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Posted Date: 9 October 2023

doi: 10.20944/preprints202310.0456.v1

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Article

Composition of the Scent in Some *Ophrys* Orchids Growing in Basilicata (Southern Italy). A Solid Phase Microextration Study Coupled with Gas Chromatography and Mass Spectrometry

Maurizio D'Auria [1,*], Richard Lorenz [2], Marisabel Mecca [1], Rocco Racioppi [1] and Vito Antonio Romano [1]

[1] Dipartimento di Scienze, Università della Basilicata, V.le dell'Ateneo Lucano 10, 85100 Potenza, Italy; marisabelmecca@libero.it (M.M.); rocco.racioppi@unibas.it (R.R.); vitoantonio_romano@libero.it (V.A.R.)
[2] AHO Baden-Württemberg, Germany; lorenz@orchids.de
* Correspondence: maurizio.dauria@unibas.it; Tel.: +39-0971-205-480

Abstract: SPME analysis of the scent *Ophrys* orchids gave the following results: *O. apifera*: benzyl benzoate, α-copaene, caryophyllene, and cyclosativene. *O. crabronifera* subsp. *biscutella*: pentadecene, pentadecane, heptadecane, nonadecane, and heinecosane. *O. bertolonii* subsp. *bertolonii*: pentadecane, heptadecane and nonadecane. *O. passionis* subsp. *garganica*: *i*-propyl palmitate, caryophyllene, pentadecane, and heptadecane. *O. holosericea* subsp. *apulica*: α-copaene, pentadecane, caryophyllene, and heptadecane. *O. lacaitae*: α-copaene, pentadecane, heptadecane, and caryophyllene. *O. bombyliflora*: cyclosativene, pentadecane, and ethyl dodecanoate. *O. insectifera*: 8-heptadecene, pentadecane, and heptadecane. *O. lutea*: heptadecane, 8-heptadecene, nonadecane, and docosane. *O. tenthredinifera* subsp. *neglecta*: α-copaene, caryophyllene, and i-propyl palmitate.

Keywords: *Ophrys*; scent composition; solid phase microextraction; gas chromatography mass spectrometry

1. Introduction

Some years ago, we started a systematic study of the scent of spontaneous orchid species growing in Basilicata (Southern Italy). The aim of this study was to create a homogeneous picture of the composition of the aroma of these species using the same methodology for all the species. In particular, we decided to use solid phase microextration (SPME) [1]. This study allowed us to identify the components of the scent of *Platanthera bifolia* subsp. *osca* [2], *Platanthera chlorantha* [3], *Cephalanthera* orchids [4], *Orchis* [5], *Serapias* [6], *Himantoglossum* [7]. *Barlia robertiana* [8], *Dactylorhiza* [9], *Gymnadenia* [10], *Neotinea* [11], and *Anacamptis* orchids [12].

In this work, the scents emitted by ten species of spontaneous orchids growing in Basilicata (Italy) belonging to the *Ophrys* genus have been determined. They are distributed between the two subgenera Fuciflorae and Ophrys and belonging to different sections and subsections (*O. apifera* Huds. 1762, *O. bertolonii* subsp. *bertolonii* Moretti 1823, *O. crabronifera* subsp. *biscutella* (O. Danesch & E. Danesch) Klaver & Kreutz 2013, *O. bombyliflora* Link (1779) 1800, *O. holosericea* subsp. *apulica* (O. Danesch & E. Danesch) Buttler 1986, *O. insectifera* L. 1753, *O. lacaitae* Lojac. 1909, *O. lutea* subsp. *lutea* Cav. 1793, *O. passionis* subsp. *garganica* (E. Nelson) H. Baumann & R. Lorenz 2005, *O. tenthredinifera* subsp. *neglecta* (Parl.) E. G. Camus, Bergon & A. Camus 1908 (Table 1 and Figures 1–5).

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Table 1. Taxonomic identification of the species utilized in this study. The nomenclature has been referred to [13].

Species	Subgenus	Section	Subsection
O. apifera	Fuciflorae	Apiferae	
O. crabronifera subsp. *biscutella*	Fuciflorae	Araniferae	Sphegodes
O. bertolonii subsp. *bertolonii*	Fuciflorae	Araniferae	Bertoloniorum
O. passionis subsp. *garganica*	Fuciflorae	Araniferae	Sphegodes
O. holosericea subsp. *apulica*	Fuciflorae	Fuciflorae	
O. lacaitae	Fuciflorae	Fuciflorae	
O. bombyliflora	Ophrys	Bomyliflorae	
O. insectifera	Ophrys	Ophrys	
O. lutea subsp. *lutea*	Ophrys	Pseudophrys	Fusci-luteae
O. tenthredinifera subsp. *neglecta*	Ophrys	Tenthrediniferae	

Figure 1. *Ophrys apifera* (left); *Ophrys crabronifera* subsp. *biscutella* (right) (Photos of V.A.R.).

