrol*	1299	1299	0.50	-	0.40	-	0.22
32	36.16	unknown	1312	-	-	-	0.41	0.08	-
33	36.51	δ-elemene	1325	1325	-	-	-	0.17	-
34	36.59	α-methylcinnamaldehyde	1328	1330	0.24	-	0.48	0.20	-
35	36.72	bicycloelemene	1333	1334	0.57	5.07	0.62	0.97	0.13
36	36.88	piperitenone	1339	1339	0.46	-	1.49	0.25	0.43
37	37.13	α-cubebene	1348	1345	0.28	-	-	0.28	-
38	37.78	piperitenone oxide	1373	1369	2.44	2.11	12.02	0.10	15.71

39	37.85	α-ylangene	1375	1377	0.24	-	-	0.30	-
40	38.07	β-bourbonene	1384	1385	0.23	1.66	1.32	0.27	0.40
41	38.23	β-elemene	1390	1391	0.29	-	0.49	0.51	0.25
42	38.49	α-copaene	1400	1400	0.15	-	0.40	0.23	0.58
43	38.59	unknown	1403	-	0.11	-	-	-	-
44	38.76	isolongipholen	1411	1406	0.30	-	1.03	0.12	-
45	38.96	β-caryophylene	1419	1418	0.09	-	0.93	0.64	0.40
46	39.19	β-cubebene	1429	1432	0.09	-	0.48	0.54	0.09
47	39.41	γ-elemene	1438	1433	0.40	-	0.18	0.19	-
48	39.63	aromadendrene	1447	1446	0.38	-	0.37	0.46	0.26
49	39.65	α-gurjunene	1448	1449	0.17	-	0.40	0.49	-
50	39.81	calarene	1455	1458	0.44	-	0.44	0.32	-
51	39.93	α-elemene	1460	1460	0.10	-	-	0.21	-
52	40.03	germacrene B	1463	1463	0.14	-	-	-	-
53	40.12	γ-muurolene	1468	1469	0.13	-	0.60	0.70	-
54	40.26	epi-bicyclosesquiphellandrene	1474	1471	0.42	-	-	-	-
55	40.36	β-selinene	1478	1479	0.22	-	0.54	0.10	0.09
56	40.45	α-amorphene	1481	1481	0.38	-	-	0.62	-
57	40.52	germacrene D	1484	1485	1.27	-	0.99	2.87	1.65
58	40.88	γ-amorphene	1489	1488	0.30	-	-	0.62	-
59	40.90	bicyclogermacrene	1500	1499	1.56	2.80	0.82	2.40	0.92
60	40.97	valencene	1503	1503	0.26	1.01	1.12	-	-
61	41.19	γ-cadinene	1512	1507	0.30	-	0.21	0.16	-
62	41.27	β-bisabolene	1516	1514	0.38	-	-	-	-
63	41.37	β-cadinene	1520	1520	0.05	-	0.63	0.83	-
64	41.49	δ-cadinene	1525	1525	0.88	1.20	1.04	1.43	0.31
65	41.72	cadina-1,4-diene	1534	1532	0.08	-	-	-	-
66	41.80	α-cadinene	1538	1538	0.18	-	0.30	0.14	-
67	41.92	α-calacorene	1543	1546	0.11	-	-	0.27	-
68	42.56	unknown	1569	-	0.12	-	-	0.19	-
69	42.85	spathulenol	1581	1582	0.13	11.08	0.31	0.06	0.27
70	43.01	globulol	1587	1587	-	-	0.15	0.08	0.31
71	44.15	unknown	1637	-	0.06	-	-	-	-
72	44.31	δ-cadinol	1644	1645	0.19	-	-	0.15	0.19
73	44.61	α-cadinol	1657	1656	0.06	2.08	0.19	-	0.21
74	45.34	unknown	1690	-	0.03	-	-	-	0.17
75	45.48	unknown	1696	-	-	-	-	-	0.08
76	46.45	bisabolol oxide A	1743	1744	-	1.50	-	-	0.12
Total					100.00	100.00	100.00	100.00	100.00
% Compound Identified					93,94	100.00	98.11	96.43	95.74
% Area Identified					99.68	100.00	99.59	99.73	99.75

[a] Rt: retention time; [b] Identified by GC-MS; [c] Kovat´s Experimental Retention Index; [d] Kovat´s Retention Index from the Literature; [e] Leaves; [f] Stems; [g] Flowers; [h] Whole Plant; [i] Essential Oil. *RI Determined using a standard sample.

