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VOLATILE COMPONENTS OF *HYPERICUM HUMIFUSUM, HYPERICUM PERFOLIATUM* AND *HYPERICUM ERICOIDES* BY HS-SPME-GC AND HS-SPME-GCMS USING NANO SCALE INJECTION TECHNIQUES

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Several products were developed, which contain *Hypericum* herb or its extracts as additives and several brands of food, beverages and yoghurts include this herb. Some Tunisian *Hypericum* species considered rare plants in Tunisia and their sampling need in the most of cases authorization from the authorities. The study of their essential oils in Tunisia was sometimes limited by operational analysis. The analysis of full dry aerial parts of these plants from Tunisia has been carried out by headspace solid phase microextraction (HS–SPME) coupled with gas chromatography–mass spectrometry (GC–MS). The obtained results showed that the non-terpene hydrocarbon fraction dominated the chemical composition of volatiles from the three *Hypericum* species with clear abundance of n-undecane, accounting 44.4%, 36.2%, and 20.2% for *H. humifusum*, *H. perfoliatum* and *H. ericoides*, respectively. This fraction was followed by terpenic hydrocarbons, and oxygenated terpenes.

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1. Introduction

The genus *Hypericum* belongs to *Hypericaceae* family [1]. It is represented by more than 450 species subdivided into 36 sections. These species are well represented in the entire Mediterranean region and unequally distributed in the other temperate areas of the world [2]. As far as we know, this genus is represented by eight species in Tunisia: *H. afrum* Desf., *H. androsaemum* L., *H. ericoides* L. ssp. Roberti (Coss.), *H. humifusum* L. ssp. Austral Rouy et Foue, *H. perfoliatum* L., *H. perforatum* L., *H. tomentosum* L., and *H. triquetrifolium* Turra [3].

Hypericum extract is used as flavouring, especially in alcoholic beverages. Maximum exposure for this use has been estimated at 6.5µg/kg bw; this is based on a maximum hypericin concentration in the beverage of 10mg/kg [4]. Dried leaves of *Hypericum* can be used in herbal teas. In this case, a daily intake of 25ug/kg bw has been estimated in the Netherlands [5]. Later, European regulation on flavourings and certain food ingredients with flavouring properties for use in and on foods has replaced earlier published regulation and directive in order to take into account

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technological and scientific developments in the area of flavourings and the developments of the food legislation in the European Community. This new regulation has mentioned that *Hypericum* extracts may only be used for the production of alcoholic beverages [6].

In addition, plants belonging to genus *Hypericum* are well known to be used in practical medicine due to their therapeutic efficacy. For centuries, *Hypericum* has been used as anti-inflammatory, sedative, analgesic, diuretic, antimalarial, and vulnerary. Traditionally, the plant was used for the treatment of several infections such as trauma, burns, rheumatism, hemorrhoids, neuralgia, gastroenteritis, snakebite, ulcers, contusions, sprains, hysteria, bedwetting, and depression [7–10].

The plants of *Hypericum* are listed in Pharmacopoeias of many countries such as Czechoslovakia, France, Germany, Poland, Romania, and Russia [11–14].

In Tunisia, several studies have been reported mainly on the morphological variability of the species of *H. triquetrifolium* [15], isozyme polymorphism of *H. humifusum* [16], fatty acid analysis of *H. triquetrifolium* [17], essential oil compositions of *H. triquetrifolium* [18], *H. perfoliatum* and *H. tomentosum* [19], antiviral activity of *H. triquetrifolium* [20], and the detection of secondary metabolites in methanol extracts of *H. perforatum*, *H. perfoliatum* and the Endemic *ericoides* Ssp *Roberti* [21].

Different chemical compositions from *Hypericum* species using gas chromatography (GC) and/or GC coupled with mass spectrometry (GC–MS) have been reported [19, 22–36].

Ultrasonic assist with headspace solid phase microextraction (HS–SPME) assay is a rapid and simple procedure successfully used to sampling the volatile components from aromatic and medicinal plants [37–39].

SPME was introduced for the first time by Arthur and Pawliszyn [40]. Later, this technique has found application in food, pesticide, and gained access to other environmental fields [41–49]. Coated with an appropriate stationary phase, it has increasingly gained attention as an effective technique for sample extraction [45, 50]. This technique is used in combination with various analytical instruments such as GC–MS for the analysis of volatile, semi-volatile, polar and non-polar plant compounds, vegetables, fruits, beverages, and dairy products [51].

