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Comparative Analyses of the Volatile Components of *Citrus Aurantium* L. Flowers Using Ultrasonic-Assisted Headspace SPME and Hydrodistillation Combined with GC-MS and Evaluation of their Antimicrobial Activities

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The volatile components of *Citrus aurantium* L. flowers were characterized by GC-MS with two different extraction techniques, hydrodistillation (HD) and ultrasonic-assisted headspace solid phase microextraction (UA-HS-SPME). In the SPME method, the volatile components of the samples, irradiated by ultrasonic radiation, were collected on a polydimethyl siloxane (PDMS) commercial fiber as well as some manually prepared nanoporous fibers from the samples headspace. To reach the better results, the extraction conditions were carefully optimized for the PDMS fiber. Under the optimized conditions (*i.e.* sonication time 15 min, extraction time 30 min and extraction temperature 55 ºC), 54 compounds were identified by the UA-HS-SPME-GC/MS method. The essential oil components of *Citrus aurantium* L. flower samples from two different regions of Iran and new and old samples from the same region were compared to one another. The major components identified for the samples with both the SPME and HD methods were linalool, linalyl acetate, limonene, β-myrcene, geranyl acetate, and neryl acetate, respectively. However, a substantial variation in the percentages of the components was identified for different samples and different extraction methods. The antimicrobial activities of the oil were also examined against six standard bacteria. There was some activity against Enterococcus fecalis, Escherichia coli, and Bacillus cereus, indicating important biological activities of the oil.

Keywords: Ultrasonic-assisted headspace SPME (UA-HS-SPME), Polydimethyl siloxane, *Citrus aurantium* L. flowers, Antimicrobial activity

INTRODUCTION

Citrus aurantium L. (*C. aurantium*), commonly known as bitter orange, belongs to the order *Geraniales* and the family *Rutaceae* [1]. Several bioactive compounds have been extracted from *C. aurantium*, such as alkaloids [2], limonoids and phytosterols [3], flavonoids [4], dietary fibers and essential oils [5] with various therapeutic effects [6-9]. Among them, *C. aurantium* oils, including neroli from flowers, have great economic, medicinal and nutritional values. *C. aurantium* oils have been generally recognized as safe because of their wide-spectrum biological activities, such as antimicrobial activity [10], antifungal activity [11], antioxidant activity [12] and anti-inflammatory and anxiolytic effects [13]. Citrus oils are highly susceptible to oxidation, resulting in significant changes in odor and flavor. Neroli oil, is extracted from the small, white, waxy flowers of the bitter-orange tree by steam distillation and yields 0.8-1%. Gas chromatography-mass spectrometry (GC-MS) and GC-olfactometry are widely used to study the composition of citrus aroma [14-19]. The main chemical components of neroli oil are linalool, linalyl acetate, and

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limonene [20]. Traditional methods of essential oil extraction are cold-pressing, hydrodistillation, steam distillation and solvent extraction. These methods, due to their simple operation and low cost are still common. However, they suffer from some specific limitations. Because of the convenience of extraction, headspace (HS) samplings, such as headspace solid-phase microextraction (HS-SPME) methods, have been attractive for both food and flavor applications. HS-SPME technique is an alternative to conventional extraction techniques in analytical scale. This extraction technique allows obtaining real aroma profiles of a plant sample.

To the best of the authors' knowledge, no study has been published on the ultrasound-assisted headspace solid phase microextraction (UA-HS-SPME) applied to the *C. aurantium* L. aroma. Therefore, the objectives of this study are to investigate the potential of UA-HS-SPME in the extraction of volatile components from *C. aurantium* and compare the results to hydrodistillation methods (HD) by GC/MS.

EXPERIMENTAL

Reagents and Materials

Helium 99.999%, as a carrier gas, was purchased from Roham Gas Company (Tehran, Iran). Alkane mixture consisting of the C_8-C_{20} alkanes (Concentration of 40 mg ml^{-1} in hexane) was purchased from Fluka. All other chemicals were of the highest purity available from Merck or Fluka.

Antibacterial evaluations were carried out against standard bacteria in the Microbiology Research Laboratory of the Razi Herbal Medicine Research Center. The tested bacteria were Staphylococcus aureus ATCC 25923, S. epidermidis ATCC 12228, Enterococcus faecalis 29212, Escherichia coli ATCC 25922, Listeria monocytogenes ATCC 1298 and a Bacillus cerusstrain which originally isolated from rice in the Foodstuff Laboratory of the Department of Health [21]. The standard bacteria were obtained from the Microbiology Reference Laboratory (BoAli Hospital, Tehran). They were grown on the Muller Hinton broth or agar (Merck, Germany) for experimental study.

