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Aroma Profiles Study in *Ocimum Sanctum L.* During Plant Growth Using Head-Space Solid-Phase Microextraction

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Abstract
A head space solid-phase microextraction (HS-SPME) method coupled to gas chromatography/mass spectrometry (GC/MS) has been developed and applied for profiling of volatile compounds released from *Ocimum sanctum L.* during plant growth. Three types of different SPME fibers including polydimethylsiloxane (PDMS),divinylbenzene/carboxen/polydimethylsiloxane(DVB/CAR/PDMS),carboxen/polydimethylsiloxane(CAR/PDMS) and carbowax/polyethylene glycol (CW/PEG) were used as an adsorption phase and the best extraction was achieved with the mixed fiber 2. A gas chromatography coupled with mass spectrometer was employed for identification of the volatiles extracted from the head space of sampling vials by SPME. As a result, 23 compounds were detected using this method. Esdragol (70.33-90.57%), L-carrol (0.07-4.66%), α-citral (0.28-6.76%), caryophyllene (0.33-2.12%), β-citral (0.14-9.63%) and methyl eugenol (0.33-3.11%) were dominate volatile components, the relative content of which was found to enable differentiating between the seasonal examined. The oxygenated terpens were the most aroma compounds of *O. sanctum L.* in head space of the sample vial.

Keywords
Ocimum sanctum L; HS-SPME; Esdragol; GC/MS; Plant growth.

1.INTRODUCTION

The genus *Ocimum* belonging to the Lamiaceae comprises annual and perennial herbs and shrubs native to the tropical and subtropical regions of Asia, Africa and South America [1]. The taxonomy of *Ocimum* is complex due to inter specific hybridization and polyploidy of the species in the genus. Pushpangadan and Bradu [2] recognized more than 150 species, however, Paton et al [3]. proposed that *Ocimum* had only 65 species and other attributions should be considered as synonyms . The *Ocimum L.* is the major essential oil crop around the world and cultured commercially in many countriesand used as medicinal herb in medical treatments such as for headaches, coughs, diarrhea, worms and kidney malfunctions. Plants of the genus *Ocimum* are also reported for many biological activities, such as mosquito reported and antimicrobial activity [4], insecticidal activity against crop pest insects, antipyretic and antioxidant activity [5-9]. It is also, essential oil of *Ocimum L.* has been utilized extensively in the food industry [10]. Several analytical methods have been developed to determine volatile constituents of essential oils present in species representing a complex mixture of volatile substances present generally at low concentrated within the analytical procedure [11].

Various methods, such as steam distillation, solvent extraction and HS-SPME have been used to extract volatile compounds in plants [12-15]. Of course, it should be noted that the use of classic distillation method, can cause some difficulties. Monoterpens are possible to undergo chemical changes under conditions of a steam distillation. On the other hand, solvent extraction may cause losses of some volatile compounds of the herbal plant during the step of solvent removal [12-13].
The SPME method combines the extraction and concentration of analytes in one step using a fiber coated with an appropriate stationary phase. The adsorbed volatiles then subsequently desorb into the hot GC injection port for the isolation of the components [12-15].
HS-SPME procedure as a solvent-less technique was combined with GC/MS to analysis of volatile constituents of *Ocimum sanctum L* . during plant growth. For this purpose, HS-SPME/GC/MS has been developed and applied for detection and identification of volatile compounds in dried *Ocimum sanctum L* . [16-17]

2.EXPERIMENTAL

2.1. Materials and samples

All samples during September to November (2018) were collected from our apartment balcony (Fig 1).

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The collected samples on 25- September, 6-October, 16-October, 26-October, 5-November, 15-November and 25-November were named S_1, S_2, S_3, and S_4, S_5, S_6 and S_7, respectively. The plant was identified in Herbarium of Payame Noor University, Mashhad and a Voucher specimen (PNUMH – Ryhan 11) was deposited. The collected plants were dried in a shadow place for one week and the dried plants were transported to GC/MS laboratory. In each growth step, 40 mg of representative sample was placed into a 15 mL head space vial, then one mL doubled distilled water was added to the vial the gas sailed cap was fixed. The sample was stirred (at 300 rpm) during adsorption step.

