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Short communication

Use of solid-phase micro-extraction as a sampling technique in the determination of volatiles emitted by flowers, isolated flower parts and pollen☆

Guido Flamini*, Pier Luigi Cioni, Ivano Morelli
Dipartimento di Chimica Bioorganica e Biofarmacia, Via Bonanno 33, 56126 Pisa, Italy

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Abstract

The volatiles emitted by fresh whole flowers or isolated flower parts of mandarin, *Citrus deliciosa* Ten. (Rutaceae), were sampled using solid-phase micro-extraction (SPME). This technique offers several advantages over dynamic headspace sampling techniques used in previous investigations. In particular, SPME requires smaller sample sizes and very short sampling times, which can minimize the formation of artifacts due to damage to the plant, and contaminations or loss of compounds. This was especially applicable to the collection of volatiles from pollen.

Keywords: Pollen; Flowers; *Citrus deliciosa*; Solid phase micro-extraction; Volatiles

1. Introduction

It is known that the color of a flower is the first and foremost cue for pollinator's attraction, but the scent of a flower also plays a major role in attracting pollinating insects [1,2]. The use of headspace technique to sample volatiles in the air surrounding a whole plant or plant organ has permitted one to ascertain that olfactory floral stimuli differ not only between species, but also between different organs within a single flower [3–8]. Distinctive volatile compounds could allow insects both to recognise specific host plants and to assess the amount of rewards in a flower, since pollen, an important food source for many flower-feeding insects, also produces odors [1].

Studies of floral scents and of their patterns within a single flower are important to better understand the chemical bases of plant–animal relationships and pollination ecology. Furthermore, they may reveal new scented molecules that could be of value to both the food industry and perfumery. Many papers deal with the analysis of flower volatiles, which are sampled mainly by means of dynamic headspace techniques [9–11]. Recently, papers have addressed the use of solid-phase micro-extraction (SPME) as a sampling technique [7,12–14], and the performance of different SPME fiber coatings for sampling the headspace of four aromatic and medicinal plants has been compared [15].

The floral scent sampling techniques employed in previous studies have the drawbacks of requiring

☆In memory of Professor Serena Catalano, 1945–2002.
*Corresponding author. Fax: +39-05-043-321.
E-mail address: flamini@farm.unipi.it (G. Flamini).

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considerable amounts of flower material, especially when sampling volatiles from pollen (50–200 mg), and very long sampling times (24–48 h for pollen, or 8–27 h for flower parts), as well as posing possible risks of sample contamination and loss of volatiles during the concentration of the solvent–volatile mixture in a water-bath in preparation for analysis by GC [4–6]. We have improved upon these sampling methods, and here we report the application of the SPME technique to collect the volatiles emitted by whole fresh flowers and different flower parts of mandarin (*Citrus deliciosa* Ten., Rutaceae).

2. Materials and methods

Flowers were picked from cultivated plants of *Citrus deliciosa* and immediately submitted to volatile collection. Five different samples were prepared:

Sample 1. Whole flowers (including sepals, petals, stamens and gynoecium). Three flowers were collected just after flower opening, cut a few mm below the calix, and the pedicels were wrapped in aluminium foil to minimize water loss. They were introduced into a 10-ml septum-cap vial and allowed to equilibrate for 20 min at 25 °C before sampling.

Sample 2. Petals only. A total of 15 petals, randomly collected from different flowers just after their opening, were placed in a vial and allowed to equilibrate as described above.

Sample 3. Stamens only. Stamens (filaments and anthers) were obtained from five flowers just after flower opening, before anther dehiscence, and prepared as above.

Sample 4. Gynoecium only. The gynoecia from five flowers were obtained and prepared as above.

Sample 5. Pollen. A sample of 3–5 mg of pollen was obtained by gently tapping flowers after anther dehiscence. It was introduced into a vial and allowed to equilibrate as described above.

Volatiles from each sample were collected by means of the SPME technique. Supelco SPME devices coated with polydimethylsiloxane (PDMS, 100 mm) were used to sample the headspace of all the samples. After the equilibration time, the fiber was exposed to the headspace for 15 min at 25 °C. Once sampling was finished, the fiber was withdrawn into the needle and transferred to the injection port of the GC and GC–MS systems, both using the splitless injection mode and with the injector temperature at 250 °C.

The GC analyses were accomplished with a HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m×0.25 mm, 0.25 μm film thickness), and with the following conditions: temperature program of 60 °C for 10 min, followed by an increase of 5 °C/min to 220 °C; injector and detector temperatures at 250 °C; carrier gas nitrogen (2 ml/min); detector dual FID; split ratio 1:30; injection of standards of 0.5 μl).

For both columns, identification of the chemicals was performed by comparing both their retention times with those of pure authentic samples and their linear retention indices (LRI) with those of the series of *n*-hydrocarbons. The relative proportions of the individual constituents, expressed as percentages, were obtained by FID peak-area normalisation (mean of three replicates).