Apparatus

For GC-MS analysis, a Shimadzu model GC-17A (Kyoto, Japan) gas chromatograph coupled to a Shimadzu Quadruple-MS model QP5050 mass spectrometer was used. Compounds were separated on a 30 m × 0.22 mm i.d. fused-silica capillary column coated with 0.25 µm film of BP-5 (Shimadzu) and a split/splitless injector with a 1 mm internal diameter glass liner. Ultra-pure helium at 0.8 ml min^{-1} was used as carrier gas.

The commercially available polydimethylsiloxane fiber (PDMS, 100 µm diameter, non-bonded) was purchased from Supelco (Bellefonte, PA, USA) and used as commercial fiber for extraction of the analytes. The fiber was handled using a manual SPME fiber holder provided by Supelco (Bellefonte, PA, USA). Ultrasonic irradiation (18 kHz, 450 W) was applied by means of a PFO100 5RS Series ultrasonicator (Italy) equipped with a water bath in which the extraction vials were placed.

Sample Preparation

Citrus aurantium flowers (blossoms) were collected from Shiraz and Babol, in the south and north of Iran, respectively. The Babol and Shiraz-new samples were collected in spring 2011 and dried in shadow in room temperature. The Shiraz-old sample was an older sample, purchased from the market at relatively the same time. The samples were ground into a coarse powder with a household grinder and stored in capped bottles in refrigerator before analysis.

Preparation of Essential Oils by HD

For the extraction of the essential oils by ordinary HD method, 40 g of the dried sample was weighted and transferred into a 500 ml round bottom flask. Then, 250 ml double distilled water was added and the mixture was boiled in a Clevenger-type apparatus for 90 min. After cooling, the essential oil was collected and dried on sodium sulphate. Finally, the essential oil was stored in refrigerator at 4 ºC.

The UA-HS-SPME Procedure

For extraction of volatiles of the plant samples using SPME fibers the optimized conditions reported elsewhere were used [23]. For this purpose, 1 g of the ground sample was weighted and placed in a 40 ml vial with a silicon tap.

The vial was capped by a septum and placed into an ultrasonic bath with a preset temperature on 55 ºC, for 15 min. Next, a PDMS fiber was exposed to the sample headspace through the septum cap for 30 min at the same temperature for SPME extraction of volatile compounds. The fiber was then retracted back into its holder and directly inserted into the injection port of the GC-MS system. The fiber was kept in the injection port for 2 min after injection for a complete desorption of the compounds from the fiber. After every 10 analyses, a GC run was performed with the fiber without sampling to assure its complete desorption. Sonication was applied to create stress in the sample matrix for better releasing of the analytes, and to control the temperature during the extraction process.

GC-MS Analysis

The injector and detector temperatures of the GC-MS instrument were set at 250 ºC and 260 ºC, respectively. The carrier gas was helium at a flow rate of 0.8 ml min^{-1}. GC was operated in a splitless mode, for SPME, or with a split ratio of 1:50, for HD analysis. The column temperature was initially set at 40 ºC and increased to 200 ºC at a rate of 4 ºC min^{-1}, then increased to 270 ºC at a rate of 35 ºC min^{-1} and retained at 270 ºC for 3 min, resulting in a total GC run time of 46 min. The ion source temperature was kept at 220 ºC, and the transfer line temperature at 250 ºC. The mass fragments were collected in the range from 35 m/z to 450 m/z with an acquisition rate of 1000 to provide satisfactory number of points per peak for the effective spectral resolution. Ionization energy of 70 eV and a detector voltage of 1700 V were applied for the qMS detector.

Antimicrobial Testing

The oil was diluted in Hexanat different concentrations to the highest possible concentration and used for antimicrobial evaluation. The solution was filter-sterilized and used for disc diffusion and broth microdilution technique [21]. Paper blank discs (Ø 4.5 mm) were saturated with 40 µl of the samples and the solvent was evaporated under a safety cabinet at room temperature. Bacterial broth cultures were incubated for 4 h to reach to the lag phase and used for microbroth dilution assay and disc diffusion. Before each, the suspension's turbidity was compared and equalized with the Mac Farland 0.5 standardand. For the disc diffusion, the suspension was spreaded over a Muller Hinton agar plate by a sterile swab and essential oil or antibiotic discs were placed on the culture plate. Ciprfloxacine antibiotic was used as positive control. The plates were then incubated at 35 ºC overnight and the inhibition zone (IZ) was measured after 24 h incubation.