The SPME fiber coated with divinylbenzene/ carboxen/ polydimethylsiloxane (50/30µm, DVB/ CAR/ PDMS) was purchased from Suppelco (USA). Before use, the fiber was conditioned following the manufacturer's recommendations. Before the samples analysis was started, a thermal cleaning of the fiber in the GC injection (10 min at 260 °C) was performed. The 15 mL head space vials (Sigma, USA) were cleaned using double distilled water and dried in an oven at 150 °C for 1h .

2.2 HS-SPME procedure

A manual SPME holder and three different fibers, polydimethylsiloxane (PDMS), divinylbenzene / carboxen/ polydimethylsiloxane (DVB/ CAR/ PDMS), and fiber carbowax/polyethylene glycol (CW/PEG) from Supelco (Bellefonte, USA), were used for the SPME procedure. All the fibers were conditioned prior to use by insertion into the GC injector at 250 °C for PDMS (0.5 h), at 270 °C for DVB/CAR/PDMS (1 h) and at 240 °C for CW/PEG (0.5 h). For each extraction, after the SPME needle pierces the septum, the fiber was extended through the needle and exposed to the headspace above the sample under a temperature 20-65 °C. After an extraction time (2-20 min), the fiber was withdrawn into the needle, and then the needle was removed from the septum and inserted directly onto the injection port of the GC. The desorption of analytes from the fiber coating was performed by heating the fiber in the injection port at 250 °C for 1-10 min. The desorbed analytes were transferred directly into the chromatographic column for analysis. Before the first daily analysis, the fiber was conditioned for 5 min at 250 °C in the GC injector. The identification of compounds in the sample was based on retention index (RI), the similarity index (SI), National Institute of Standards and Technology (NIST) MS spectral library and literature survey [18-23].

2.3. GC–MS conditions

Chromatographic separation was performed on a DB-5 column (30 m × 0.32 mm, film thickness 0.25 µm) (Australia) using a Shimadzu GC-MS model QP5050 (Kyoto, Japan). The analysis program and conditions were as follows: helium at 1.7 ml min^{-1} as a carrier gas; ionization potential, 70 eV; injector and detector temperatures were at 260 °C and 280 °C, respectively. The initial temperature of the column was kept at 60 °C for 1 min and programmed to 140 °C at a rate of 3 °C min^{-1}, then to 250°C at a rate of 50 °C min^{-1} and kept constant at 250°C for 3 min. The split ratio was 1:28. The mass spectra were acquired across a mass range, *m/z* 30 to 450 amu in full scan acquisition mode. *N*-alkene mixtures were also performed under the same temperature condition program to calculate the retention indexes (RI). Volatile compounds were identified by comparison of mass spectra obtained from analytes to those of authentic reference standards from the NIST and Wiley libraries with a resemblance above 85% .The calculated RI for each component was compared with literature

Fig. 1. The photos of *Ocimum sanctum L* during plant growth.

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3.RESULTS AND DISCUSSION

3.1 SPME fibers

Three types of fibers (PDMS 100 µm, DVB/CAR/PDMS 2 Cm 50/30 µm , and CW/PEG 60 µm) were used to evaluate the effect of fiber types on the extraction of volatile compounds in *Ocimum sanctum L* . Fig. 2 shows the total peak areas of the obtained compounds by the three types of fibers. As it is shown in Fig2, DVB/CAR/PDMS fiber achieved higher extraction of the analytes than the other fibers. This suggested that the retention ability of the DVB/CAR/PDMS fiber for the volatile compounds in the plant is much stronger than the rest two fibers. As shown in Fig 2, the fibers with a medium polar coating appeared to be more efficient for the extraction of *Ocimum sanctum L* . compounds. It probably resulted from the fact that most of the analytes in the sample are of medium polarity. The polarity of the two fibers were supposed to be in the order of PDMS<DVB–CAR–PDMS. In this case, the fiber DVB–CAR–PDMS has higher extraction ability than the others. On the basis of the above results, the DVB–CAR–PDMS fiber was selected for the extraction of the volatile compounds in this medicinal plant.