Figure 2. *Ophrys bertolonii* subsp. *bertolonii* (left); *Ophrys passionis* subsp. *garganica* (right) (Photos of V.A.R.).

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Figure 3. *Ophrys holosericea* subsp. *apulica* (left); *Ophrys lacaitae* (right) (Photos of V.A.R.).

Figure 4. *Ophrys bombyliflora* (left); *Ophrys insectifera* (right) (Photos of V.A.R.).

Figure 5. *Ophrys lutea* subsp. *lutea* (left); *Ophrys tenthredinifera* subsp. *neglecta* (right) (Photos of V.A.R.).

It is known that *Ophrys* flowers imitate the mating signals of some insect species and are pollinated by sexually excited males who mistake the flower for a female of the same species and pollinate it during a "pseudocopulation".

Sexually deceptive orchids are unique in their exclusive and effective use of male insects, primarily aculeate Hymenoptera, but also other Hymenoptera and some Diptera [14].

 doi:10.20944/preprints202310.0456.v1

In most European *Ophrys* species studied so far, male copulation attempts can only be elicited by a scent identical to the female sex pheromone of the pollinating species, substances inducing pseudocopulation however are extractable longchain alkanes/alkenes, visual cues appear to be less important [15].

Often the differences in odor between similar orchid species are small. Small variations have been found between the bouquets of *Ophrys fusca* and *O. bilunulata*, as well as between the similar *O. sphegodes* and *O. exaltata* [15,16].

Sexually deceptive orchid species typically exploit one or a few specific species of pollinators and may have different pollinators in different regions. A single insect species can also pollinate more than one sexually deceptive orchid species in different regions [16].

All the species examined in this work are sexually deceptive with the sole exception of *O. apifera*, which is notoriously an autogamous (self-pollinating) species.

With this work we wanted to test, with a rapid method (SPME), species belonging to different sections and subsections of the *Ophrys* genus in order to verify whether the scent they emit is very similar or different within the different groups they belong to.

The scent of *Ophys* orchids has been extensively studied. Most of the studies has been performed through the identification of the components of the extracts of labella. Thus, *O. insectifera* showed the presence as main components of pentacosane, tetracosane, nonanoic acid and nonanal, in a study of 1987 [17], and tricosene, pentacosane, 9-heptacosene, and 9-nonacosene, in a study of 2017 [18]. However, the absorption of the scent of *O. insectifera* subsp. *insectifera* on Porapak Q showed as main components pentadecane, heptadecane, and cyclosativene [19], then alkanes with a lower molecular weight than those determined in the other studies on the orchids, and a terpene. In the scent of *O. sphegodes* pentacosane and tricosane were found in labella extracts [20], while tricosane, pentacosane, and *p*-cresol were the main components of the scent obtained by steam distillation of the flowers [21]. The scent of *O. lupercalis* and *O. iricolor* was due to the presence of tricosane, pentacosane and heptacosane [22]. Together with the same compounds, nonanal was found in *O. lupercalis*, *O. bilunulata*, and *O. fabrella* [23]. SPE collection of the scent of *O. normanii* showed the presence of octadecanal, tricosane, tricosene, and pentacosene [24]. SPE absororption of the scent of *O. apifera* showed the presence of butanol, butyl ether, and caryophyllene [25]. Pentacosene and tricosene were found in the labella extracts of *O. holosericea* [26], while nonanal was the main component of the scent of *O. lutea* [27]. These studies showed that the extraction of the labella leads to the identification of high molecular weight alkanes and alkenes as the main components of the aroma of these orchids. However, when SPME was used to determine the composition of the scent of *O. bertolonii* subsp. *benacensis* 4-methyl tetradecane, nonanal, decanal, dodecanal, 3,5-octadiene-2-one, and caryophyllene were found as the main components of the aroma [28].