Minimum inhibitory concentration (MIC) was determined in a 96 well flat-bottom sterile plates (Nunc, Denmark). The bacterial inoculums were grown in Muller-Hinton broth to the lag phase and then adjusted to the turbidity of McFarland 0.5 Standard. The oil was serially diluted with the medium in the wells and then, 100 µl of bacterial suspension was added to obtain a final concentration of 5×10^{-5} cfu/ml [22]. In growth control well, uninoculated and antibiotic controls were included on each plate. The plates were incubated at 35 ºC and the turbidity was observed on a tray-reading stand. Samples from clear wells were cultured on nutrient agar (Merck, Germany) for determination of the MBC. The MIC is defined as the lowest concentration of the test which inhibits bacterial growth. The lowest concentration that did not grow on the nutrient agar plate was taken as the MBC. All experiments were repeated three times and average values were presented as the result.

RESULTS AND DISCUSSION

In order to provide a complete peak separation of extracted compounds, some preliminary SPME-GC-MS experiments were performed. The experimental parameters such as sonication time, extraction time and temperature, desorption time and water content of sample were optimized based on the results of a previous study in our department [23].

Comparison of UA-HS-SPME and HD

It has been reported that the humidity or addition of water to the samples may have significant influence on the extraction in an SPME experiment [24]. Therefore, the effect of humidity was studied by addition of 200 µl of water to the samples in the optimized conditions. As shown in Fig. 1, for some of the components, there is a moderate improvement in the extraction efficiency after the addition of water.

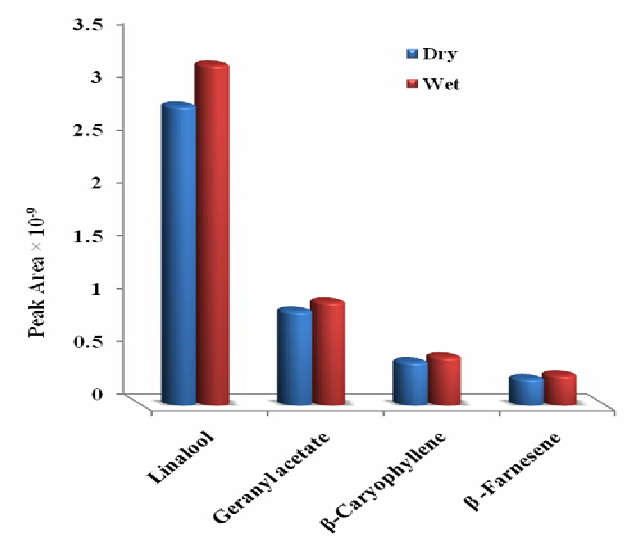

Fig. 1. Effect of water addition on the extraction efficiency of the SPME method.

The components were identified and quantified by GC-MS. Identification of the components was carried out by comparison of their spectra with standard spectra in the NIST Library (Wiley) and calculation of the time and retention indicators of the components. Retention indexes (RIs) of the analytes of interest were calculated based on the linear temperature program retention index method using the mixture of alkanes (C_8-C_{20}) as retention index prob.

Relative percentage of each component of the essential oil was obtained according to the surface area of the corresponding peak on GC chromatogram by normalizing of the surface and ignoring the response coefficient.

After obtaining the chromatograms and mass spectra, retention indicators and the yield of the components were calculated. The number of components and their percentages obtained with the PDMS fibers were compared to those obtained by a classic hydrodistillation (HD) technique. The results are summarized in Table 1.

The volatile components of *Citrus aurantium* L., extracted by UA-HS-SPME and analyzed by GC-MS, were compared with each other and with those obtained by using a conventional HD method. Figure 2 shows the TIC chromatograms of the samples using PDMS fiber.

As shown in Table 1, almost all the volatile components monitored by HD, were identified by the SPME method as well. Therefore, the PDMS fiber can provide more or less, similar information to those of the HD method by using much less amounts of the sample and in a much shorter time. The major components identified in both methods were linalool, linalyl acetate, limonene, β-myrcene, geranyl acetate and neryl acetate, respectively. However, a substantial variation in the percentages of the components identified by the two methods was observed.