GC–EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (both 30 m×0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures at 220 and 240 °C, respectively; oven temperature was programmed from 60 to 240 °C at 3 °C/min; carrier gas helium at 1 ml/min; injection of standards of 0.2 μl (10% hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparison of their linear retention indices relative to the series of *n*-hydrocarbons, and computer matching of their mass spectra against commercial (NIST 98 and Adams 95) and home-made library mass spectra built up from pure substances and components of known essential oils and MS literature data [16–21]. Moreover, the molecular mass of all the identified substances were confirmed by GC–CIMS, using MeOH as CI ionizing gas.

3. Results and discussion

SPME is a fast, solventless technique that is based on the partitioning of volatiles between the sample matrix, the headspace above the sample, and a

Table 1
Composition of the volatiles sampled by SPME from mandarin flowers and flower parts

Compounds	LRI	Percentages obtained by GC–FID					ID method
		Whole flowers	Petals	Gynoecea	Stamens	Pollen	
(1) Isoprenoids							
(a) Monoterpenes							
α-Thujene	932	–	0.3	–	–	–	S
α-Pinene	940	0.6	1.3	–	–	–	S
Sabinene	977	35.1	37.6	1.6	2.4	5.8	S
β-Pinene	981	0.9	1.6	–	Trace	–	S
Myrcene	992	19.2	23.2	1.6	49.7	–	S
α-Phellandrene	1011	–	0.1	–	–	–	S
α-Terpinene	1020	–	0.1	–	–	–	S
o-Cymene	1023	0.5	–	–	–	–	S
p-Cymene	1028	0.4	0.1	–	–	–	S
Limonene	1032	1.6	2.4	1.7	0.5	0.8	S
β-Phellandrene	1033	Trace	–	–	–	–	M [21]
(*Z*)-ocimene	1040	0.2	0.4	–	0.3	–	S
(*E*)-ocimene	1051	10.4	17.8	3.3	15.6	0.5	S
γ-Terpinene	1063	Trace	0.2	–	–	–	S
cis-Sabinene hydrate	1070	2.3	0.7	Trace	0.8	1.7	S
Terpinolene	1089	0.1	0.2	–	Trace	–	S
p-Cymenene	1091	–	Trace	–	–	–	M [21]
Linalool	1098	18.7	7.1	1.9	2.3	11.1	S
trans-Sabinene hydrate	1101	Trace	–	1.4	–	–	M [21]
β-Thujone	1116	–	Trace	–	–	–	S
allo-Ocimene	1130	Trace	Trace	–	–	–	S
cis-Dihydro-α-terpineol	1152	Trace	–	–	–	–	M [21]
4-Terpineol	1179	Trace	Trace	–	–	–	S
α-Terpineol	1195	0.5	0.2	Trace	0.3	2.0	S
(*E*)-geranyl acetone	1454	–	Trace	1.1	0.6	–	S
(b) Sesquiterpenes							
δ-Elemene	1339	0.7	0.3	–	Trace	1.5	M [21]
α-Copaene	1378	–	Trace	–	–	–	S
β-Elemene	1392	0.3	0.1	–	–	0.8	M [21]
β-Caryophyllene	1419	1.0	0.8	–	Trace	0.6	S
γ-Elemene	1435	0.4	0.2	–	Trace	1.9	M [21]
α-Guaiene	1440	Trace	Trace	–	–	–	M [21]
α-Humulene	1456	0.1	0.3	–	–	–	S
Germacrene D	1481	0.4	Trace	–	Trace	0.5	M [21]
Bicyclogermacrene	1496	Trace	–	–	–	0.8	M [21]
δ-Cadinene	1523	–	Trace	–	–	–	S
(2) Fatty acid derivatives							
Heptanol	970	1.7	–	–	–	–	S
6-Methyl-5-hepten-2-one	988	–	–	1.8	0.7	0.4	S
Octanal	1004	–	–	–	0.1	–	S
(*E*)-3-hexenyl acetate	1006	–	–	2.1	–	–	S
Nonanal	1103	Trace	Trace	–	0.3	0.6	S
(Z)-3-hexenyl butyrate	1188	–	–	1.2	–	–	S
(*E*)-2-hexenyl butyrate	1193	–	–	0.5	–	–	S
Decanal	1205	–	Trace	1.2	0.2	0.5	S
Hexyl-2-methyl butyrate	1236	–	–	0.4	–	–	S