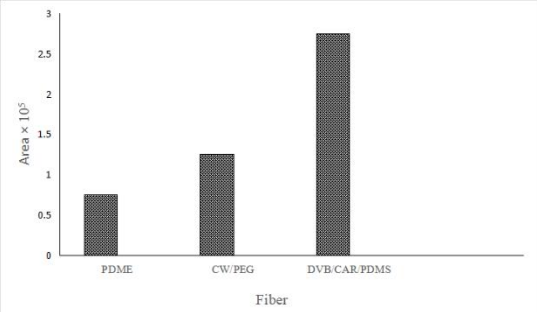

Fig. 2. Effect of fiber type on the total peak areas of all the obtained compounds from *Ocimum sanctum L* .3.2. Extraction temperature

As shown in Fig 3 it was found that the total peak areas increased steadily with the temperature increasing. The extraction temperature had a significant influence on the extraction because it can influence the distribution coefficients of the compounds between the sample and the headspace and between the headspace and the fiber. From these results, the temperature of 50 ◦C was finally used for the present work as the optimal extraction temperature. Regarding temperature, there is a compromise in the behavior between two main processes. This means, an increase of this variable will promote the analyte distribution to the HS from the sample since it is an endothermic process, meanwhile, it will disfavor the analyte distribution from the HS to the extractant phase (extraction) due to the process is exothermic. Consequently, a temperature optimization is highly encouraged[24].

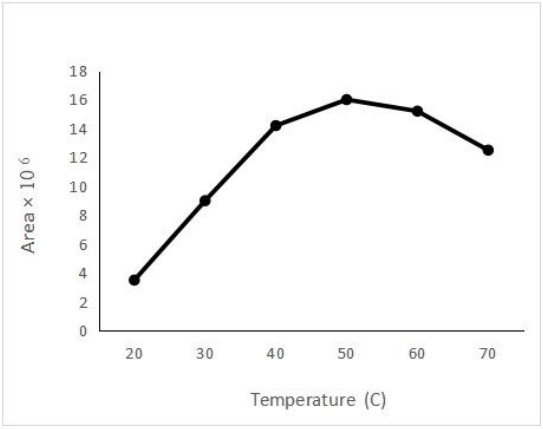

Fig. 3. The results of extraction temperature optimization for HS-SPME analysis of *Ocimum sanctum L* .

3.3 Extraction time

The extraction time varying from 2 to 20 min was investigated and the results are shown in Fig 4. Fig 4, shows the effect of extraction time on the total peak areas of all the obtained compounds for the medicinal plant. The profile for the total peak area shows a highest total peak area at 10 min. So, the extraction time of 10 min was finally selected for the analysis. As SPME is an equilibrium process there is no more extraction after 10 min.

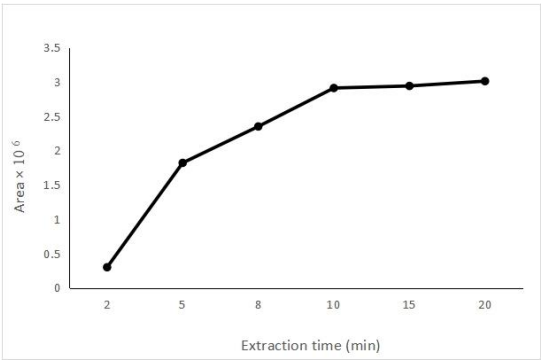

Fig. 4. The result of extraction time for HS-SPME analysis of *Ocimum sanctum L* .3.4. Desorption time