The percentages of the components in different *C. aurantium* samples were also different. For example, camphor and borneol were only found in the old sample from Shiraz and thymol, which existed only in the Babol sample. In addition, neo-allo ocimene, plinol-d and zingiberene were identified by the UA-HS-SPME method in all the samples but they were not found in the HD essences of them. Heptadecane, tridecanal, benzyl benzoate, triatetracontane were found only in the new samples from Shiraz. The variations in chemical composition of the samples, may be due to the nature of the soil [25], the amount of sunlight, and temperature variations or the occurrence of chemotypes [26]. The substantial difference between the composition of the new and old samples from Shiraz indicated that the composition of the flowers changes during the drying procedure.

Screening for Antibacterial Activity

The broth microdilution assay was used for evaluation of antimicrobial activity of the oil. Interestingly, the essential oil was antibacterial against species of Gram positive as well as Gram negative bacteria. There was an inhibitory effect as well as bactericidal effects against E. coli, Entrocuccusfecalis, and Bacillus cereus (Table 2). The dilution of 45 µg ml^{-1} showed control on the growth of E. coli. This antibacterial activity was also confirmed by the disc diffusion technique as there was an inhibition zone of 12 mm around the tested oil's disc (Table 2). Antibacterial activities against L. monocytogenes (MIC = 194, MBC = 388 µg ml^{-1}; IZ = 8 mm), E. fecalis (MIC = 194, MBC = 194 µg ml^{-1}; IZ = 10 mm) and Bacillus cereus (MIC = 194, MBC = 388 µg ml^{-1}) were observed. The reaction to the other bacterial species was insignificant at the concentration tested.

Antibacterial properties of Citrus aurantium have been reported recently [27-29]. Siddique *et al*. have reported