Several products were developed, which contain *Hypericum* herb or its extracts as additives and several brands of food, beverages and yoghurts include this herb [52, 53].

Hypericum species are generally classed as essential oil-poor plants (generally oil yield <1%, w/w) [54, 55]. In addition, *H. humifusum*, *H. perfoliatum* and *H. ericoides* were considered rare plants in Tunisia and their sampling need in the most of cases authorization from the authorities. The study of their essential oils in Tunisia was sometimes limited by the above discussed reasons. This limitation was justifiable for operational reasons. To overcome these difficulties, we have tried to analyze the chemical composition of these species by rapid and cost-effective methods such as HS–SPME.

Thus, the main aims of the present study were to provide a description of volatile profiles and its variability among the less studied *Hypericum* species in Tunisia. The full dry aerial parts of *H. humifusum, H. ericoides* Ssp *Roberti*, and *H. perfoliatum* from Tunisia were collected by HS–SPME. Their analusis was performed using GC and GC–MS, dual flame ionization detector (FID), Electron Ionization Mass Spectrometry (EIMS), and Chemical Ionization Mass Spectrometry (CIMS).

2. Experimental

2.1. Plant collection

Specimen's collection was carried out under the permission of the Tunisian Ministry of Agriculture, Water Resources and Fisheries. Plant samples of *H. perfoliatum, H. humifusum,* and *H. ericoides* sp *Roberti* were collected in June 2008 from the 'El Feidja' National Natural Reserve in the North West of Tunisia (36°30'N, 8°19'E, altitude 812 m). Specimens were identified by Professor Mohammed El Hedi El Ouni (Department of Biology, Faculty of Sciences of Bizerte, Tunisia). Voucher specimens were deposited in the Herbarium of the Laboratory of Transmissible

Diseases and Biological active Substances (Faculty of Pharmacy of Monastir, Monastir, Tunisia).

2.2. Instruments, operating conditions and procedure

For HS–SPME a Supelco SPME device coated with polydimethylsiloxane (PDMS, 100 m) was used. The studied plants were introduced in a 10 mL septum–cap vial and allowed to equilibrate for 20 min at 25°C before sampling. The fiber was pre-conditioned according to the manufacturer instructions. At equilibrium, the fiber was exposed to the headspace for 1 min at room temperature. Once sampling was finished, the fiber was withdrawn into the needle and transferred to the injection port of GC or GC–MS system. For GC a Hewlett Packard gas chromatograph HP-5890 Series II instrument equipped with HP-WAX and DB-5 capillary columns (30 m×0.25 mm, 0.25 μm film thickness) was used. The temperature was programmed at 60°C for 10 min, ramp of 5°C/min up to 220°C. Injector was at 250°C. Helium was used as a carrier gas with a constant flow at 2 mL/min; FID, split ratio 1:30.

For GC-EIMS, a Varian CP-3800 gas–chromatograph equipped with a DB–5 capillary column (30 m×0.25 mm; coating thickness 0.25 m) and a Varian Saturn 2000 ion trap mass detector were used. Injector and transfer were at 220°C and 240°C, respectively. The temperature was programmed from 60°C to 240°C at a rate of 3°C min^{-1}.The carrier gas used was helium, at a flow rate of 1 mL/min. Injection of 0.2 μL (10% hexane solution); split ratio 1:30. Moreover, the molecular weights of all the identified substances were confirmed by GC–CIMS, using methanol (MeOH) as chemical ionizing gas.

2.3. Qualitative and quantitative analyses

Identification of the constituents was based on comparison of the retention times (l.r.i.) with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, and on computer matching against commercial [56, 57] and home-made library mass spectra built up from pure substances and components of known oils and MS literature data [57–61].

3. Results and discussion

A total of 127 volatile components were characterized, representing 96.5%, 90.4%, and 94.5% of the total volatile components detected for *H.humifusum*, *H.perfoliatum*, and *H. ericoides*.