2. Materials and Methods

2.1. Plant Material

The sample of *O. apifera* was collected at Piani del Mattino (PZ), on June 8, 2017. The sample of *O. crabronifera* subsp. *biscutella* was collected at Valico Faggeto in the municipality of Moliterno (PZ), on March 11, 2018. The sample of *O. bertolonii* subsp. *bertolonii* was collected at Monte Grosse (PZ), on April 18, 2018. The sample of *O. bombyliflora* was collected at Contrada Macchia Orsino in the municipality of Tolve, on April 9, 2018. The sample of *O. holosericea* subsp. *apulica* was collected at Scalo di Grassano, on April 19, 2018. The sample of *O. insectifera* was collected at Monte Zaccana in the municipality of Castelluccio Superiore, on May 2, 2018. The sample of *O. lacaitae* was collected at Contrada l'Aia Antica in the municipality of Calvello, on June 6, 2018. The sample of *O. lutea* subsp. *lutea* was collected at Scalo di Albano, on April 11, 2018. The sample of *O. passionis* subsp. *garganica* was collected at Scalo di Campomaggiore, on April 16, 2018. The sample of *O. tenthredinifera* subsp. *neglecta* was collected at Torrente Serrapotomo in the municipality of Laurenzana, on April 8, 2018. The plants were collected by Vito Antonio Romano.

 doi:10.20944/preprints202310.0456.v1

The plants were harvested taking all the clod of earth, taking care not to damage the root system. All the plants had closed flowers to avoid using flowers that were already fertilized but not visible because they were at the beginning of fertilization. The plants were planted in special pots in the greenhouse of the University of Basilicata (Potenza 650 m. a.s.l.), in closed boxes with transparent cloth to avoid fertilization (even if occasional). The correct classification of the species was carried out on flowering plants. The plants were tested when the flowers were all open except the last two.

The plants were tested, whole without being damaged, under a cylindrical glass bell (12cm x 45cm) in which only the inflorescence and the SPME probe are inserted.

To avoid contamination, the interior of the bell was isolated from the external environment with appropriate closing and sealing systems during the 24 hours of the test (from eight in the morning to 8 the following day).

In order to be sure that the internal environment of the bell was isolated from the external environment, various blank tests were carried out.

After the tests the plants remained closed in the boxes to verify that at the end of flowering there were no fertile ovaries and for this reason no herbarium samples were taken. The earthen bread with the bulbs were brought back to the site.

In view of the fact that the investigated taxa are rare wild plants, in order to preserve the species, we have chosen to use a single plant for our analysis.

2.2. Analysis of Volatile Organic Compounds

The SPME analysis of ten different samples of *Ophrys* has been performed. This way, the identified plants were collected and inserted in glass jar for 24 h where was present also the fiber (DVB/CAR/PDMS) of and SPME syringe. After this time the fiber was desorbed in a gas chromatographic apparatus equipped with a quadrupole mass spectrometer detector. A 50/30-µm DVB/CAR/PDMS module with 1 cm fiber (57328-U, Supelco, Milan, Italy) was employed to determine VOCs. SPME fiber was maintained in the bell jar for 24 h. The analytes were desorbed in the splitless injector at 250 °C for 2 min. Analyses were accomplished with an HP 6890 Plus gas chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30-m × 0.25-mm i.d. × 0.25 µm FT) (Agilent, Milan, Italy). An HP 5973 mass selective detector in the range 0-800 m/z (Agilent) was utilized with helium at 0.8 mL/min as the carrier gas. The EI source was used at 70 eV. The analyses were performed by using a splitless injector. The splitless injector was maintained at 250 °C and the detector at 230 °C. The oven was held at 40 °C for 2 min, then gradually warmed, 8 °C/min, up to 250 °C and held for 10 min. Tentatively identification of aroma components was based on mass spectra and Wiley 11 and NIST 14 library comparison. Single VOC peak was considered as identified when its experimental spectrum matched with a score over 90% that present in the library. All the analyses were performed in triplicate.

3. Results and Discussion

The SPME-GC-MS analysis of *Orphys* samples gave the results reported in Table 2. The main component of the scent of *O. apifera* was benzyl benzoate (22.52%), while other important components were a-copaene (9.11%), caryophyllene (8.07%), and cyclosativene (6.97%) (Table 2). It is noteworthy the significant difference in the scent in comparison with that obtained by using SPE absorption. In that case, butanol, butyl ether, and caryophyllene were the main components of the aroma [25,29]. The observed difference can be due to the different analyzed species, to the different harvesting places (Basilicata and Catalonia), to different pollinator insects, to the different analytical procedures.

Table 2. Volatile organic compounds detected by using SPME-GC-MS in *Ophrys* species.