Desorption time in the injection port was investigated in the range of 2–10 min while keeping the DVB/CAR/PDMS fiber at the same injection. The effects of desorption time on the total peak area are shown in Fig 5. The profile for the total peak area shows an almost complete desorption of the compounds at 3 min. Taking into consideration that a longer desorption time may hurt the fiber lifetime, desorption time of 3 min was employed for the purpose. The results show that the sample preparation time was 13 min (10 and 3 min for adsorption and desorption, respectively). Also, the total analysis time including sample preparation and GC/MS analysis was less than 1h. The qualitative and quantitative

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analytical results are show in Table1 [25]. A total of 23 components were identified by GC/MS, representing more than 90% of the volatile components in the head space of the samples.

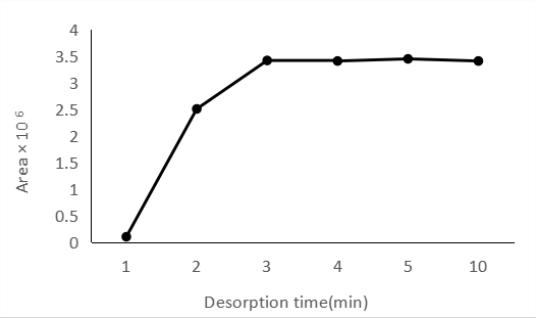

Fig. 5. The result of desorption time for HS-SPME analysis of *Ocimum sanctum L* .

Comparing the oil composition of these stages of the plant growth showed some differences. A comparison between the percentages of some main components in the plant is shown in Fig.6. The first major compound obtained from all of the studied samples was esdragol. The highest and lowest percentages of esdragol were found 90.57% and 70.33%, respectively. The next main components with large variation amounts during the plant growth were (Fig 6) L-carrol (0.07-4.66%), α-citral (0.28-6.76%), caryophyllene (0.33-2.12%), β-citral (0.14-9.63%) and methyl eugenol (0.33-3.11%). Comparing the volatiles profile among different stages of plant growth did not show any correlation between life time and the major component (estragol) of the plant. On the other hand the maximum amount for β-citral was found in S_3 step. However, the total amount of the volatiles depends on the stages of the plant growth (Table 1). It could be conclude that for the effectiveness of estragol in the plant there is no using step of the medicinal herb. Literatures showed high content of methyl chavicol (44.63%) and linalool (21.84%) in the O. sanctum essential oil from India.[26] In another study reported from India, eugenol (61.30%) was found as major component in the essential oil of O. sanctum followed by β-caryophyllene (11.89%) and germacrene D (9.14%).[27] Joshi [28] also reported that mehyl eugenol is detected as major (92.4%) component of *O. sanctum* essential oil from Belgaum.