3.1. Chemical composition of volatiles from *H. humifusum*

As shown in table 1, the non-terpene hydrocarbons (HC) fraction represented the main fraction (47.0%) and it was represented mainly by *n*-undecane (44.4%), whereas *n*-nonane is less represented and it constituted only 2.2% of the total volatiles. The second major chemical fraction was represented by monoterpene hydrocarbons (MH, 36%). Eleven compounds within this group of chemicals were identified in which 5 compounds were represented as traces. The main components were *α*-pinene (22.8%), *β*-pinene (11.2%) and limonene (1.2%), myrcene (0.4%), □-cymene (0.3%), and □-terpinene (0.1%). The minor components were *α*-fenchene (tr), *α*-terpinene (tr), lavender lactone (tr), (E) *β*-ocimene (tr), and bergamal (tr). The sesquiterpene hydrocarbons fraction (SH), the third main chemical group, was represented by 20 components and constituted 10.14% of the total volatile compounds. This fraction was represented mainly by 1,7 di-epi-*β*-cedrene (4.4%), □-muurolene (0.8%), *α*-copaene (0.8%), *δ*-cadinene (0.6%), and khusimene (0.4%). The oxygenated monoterpenes fraction (OM) was the least represented (2.6%) and constituted by 12 compounds. It was represented by cis and trans-linalool oxide (0.8% and 0.5%, respectively), pinocarvone and trans-pinocarveol in equal content (0.3%). In addition, *H. humifusum* showed a low content of oxygenated sesquiterpene (OS, 0.1%) which was represented by caryophellen oxide.

Table1. Composition and relative percentage concentrations of volatiles constituent of studied Hypericum species according to their elution order in the GC analysis.

No	Fractions	Compounds	l.r.i.	***H. humifusum***	***H. perfoliatum***	***H. ericoides***
				Area %	Area %	Area %
1	Other	n-hexanal*	800	0.3	0.3	0.2
2	Other	(E)-2-hexanal*	854	tr	tr	0.8
3	Other	2-heptanone*	889	tr	0.2	tr
4	HC	n-nonane	900	2.2	21.1	42.2
5	Other	2-methyl-4-heptanone	921	tr	tr	tr
6	MH	α-pinene	938	22.8	9.6	3.8
7	MH	α-fenchene	952	tr	tr	0.4
8	Other	(Z)-Hept-4-en-1-ol	973	tr	tr	1.4
9	MH	β-pinene	982	11.2	0.7	0.6
10	Other	6-methyl-5-hepten-2-one	988	0.4	0.4	0.4
11	MH	myrcene	992	0.4	0.6	tr
12	HC	n-decane	1000	0.1	1.2	0.5
13	Other	n-octanal	1002	tr	tr	0.3
14	MH	α-phellandrene	1008	tr	tr	0.3
15	MH	α-terpinene	1020	tr	tr	tr
16	MH	□-cymene	1028	0.3	0.4	0.1
17	MH	limonene	1033	1.2	1.5	1.7
18	Other	benzyl alcohol	1040	tr	tr	tr
19	OM	lavender lactone	1049	tr	tr	tr
20	MH	(E)β-ocimene	1052	tr	tr	tr
21	MH	bergamal	1058	tr	tr	0.1
22	MH	□-terpinene	1063	0.1	tr	tr
23	OM	cis-linalool oxide	1077	0.8	0.4	0.5
24	OM	cis-sabinene hydrate	1079	tr	tr	tr
25	OM	trans-linalool oxide	1092	0.5	tr	0.3
26	Other	2-nonanone	1094	tr	tr	0.2
27	HC	n-undecane	1100	44.4	36.2	20.2
28	OM	cis-thujone	1116	tr	tr	tr
29	OM	exo-fenchol	1116	0.1	tr	tr
30	OM	cis-□-menth-2-en-1-ol	1124	tr	tr	tr
31	Other	methyl octanoate	1128	tr	tr	tr
32	OM	α-campholenal	1130	0.1	tr	tr
33	Other	octyl formate	1136	tr	tr	0.1
34	OM	trans-pinocarveol	1142	0.3	tr	tr
35	OM	camphor	1149	tr	tr	0.2
36	OM	isopolegol<neo>	1152	tr	tr	tr
37	OM	(E,Z)-2,6-nonadienal	1157	tr	tr	tr

Table 1 (cont.)