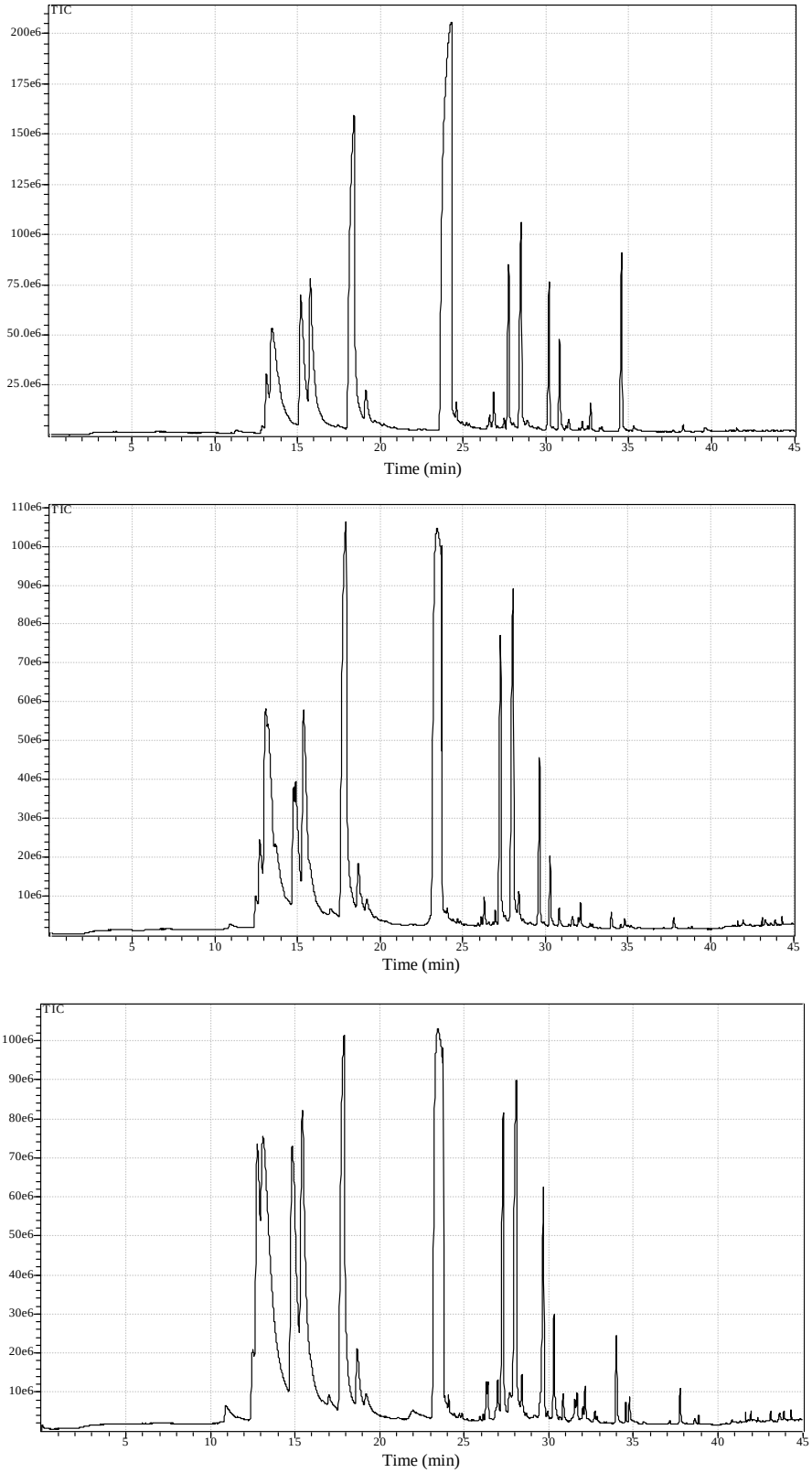

Fig. 2. GC-MS chromatograms of the extracted volatile compounds of *Citrus aurantium* L obtained by UA-HS-SPME using PDMS fiber. (a), Shiraz-fresh (b), Shiraz-old and (c), Babol extracts. Experimental conditions: sample weight, 1 g; temperature, 55 °C; sonication time, 15 min; extraction time, 30 min.

Table 1. Comparison of Compounds Identified in the Headspace of *C. aurantium* L. by Use of HD and UA-HS-SPME-GC/MS

No.	Compound	RI	*Shiraz-fresh*	*Babol*	*Shiraz-old*			
			SPME	HD	SPME	HD	SPME	HD
1	*α*-Pinene	11.35	0.093	0.55	0.38	1.26	0.05	0.24
2	Camphene	12.07	-	0.04	-	-	-	-
3	Sabinene	12.98	0.25	0.85	1.16	2.98	0.75	0.53
4	*2-β*-Pinene	13.32	2.75	7.34	6.76	10.97	2.71	3.32
5	*β*-Myrcene	13.55	14.31	3.56	17.92	2.95	8.13	1.8-
6	*α*-Pentylfuran	13.64	-	0.12	-	0.387	-	-
7	*α*-Terpinene	14.74	-	0.11	-	-	-	-
8	Limonene	15.46	9.25	13.27	17.92	-	2.58	4.97
9	*β*-Ocimen	15.99	10.75	7.79	9.64	-	4.44	2.92
10	*γ*-Terphnene	16.47	-	0.31	-	-	-	-
11	*trans*-Linaloloxid	17.07	-	0.25	-	0.9	0.06	0.5
12	Terpinolene	17.54	0.053	0.72	0.17	-	0.14	0.44
13	Linalool	18.75	26.19	35.78	11.01	40.65	18.53	31.34
14	Lilac aldehyde	20.39	0.093	0.07	-	-	-	0.16
15	Terpineol-4	21.69	0.21	0.19	-	1.13	-	-
16	*α*-Terpineol	22.42	-	3.85	-	8.6	-	9.19
17	Linalyl acetate	23.97	-	11.15	20.39	11.65	30.85	21.68
18	Geraniol	24.534	-	0.92	0.04	5	-	4.77
19	Bornyl acetate	25.42	0.039	0.06	0.03	-	0.08	-
20	Linalyl propionat	26.72	0.42	0.05	0.04	-	0.1	-
21	*delta*.-Elemene	27.02	-	0.2	0.28	-	-	-
22	*α*-Terpinyl acetate	27.60	0.22	0.1	0.26	-	0.21	-
23	Neryl acetate	27.86	4.36	2.71	3.88	3.61	5.35	3.83
24	Geranyl acetate	28.60	6.95	4.71	6.13	5.16	8.06	5.95
25	*β*-Elemene	29.025	0.42	0.04	0.57	-	0.73	-
26	*β*-Caryophyllene	30.29	2.66	0.94	2.86	-	2.46	0.65
27	*β*-Farnesene	30.93	2.25	0.49	1.08	0.525	0.9	0.31
28	*α*.-Humulene	31.52	0.28	0.08	3.88	-	0.32	0.19
29	Germacrene-*d*	32.35	0.19	0.09	0.28	-	0.2	0.06
30	*α* -Farnesene	32.66	0.10	0.03	0.57	-	0.21	-
31	Bicyclogermacrene	32.83	0.59	0.36	0.29	-	0.46	0.34
32	*delta*-Cadinene	33.41	0.079	0.04	0.1	-	0.05	0.1
33	*β*-Sesquiphellandrene	33.54	0.079	0.02	-	-	0.05	-
34	Nerolidol isomer	34.70	4.41	1.5	0.77	3.72	0.24	1.76
35	Hexadecane	35.38	0.093	0.74	0.57	0.28	0.07	-