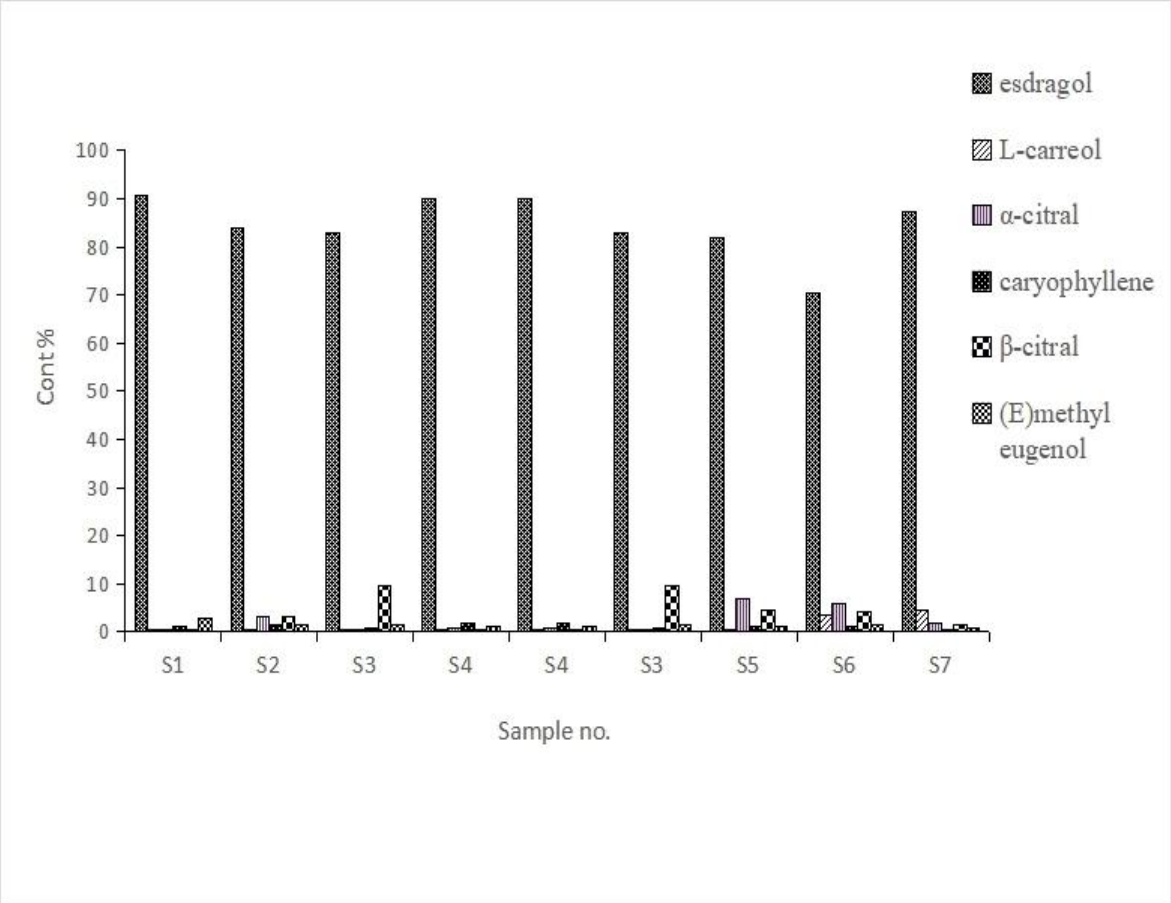

Fig.6. The variation of five major components of *Ocimum sanctum L* during the plant growth.

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Table 1. Chemical variation of *Ocimum sanctum L.* volatiles (%) during plant growth.

RI[1]	Compound	SI[2]%	S_1	S_2	S_3	S_4	S_5	S_6	S_7
1033	1,8-cineol	85	0.15	0.05	0.03	0.14	0.10	0.33	0.43
1114	fenchol	90	0.76	0.21	ND	0.24	0.41	0.40	0.05
1195	esdragol	98	90.57	83.83	82.93	90.06	81.74	70.33	87.29
1223	L-carreol	80	0.18	0.11	0.21	0.07	0.05	3.46	4.66
1254	*α*-citral	90	0.52	3.16	0.28	0.33	6.76	5.72	1.86
1268	anethole	86	0.05	0.3	ND	0.28	0.45	ND	ND
1290	m-thymol	89	0.13	0.1	ND	0.33	ND	0.70	ND
1351	*α*-longipinene	90	1.19	0.27	0.09	0.49	0.28	0.47	0.32
1402	longifolene	85	0.10	0.07	0.09	0.10	0.07	0.88	ND
1411	caryophyllene	95	1.14	1.37	0.33	1.96	1.23	1.17	0.61
1413	cedrene	85	0.09	0.04	0.90	0.09	0.67	0.69	0.33
1425	*α*- bergamotene	88	0.15	0.15	0.17	0.25	0.13	ND	ND
1429	thujopsene	85	0.04	0.06	0.08	0.12	0.03	ND	0.23
1447	humulene	98	ND	1.2	ND	1.01	0.17	ND	ND
1480	germacrene D	97	0.38	0.53	0.79	1.22	0.79	ND	ND
1488	muurolene	85	0.05	0.09	0.09	ND	0.07	ND	ND
1509	*β*-bisabolene	90	0.94	1.17	0.93	1.49	0.84	ND	0.40
1590	viridiflorol	85	0.01	0.04	0.22	ND	ND	0.46	0.16
1688	cedrenol	85	0.04	0.05	ND	ND	ND	ND	0.54
1692	*β*-citral	90	0.14	3.16	9.63	0.19	4.52	4.05	1.66
1697	(E)methyl eugenol	90	2.98	1.46	1.45	1.29	1.06	1.34	0.33
1709	isocaryophillene	85	ND	0.15	0.15	0.24	0.13	ND	ND
1593	*cis*-geranic acid methyl ester	88	0.05	0.24	0.17	0.06	0.39	ND	0.16
	total		98.52	97.81	98.54	99.96	99.89	90.00	99.03