38	OM	iso-borneol	1159	tr	tr	tr
39	OM	trans-pinocamphone	1160	tr	tr	tr
40	OM	pinocarvone	1166	0.3	tr	tr
41	OM	□-cymen-8-ol	1187	tr	tr	tr
42	OM	α-terpineol	1192	0.1	tr	tr
43	Other	methyl salicylate	1195	tr	tr	tr
44	OM	myrtenol	1196	0.2	tr	tr
45	HC	n-dodecane	1198	tr	tr	tr
46	OM	safranal	1201	0.2	tr	0.2
47	Other	n-decanal	1207	tr	tr	tr
48	OM	verbenone	1210	tr	tr	0.1
49	OM	trans-carveol	1222	tr	tr	0.3
50	OM	carvone	1242	tr	tr	tr
51	Other	butyrophenone	1254	tr	tr	tr
52	OM	geranial	1274	tr	tr	0.1
53	Other	nonanoic acid	1285	tr	tr	tr
54	Other	n-undecanone	1295	tr	tr	tr
55	HC	n-tridecane	1300	0.3	1.3	0.7
56	SH	α-cubebene	1353	0.2	10.5	7.0
57	Other	n-undecanol	1360	tr	tr	tr
58	SH	cyclosativene	1368	tr	0.3	0.3
59	SH	α-ylangene	1373	0.2	tr	tr
60	SH	α-copaene	1378	0.8	0.3	0.7
61	SH	β-patchoulene	1380	tr	tr	1.8
62	SH	β-maaliene	1381	tr	1.9	tr
63	SH	α-duprezianane	1383	tr	tr	tr
64	SH	β-bourbonene	1386	tr	tr	0.6
65	SH	β-cubebene	1393	tr	tr	tr
66	SH	β-elemene	1395	0.2	tr	1.0
67	SH	β-longipinene	1399	tr	1.0	tr
68	SH	longifolene	1405	0.3	0.2	tr
69	SH	iso-caryophyllene	1408	tr	tr	0.5
70	SH	1,7 di-epi-β-cedrene	1414	4.4	0.4	tr
71	SH	α-cedrene	1417	tr	tr	tr
72	SH	β-caryophyllene	1421	tr	tr	1.2
73	SH	β-copaene	1431	tr	tr	0.4
74	SH	β-gurjunene	1430	0.2	tr	0.5
75	SH	cis-thujopsene	1432	tr	0.5	tr
76	SH	□-elemene	1437	0.2	tr	tr
78	SH	α-guaiene	1440	tr	tr	0.3
79	SH	cis-muurola-3,5-diene	1446	tr	tr	0.3

Table 1 (cont.)

80	SH	α-himachalene	1450	tr	tr	tr
81	SH	khusimene	1451	0.4	0.4	0.5
82	OM	geranyl acetone	1453	tr	tr	tr
83	SH	α-humulene	1455	tr	tr	tr
84	SH	β-farnesene	1458	0.1	tr	tr
85	SH	allo-aromadendrene	1460	0.1	tr	0.3
86	SH	cis-muurola-4(14),5-diene	1464	tr	tr	tr
87	SH	dehydro-aromadendrene	1467	tr	tr	tr
88	SH	β-acoradiene	1467	0.3	tr	tr
89	SH	□-muurolene	1478	0.8	0.5	1.7
90	SH	germacrene-D	1480	0.2	tr	0.2
91	SH	AR-curcumene	1483	0.3	tr	tr
92	SH	β-selinene	1484	tr	tr	tr
93	Other	(E)-β-ionone	1485	tr	tr	0.2
94	SH	cis-β-gaiene	1493	0.2	tr	0.2
95	SH	valencene	1491	tr	tr	tr
96	SH	α-muurolene	1500	0.2	tr	0.4
97	HC	n-pentadecane	1507	tr	0.2	tr
98	SH	trans-□-cadinene	1514	0.3	0.2	tr
99	SH	δ-cadinene	1525	0.6	tr	0.7
100	SH	zonarene	1526	tr	tr	tr
101	SH	cis-calamenene	1532	0.1	tr	tr
102	SH	α-cadinene	1539	tr	tr	tr
103	SH	α-calacorene	1544	tr	tr	tr
104	SH	β-calacorene	1565	tr	tr	tr
105	OS	spathulenol	1578	tr	tr	tr
106	OS	caryophellen oxide	1583	0.1	tr	tr
107	HC	1-hexadecene	1592	tr	tr	tr
108	HC	n-hexadecane	1603	tr	tr	tr
109	Other	tetradecanal	1613	tr	0.2	tr
110	OS	1-epi-cubenol	1629	tr	tr	tr
111	SH	hinesol	1640	tr	tr	tr
112	OS	epi-α-cadinol	1643	tr	tr	tr
113	OS	α-cadinol	1656	tr	tr	tr
114	OS	cadalene	1675	tr	tr	0.3
115	OS	khusinol	1680	tr	tr	tr
116	Other	apiol	1682	tr	tr	tr
117	HC	n-heptadecane	1700	tr	0.2	tr
118	HC	n-octadecane	1800	tr	tr	tr
119	Other	musk xylol	1832	tr	tr	tr

Table 1 (cont.)