Table 1. Continued

36	*β*-Eudesmol	38.20	-	0.09	-	-	-	-
37	Heptadecane	38.42	0.15	0.62	0.28	0.325	0.16	-
38	Tridecanal	39.27	-	0.06	-	-	-	-
39	*Trans-2*-Tridecanal	40.84	-	0.04	-	-	-	-
40	Benzyl benzoate	41.16	0.053	0.21	-	-	-	-
41	Triatetracontane	42.56	-	0.08	-	-	-	-
42	Eicosanoic acid methyl ester	42.71	-	0.02	-	-		-
43	Bicycloelemene	26.85	0.98	-	-	-	0.34	-
44	Zingiberene	29.16	0.11	-	0.02	-	0.2	-
45	Spathulenol	35.20	-	-	-	-	0.06	0.43
46	*neo-allo*-Ocimene	19.13	2.20	-	1.58	-	0.67	-
47	*cis*-Jasmone	29.51	0.053	-	-	-	0.16	0.1
48	*β*-Cubebene	29.76	-	-	0.12	-	0.08	-
49	Thymol	25.27	-	-	1.29	-	-	-
50	Plinol-*d*	25.01	0.026	-	0.07	-	0.13	-
51	Camphor	20.23	-	-	-	-	-	0.34
52	Borneol	21.12	-	-	-	-	-	0.46

Table 2. The MIC and MBCs (μg ml^{-1}) and Inhibitin Zone (mm) of *Citrus aurantium* Against some Standard Bacteria

Bacteria	(μg ml^{-1}) Essential oil MIC	MBC		Ciprofloxacine (μg ml^{-1}) MIC	MBC	Disc Difussion CP	Oil	-
E. coli	45	45		1	3.5	12	22	
S. aureous	>388*	> 388	>388	1	1	>5	15	
S. epidermidis		> 388		1	1	>5	30	
L.	194	>388		0.5	2	8	28	
monocytogenes	1 94	194		1	2	10	29	
E. fecalis	194	388		1	1	8	29	
B. cereus								

antibacterial activities of essential oil extracted from fruit peel to be effective only against Bacillus subtilis and Staphylocuccus aureus but not Gram negative bacteria [27]. Sokovic and her colleagues have reported that commercially prepared oil has third lowest antibacterial activity amongst ten tested herbal essential oil [28]. The report also indicated that limonene is the most abundant compound in the oil, but it has the second lowest antibacterial activity against a battery of Gram positive and negative bacteria. These reports might also indicate the reason for moderate antibacterial activity of the Citrus auratium essential oil we have found. This study extracted essential oil from

flowering tips which has not been studied for antibacterial activity before. The starting dilution of the oil for antibacterial assays was low due to the low yield of the extraction. This also might have contributed to the lack of activity in some bacteria. Nevertheless, Citrus auratium essential oil has antibacterial property which would contribute to the application of this oil in perfumery or as a food preservative.

CONCLUSIONS

The UA-HS-SPME-GC/MS method provided an effective combination to perform a rapid analytical method for the direct profiling of the volatile compounds without manipulation of samples. A large number of compounds can be identified in the headspace above various plant samples using this system. These collected volatile profiles can be eventually used for classification of plants for different purposes. Because of the limited capacity of fiber coating, an exhaustive analysis such as HD, is needed to acquire the absolute weight percentage of constituents. In comparison to HD method, the proposed method could equally monitor almost all the components of the sample, in an easier way, shorter time and requiring much less amount of the sample. SPME is a powerful method for chemical screening of many multi-component samples, such as foods and plants. In the present study, we identified 52 compounds in the headspace of *C. aurantium* flowers using SPME coupled to GC/MS. Among them, five compounds were not found in the HD essence. The essential oil of the flowers showed antibacterial property, which would contribute to the application of this oil in perfumery or as a food preservative.

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