1 retention index(RI)
2 similarity index (SI%)
3 not detected

4.CONCLUSION

The results showed, the fibers with a medium polar coating appeared to be more efficient for the extraction of *Ocimum sanctum L* . compounds. Besides esdragol, L-carrol , α-citral , caryophyllene , β-citral and methyl eugenol were also found as major constituents of the plant. The chemical composition shows a great variation during plant growth for the most of the major constituents. The content of β-citral, goes from 0.14% (in S_1) until 9.63% (in S_3), while the third major component, α-citral, shows variation from 0.33% (in S_4) until 6.76% (in S_5). The similar results were found for the other major components of *Ocimum sanctum L*. These types of variation for the *Ocimum* species are reported previously by hydrodistillation method in the literature.

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مطالعه روش میکرو استخراج فاز جامد فضای فوقانی ترکیبات فرار گیاه ریحان (*Ocimum Sanctum L*) در طول رشد آن

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چکیده

روش میکرو استخراج فاز جامد فضای فوقانی (HS-SPME) کوپل شده با کروماتوگرافی گازی / اسپکترومتری جرمی (GC/MS) توسعه داده شده و برای ترکیبات فرار آزاد شده از گیاه ریحان (*Ocimum Sanctum L*) درطول رشد آن به کار برده شد. سه نوع فیبر متفاوت SPME شامل پلی دی متیل سیلوکسان (PDMS) ، دی وینیل بنزن/ کربوکسن/ پلی دی متیل سیلوکسان (DVB/CAR/PDMS) و کربوواکس/ پلی اتیلن گلیکول (CW/PEG) به عنوان فاز جاذب استفاده شدند و بهترین استخراج با استفاده از فیبر (DVB/CAR/PDMS) بدست آمد. دستگاه کروماتوگرافی گازی کوپل شده با اسپکترومتر جرمی برای جداسازی و شناسایی ترکیبات فرار استخراج شده از فضای فوقانی ویال های نمونه به کمک SPME به کار برده شد. بدین ترتیب، ۲۳ ترکیب با این روش استخراج و شناسایی شد. استراگول (Estragol 70.33-90.57%)، ال کارول (L-Carrol 0.07-4.66%) ، آلفا سیترال (α-Citral 0.28-6.76%)، کاریو فیلن (Caryophyllene 0.33-2.12%)، بتا سیترال (β-Citral 0.14-9.63%) و متیل ایگنول (Methyl eugenol 0.33-3.11%) ترکیبات فرار را شامل بودند که نسبت آنها در بازه زمانی مطالعه شده متفاوت بود. ترپن های اکسیژنه بیشترین ترکیبات معطر ریحان در فضای فوقانی ویال نمونه بودند.

کلید واژه ها

ریحان (*Ocimum Sanctum L*); HS-SPME; استراگول ;GC/MS ; طول رشد گیاه.

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