120	HC	n-nonadecane	1900	tr	tr	tr
121	Other	cembrene	1930	tr	tr	tr
122	Other	beyerene	1930	tr	tr	tr
123	Other	phytol	1950	tr	tr	tr
124	HC	heneicosane	2100	tr	tr	tr
125	HC	n-decosane	2200	tr	tr	tr
126	HC	n-tricosane	2300	tr	tr	tr
127	HC	n-tetracosane*	2400	tr	tr	tr

MH	Monoterpene hydrocarbons	36.0	12.7	7.0
OM	Oxygenated monoterpenes	2.6	0.4	1.7
SH	Sesquiterpenes hydrocarbons	10.1	16.0	18.6
OS	Oxygenated sesquiterpenes	0.1	0.0	0.3
HC	Non-terpene hydrocarbons	47.0	60.2	63.5
Others		0.7	1.1	3.4
Total		96.5	90.3	94.5

l.i.r: linear retention index relative to C9-C23 n-alkanes on the DB-5 capillary column; tr= trace (<0.1%).
* tentatively identified.

3.2. Chemical composition of volatiles from *H. perfoliatum*

The HC was the major fraction (60.2%) which was represented by 13 compounds, 7 of which were revealed as traces. The major compounds were n-undecane (36.2%) and n-nonane (21.1%). The SH was the second main fraction (16.0%). It was represented by 19 compounds, 8 of which were revealed as traces. This fraction was represented mainly by α-cubebene (10.5%). The fourth main fraction was represented by MH (12.7%). Six compounds were identified, accounting 12.8%, with α-pinene (9.6%) as the major component. The OM fraction (0.4%) was represented by 11 compounds. Among this group of chemicals, cis-linalool oxide was revealed at a level above trace amounts (0.4%). Finally, the OS fraction was represented by 3 chemicals, all at trace amounts (Table 1).

3.3. Chemical composition of volatiles from *H. ericoides* Ssp *Roberti*

The volatiles of this plant were predominantly composed of alkanes (63.5%), 7 within this fraction were identified as *n*-nonane, *n*-decane, *n*-undecane, *n*-hexadecane, *n*-heptadecane, *n*-octadecane, and *n*-nonadecane and 4 compounds were revealed as traces. *n*-nonane (42.2%) and *n*-undecane (20.2%) were the major compounds of this fraction. The second main fraction, SH (18.6%), was represented by 28 compounds, 13 within these compounds were revealed as traces. The representative compounds of this fraction were identified as *α*-cubebene (7%), □-muurolene (1.7%), *β*-caryophyllene (1.2%), *β*-elemene (1%). The MH fraction were represented in less amounts (7%) and it was represented by 7 compounds, one within these compounds was revealed as trace. The MH fraction was represented mainly by *α*-pinene (3.82%), limonene (1.70%), and *β*-pinene (0.55%). The content of OM was relatively low (1.7%). The OS fraction (0.3%) was represented by cadalene (0.3%) (Table 1).

Taken the above discussed results, the HC fraction dominated the chemical composition of volatiles from the three *Hypericum* species, accounting 47.0%, 60.2% and 63.5% for *H. humifusum, H. perfoliatum*, and *H. ericoides* Ssp Roberti, respectively (Figure 1). This fraction was represented mainly by *n*-nonane and *n*-undecane, which both constituted 57.3% for *H. perfoliatum* and 62.4% for *H. ericoides* Ssp Roberti, whereas *n*-nonane is less represented for *H. humifusum* (2.2%). The HC was represented for *H. humifusum* mainly by *n*-undecane (44.4%). Terpenic hydrocarbons constituted the second main chemical group in the *Hypericum* samples. This fraction was dominated by MH fraction for *H. humifusum* (36%) followed by SH fraction (10.1%), whereas terpenic hydrocarbons for *H. perfoliatum* and *H. ericoides* Ssp Roberti were represented mainly by SH fraction (16.0% and 18.6%, respectively) followed by MH fraction (12.7% and 7.0%, respectively) (Figure 1). The less abundant chemicals were oxygenated terpenes which represented only 0.4%, 2.0%, and 2.7% for *H. perfoliatum*, *H. ericoides* Ssp Roberti, and *H. humifusum*, respectively. The OM was the dominated fraction in this group of chemicals and represented 2.6%, 1.7%, and 0.4% for *H. humifusum*, *H. perfoliatum*, and *H. ericoides* Ssp Roberti, respectively.