Table 1. Continued

Compounds	LRI	Percentages obtained by GC–FID					ID method
		Whole flowers	Petals	Gynoecea	Stamens	Pollen	
cis-3-Hexenyl isovalerate	1238	–	–	0.5	–	–	S
Indole	1295	0.3	0.7	5.5	9.4	1.2	S
(Z)-jasmone	1395	–	Trace	–	0.3	2.1	S
n-Tetradecane	1400	–	–	–	–	0.8	S
Dodecanal	1410	0.1	–	–	–	–	S
n-Pentadecane	1500	–	Trace	Trace	0.2	–	S
1-Hexadecene	1579	–	–	1.2	0.1	1.8	S
n-Hexadecane	1600	–	Trace	0.6	0.2	1.1	S
1-Heptadecene	1682	0.2	0.3	49.4	5.5	45.9	S
n-Heptadecane	1700	0.1	0.2	8.6	3.0	10.4	S
1-Octadecene	1786	–	–	1.3	–	–	S
n-Octadecane	1800	–	–	–	Trace	–	S
n-Nonadecane	1900	–	–	–	0.1	–	S
(3) Benzenoids							
Phenylethyl alcohol	1111	–	0.1	–	–	–	S
Phenylacetic acid methyl ester	1177	–	–	–	Trace	–	S
Methyl salicylate	1191	–	–	–	Trace	–	S
(4) Nitrogen derivatives							
Methyl anthranilate	1342	0.7	1.5	1.4	5.1	–	S
Benzyl nitrile	1145	–	0.5	1.4	1.0	–	S
Total identified (%)		96.5	98.3	89.7	98.7	92.8	

LRI=linear retention indices (HP-5 column)
Trace: <0.1%.
Identification method: S=MS and LRI using authentic samples, M=MS and LRI using literature (reference in square brackets).

stationary phase coated on a fused-silica fiber. The adsorbed volatiles are then thermally desorbed from the fiber in the injector port of a gas chromatograph. This technique permits the sampling of volatiles emitted by living plants in a fast and easy manner. Our method shows noteworthy improvements over those used in previous papers, especially with respect to pollen [4–6]: (1) because of its high concentration capability, SPME permits the use of considerably smaller amounts of pollen than in methods using dynamic headspace adsorption techniques (3 mg instead of 50–200 mg); (2) the sampling time for pollen is very reduced (15 min instead of 24–48 h), minimizing the possibility of sample contamination due to the forced flow of air required by the former method; (3) the absence of solvents prevents the loss of volatiles during the concentration of the extractive solutions; and (4) the higher concentration capability of this technique permits the identification of many compounds (62 different volatiles) (Table 1).

SPME analysis permitted us to confirm the existence of spatial fragrance patterns within the flowers of *Citrus deliciosa*, due to different qualitative and/or quantitative emissions of volatiles by different floral parts. Indeed, monoterpenes were the main class of volatiles in whole flowers, petals and stamens (90.5, 93.3 and 72.5%, respectively), while fatty acid derivatives were the principal components of volatiles from gynoecium and pollen (74.3 and 64.8%, respectively). However, this class of compounds was represented in substantial amounts also in stamens (20.1%). Stamens emitted also the greatest amount of nitrogen derivatives (6.1%).

The fragrance of the whole flowers, which consisted mainly of sabinene (35.1%), myrcene (19.2%), linalool (18.7%) and (*E*)-ocimene (10.4%), seemed to depend largely on the volatiles emitted from petals, which included all these compounds among their major volatiles. The inner part of the flower showed quite a different blend of compounds, which included nitrogen derivatives. About half of the fragrance from the stamens consisted of myrcene (49.7%); other important compounds were (*E*)-ocimene (15.6%), indole (9.4%), 1-heptadecene (5.5%) and methyl anthranylate (5.1%). The gynoecium showed a different composition, domi-

nated by 1-heptadecene (49.4%); other main compounds were *n*-heptadecane (8.6%) and indole (5.5%).

Pollen in particular contributed to the fragrance pattern of the whole flower by its high content of linalool (11.1%), together with the petals. Pollen emission was similar to the gynoecium because of the dominance of 1-heptadecene (45.9%) and the notable amounts of *n*-heptadecane (10.4%). Noteworthy in pollen scent, however, was the lack of myrcene, a compound that dominated the fragrance of stamens and, in lesser amounts, of petals and the whole flower, and the very low amounts of (*E*)-ocimene (0.5%), which was another important compound of the rest of the flower. Furthermore, pollen emitted the greatest amounts of nitrogen derivatives, especially methyl anthranilate (5.1%). From these results it can be concluded that pollen scent in *C. deliciosa* resembled more the fragrance of the gynoecium than that of the whole stamens, from which it was produced. This situation could be related to pollination of the flower: spatial scent patterns within a flower may function like visual patterns. Thus, differences in the strength and/or quality of emitted volatiles between flower organs may serve as guides to insects, assisting them in finding food rewards or leading them to position themselves suitably on a flower for effective pollination [6].

In summary, application of SPME technique permitted us to collect and analyze the volatiles emitted from the whole fresh flowers and from different fresh flower parts of *C. deliciosa*. It required very short sampling times, thus avoiding the formation of artifacts due to damage to the plant.

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