From measurements made on leaves of *C. odorum* (Table Nº 1), the major components were found to be pulegone (35.46%) and carvone oxide (11.44%), with lower proportions being observed for cis-limonene oxide (9.95%), piperitone (5.84%), 1-octenyl-3-acetate (4.89%), *cis*-isopulegone (3.66%), menthone (2.82%), limonene (2.73%), piperitenone oxide (2.44%), *trans*-anethole (2.35%), bicyclogermacrene (1.56%), linalool (1.36%), germacrene D (1.27%) and *trans*-sabinyl acetate (1.04%). The rest of the components were present at amounts ranging from 0.88% (for example δ-cadinene) to 0.03% (for example β-cadinene, among others).

HS-SPME of stems

As can be seen in Table Nº 1, the existence of 17 different components in the volatile fraction of the

leaves of *C. odorum* was established and was successfully identified. Thus, positive identification was achieved in 100.00% of the total area observed in the chromatogram.

In stems of *C. odorum* (Table Nº 1), pulegone (35.10%), limonene (16.37%) and spathulenol (11.06%) were observed to be the main components. All other compounds were found at appreciable amounts (>1%), for example: menthone (7.99%), bicycloelemene (5.07%), *cis*-isopulegone (4.16%), bicyclogermacrene (2.80%), *trans*-sabinyl acetate (2.38%), piperitenone oxide (2.11%), α-cadinol (2.08%), piperitone oxide (2.05%), isomenthone (1.79%), β-bourbonene (1.66%), α-terpineol (1.65%), bisabolol oxide A (1.50%), δ-cadinene (1.20%) and valencene (1.01%).

HS-SPME of flowers

As can be seen in Table Nº 1, the existence of 53 different components in the volatile fraction of the inflorescence of *C. odorum* were established, of which 52 were successfully identified (98.11%). Thus, a positive identification was achieved in 99.59% of the total area observed in the chromatogram.

Pulegone (16.06%) and piperitenone oxide (12.02%) were found to be the major components. Additionally, appreciable amounts of eucalyptol (9.96%), piperitone oxide (7.23%), carvone oxide (6.18%), *trans*-sabinyl acetate (4.13%) and limonene (4.02%) were found. The presence of α-terpineol (3.20%), menthone (2.78%), linalool (2.01%), *cis*-isopulegone (1.59%), *trans*-isopulegone (1.56%), piperitenone (1.49%), diosphenol (1.41%), α-pinene (1.39%), β-bourbonene (1.32%), *cis*-sabinyl acetate (1.26%), valencene (1.12%), δ-cadinene (1.04%), and isolongipholen (1.03%) were also observed. The rest of the components were present at amounts ranging from 0.99% (germacrene D) to 0.15% (globulol).

Differences between the composition of flowers, stems and leaves

The data summarized in Table Nº 1 show some interesting differences between the results of the HS-SPME analysis of the flowers, stems and leaves of *C. odorum*:

* There was a significant difference in the number of compounds produced by each part of the plant. Both the flowers and the leaves were responsible for the greatest number of volatile organic compounds (53 and 66 respectively), while the stems contributed a smaller number (only 17 compounds).

*The main component, pulegone, was found at greater proportions in leaves and stems than in flowers. However, the flowers were the main source of piperitenone oxide and piperitone oxide.

*The contribution of limonene, spathulenol, menthone, *cis*-isopulegone, bicycloelemene and bicyclogeremacrene were primarily due to the stems while *cis*-limonene oxide, 1-octenyl-3-acetate and *trans*-anethol were supplied solely by the leaves.

*Moreover, eucalyptol was observed exclusively in inflorescence and bisabolol oxide A was exclusive component of the stems.

HS-SPME of combined aerial parts of the whole plant

As can be seen in Table Nº 1, the existence of 56 different components in the volatile fraction of *C. odorum* were established, of which 54 were successfully identified (96.43%). Thus, a positive identification was achieved in 99.73% of the total area observed in the chromatogram.