In addition, some chemical compounds were detected in *H. humifusum* with a content above the cutoff determined for traces (<0.1%), whereas these compounds were revealed as traces for the remaining species. These compounds were □-terpinene, exo-fenchol, *α*-campholenal, trans-pinocarveol, pinocarvone, *α*-terpineol, myrtenol, *α*-ylangene, □-elemene, *β*-farnesene, *β*-acoradiene, *AR*-curcumene, *cis*-calamenene, and caryophellen oxide. Other compounds were more related to *H. perfoliatum* such as 2-heptanone, *β*-maaliene, *β*-longipinene, *cis*-thujopsene, *n*-pentadecane, *n*-heptadecane, tetradecanal, and *n*-heptadecane, whereas other compounds were more related to *H. ericoides* such as (E)-2-hexanal, *α*-fenchene, (Z)-Hept-4-en-1-ol, *n*-octanal, *α*-phellandrene, bergamal, 2-nonanone, octyl formate, camphor, trans-carveol, geranial, *β*-patchoulene, *β*-bourbonene, iso-caryophyllene, *β*-caryophyllene, *β*-copaene, *α*-guaiene, *cis*-muurola-3,5-diene, cadalene, and (E)-*β*-ionone.

Other chemical compounds were detected in two Hypericum species, whereas they were revealed as traces for the remaining plant. For *H. humifusum* and *H. perfoliatum*: longifolene, myrcene, 1,7 di-epi-*β*-cedrene, and trans-□-cadinene; for *H. humifusum* and *H. ericoides*: trans-linalool oxide, safranal, *β*-elemene, *β*-gurjunene, *allo*-aromadendrene, germacrene-D, cis-*β*-gaiene, *α*-muurolene, *δ*-cadinene; for *H. perfoliatum* and *H. ericoides*: cyclosativene.

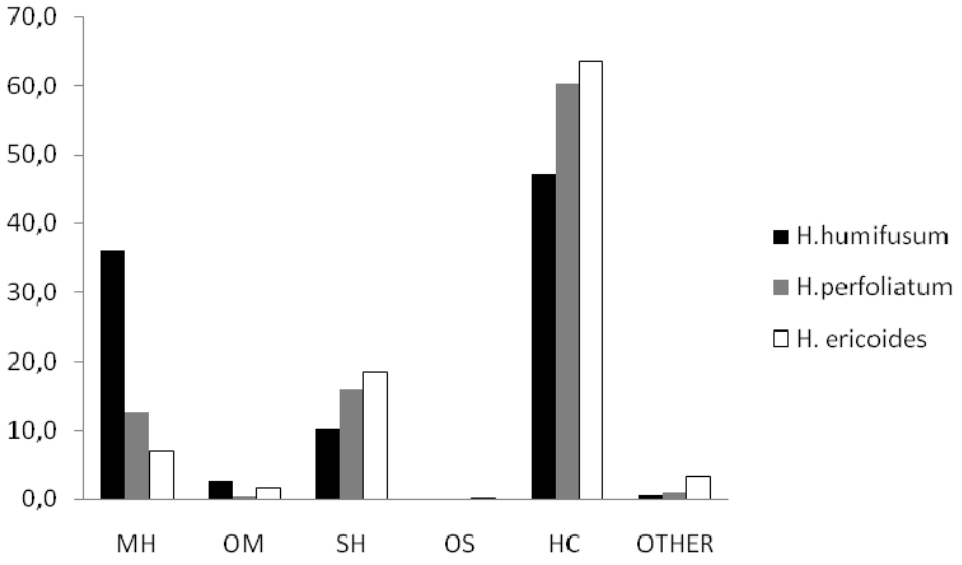

Fig. 1. Percentage of major volatile groups of studied Hypericum species.

4. Conclusion

In summary, the coupling of HS–SPME sampling with GC–MS has been shown to be very fast, handy, reliable and inexpensive extraction tool for organic volatiles. SPME is capable to analyze the volatiles with the least sample amount and sample preparation steps. In addition, significant ability of trapping and extracting of compounds which are more volatile mainly for Tunisian rare plants such as *Hypericum* species, which their sampling is under strict governmental regulations.

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