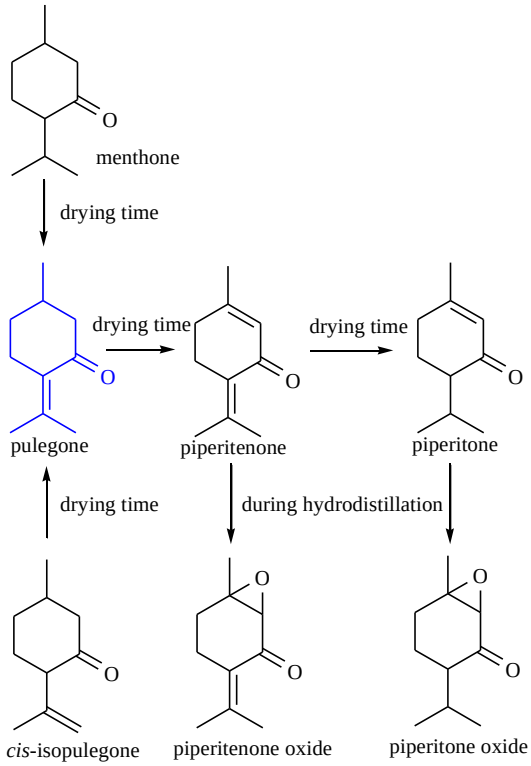
Table Nº 1 summarizes the main components provided by the HS-SPME analysis of the aerial parts of the whole plant, with pulegone (51.76 %), *iso*-pulegone (12.10%) and menthone (9.15%) being found at the greatest proportions. In addition, there were also appreciable amounts of germacrene D (2.87%), bicyclogermacrene (2.40%), isomenthone (2.18%), δ-cadinene (1.43%), linalool (1.40%), piperitone oxide (1.32%) and limonene (1.16%). The rest of the observed components were present at amounts ranging from 0.83% (β-cadinene) to 0.05% (*cis*-limonene oxide).

Essential oil analysis

As can be seen in Table Nº 1, the existence of 47 different components in the essential oil obtained by hydrodistillation of desiccated aerial parts of *C. odorum* were established, of which 45 were successfully identified (95.74%). Thus, a positive identification was achieved in 99.75% of the total area observed in the chromatogram.

The major components present in the essential oil of *C. odorum* were pulegone (40.96%), piperitenone oxide (15.71%) and piperitone oxide (13.93%). In addition, there were also significant amounts of: menthone (4.23%), limonene (2.68%), eucalipthol (2.43%), linalool (1.92%), *cis*-isopulegone (1.88%), α-terpineol (1.72%), germacrene D (1.65%) and α-pinene (1.20%). Other components were found at amounts ranging from 0.92% (bicyclogermacrene) to 0.09% (β-selinene).

Figure Nº 5
Possible pathway transformations of the principal volatile organic compounds of *C. odorum* during the drying and essential oil processes



HS-SPME analysis vs. essential oil analysis

Using the HS-SPME analysis in the previously established conditions, the existence of 56 different components in the volatile fraction of *C. odorum* were established, while in the essential oil analysis 47 components were determined (see Table Nº 1). This situation represents an observation of 16% more components using HS-SPME analysis. Moreover, the data summarized in Table Nº 1 show some differences between the results of the HS-SPME analysis of the whole plant and those from the essential oil. Comparing the essential oil and the HS-SPME data revealed the main component (pulegone) to be the same. However, the relative percentages of the other principal components were remarkably different. Whereas piperitenone oxide and piperitone oxide were the other main components in the essential oil, in HS-SPME *cis*-isopulegone and menthone prevailed. This observed difference between the results obtained by HS-SPME analysis of fresh aerial parts and the essential oil analyses could perhaps be explained through enzymatic processes (Croteau *et al.*, 1991) and microbiological changes (Madyastha and Thulasiram, 1999) occurring during the drying process, in addition to possible chemical reactions during hydrodistillation (Babu *et al.*, 2004; Babu *et al.*, 2005; Babu and Kaul, 2007). In this way, it's possible that pulegone was transformed into piperitenone oxide and piperitone oxide through the path shown in Figure Nº 5. Our working group is currently conducting studies to try to establish kinetic data to reinforce this hypothesis.

Moreover, at short retention times (0 - 35 min), there was a greater number of minor components in the essential oil than in HS-SPME, whereas at retention times ranging from 35 to 47 min, minority components were more frequent using HS-SPME.

CONCLUSIONS

A simple, rapid and solvent-free technique to determine the volatile components in *C. odorum* using the HS-SPME/GC-MS method was developed, and differences between the essential oil profiles and the data obtained by HS-SPME were established.

Using fewer samples, the shorter extraction time and much simpler procedure of the HS-SPME method can achieve comparable results to those obtained by essential oil analysis. Therefore, the developed method provides a fast and easy characterization of the volatile compound profiles, which can be used in further studies aimed at characterizing different populations of *C. odorum* using a micro scale analytical methodology. Moreover, the analytical method presented here, can be used to make quality control of commercial samples of *C. odorum*.

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