Compound	r.t. [min.]	KI	Area % ± 0.03									
			Ophrys									
			apifera	*crabronifera* subsp. *biscutella*	*bertolonii subsp. bertolonii*	*passionis* subsp. *garganica*	*holosericea* subsp. *apulica*	*lacaitae*	*bombyliflora*	*insectifera*	*lutea* subsp. *lutea*	*tenthredinifera* subsp. *neglecta*
Octanol	10.82	1072									4.69	
Undecane	11.31	1100		1.05								
Decanal	13.17	1195	0.70	0.74						0.48		
Dodecane	13.27	1200			0.58					0.45		0.53
Nonanoic acid	14.38	1272	2.00									
Isobornyl acetate	14.97	1285							1.65	0.96		1.27
2-Undecanone	15.01	1291			5.18							
Tridecane	15.05	1300		3.37	4.48		3.63	0.56	3.08	1.90	1.56	2.32
Decanoic acid	16.02	1335	3,30									
Cyclosativene	16.38	1344	6.97		5.15	6.79			10.09	0.96	1.09	
a-Copaene	16.52	1353	9.11	3.81			11.30	12.08	3.15			11.61
Tetradecane	16.74	1400	0.86	1.81	6.36	3.72	4.47	3.17	3.47	2.13	1.09	3.60
Dodecanal	16.86	1407		3.49								
Caryophyllene	17.28	1428	8.07	1.08	1.72	8.34	7.90	6.68	4.38		1.23	11.73
Geranylacetone	17.46	1451	0.88						1.62			
b-Farnesene	17.62	1454								3.58		
Alloaromadendrene	17.76	1456	0.86									
Epi-b-santalene	17.80	1460		0.74								
2,6-di-*t*-butyl-*p*-benzoquinone	17.96	1458					1.97	1.69	1.77	0.80		1.06
1-Pentadecene	17.99	1489		6.43								
Pentadecane	18.30	1500	2.52	8.06	28.62	8.69	11.48	13.40	10.33	13.73	5.53	5.04
b-Cadinene	18.65	1507	2.52									
Methyl dodecanoate	18.68	1509			1.00					1.09		
d-Cadinene	18.82	1524						1.27	6.94			1.04
Dodecanoic acid	19.11	1559	1.78	0.71				0.98				

doi:10.20944/preprints202310.0456.v1

Cyclotridecane	19.46	1565								0.64		
Ethyl dodecanoate	19.75	1579		1.40	4.16	1.83	5.31		9.46	5.18	0.99	4.16
Hexadecane	19.80	1600		2.86	3.58	6.38	6.16	4.17	6.97	3.38	2.08	4.03
Tetradecanal	20.01	1611	1.63	2.03	4.10		3.72	2.93		1.02		
i-Propyl dodecanoate	20.06	1618	0.86									
Isolongifolen-5-one	20.17	1622		3.81								
Benzophenone	20.28	1625	1.97									
Unidentified	20.62								8.52			
Methyl dihydrojasmonate	20.66	1648		3.15						2.93		
8-Heptadecene	20.91	1664		3.12	2.20		4.61	3.91		18.88	7.25	
Heptadecane	21.22	1700	4.78	8.37	7.23	9.22	8.15	14.43	6.67	7.98	39.37	4.40
Pristane	21.30	1709	1.14	1.89		5.49	2.39	1.86	2.12	1.90		1.57
2-(Phenylmethylene)-octanal	21.87	1728	1.34									
Farnesal	21.89	1738									0.54	
Tetradecanoic acid	21.91	1761	1.89	1.60								
Benzyl benzoate	22.16	1768	22.52									
3,5-di-*t*-butyl-4-hydroxybenzaldehyde	22.24	1771								0.80		
Ethyl tetradecanoate	22.51	1774			0.90		1.08		1.53	1.49	0.59	1.47
Octadecane	22.57	1800	2.18	1.89	1.66	2.07	2.07	3.51	2.91	1.81	1.55	1.71
Phytane	22.64	1814								0.99		
Hexadecanal	22.82	1819	1.60		1.24		1.08	4.04		0.83		
Farnesyl acetaldehyde	23.18	1855		1.81						2.26		1.09
Nonadecane	23.85	1900	1.85	8.18	6.30	1.72	4.28	6.81	2.80	2.99	8.87	1.65
b-Springene	24.16	1922										1.60
Methyl hexadecanoate	24.20	1927										0.74
Hexadecanoic acid	24.58	1935		1.95						0.83		
a-Springene	24.78	1940										5.45
Ethyl 11-Hexadecenoate	24.80	1974									2.58	
Eicosane	25.06	2000	1.82	1.15	1.32		1.18	1.57	1.06	1.56	0.74	
Geranyl-a-terpinene	25.15	2005										5.66

doi:10.20944/preprints202310.0456.v1

i-Propyl palmitate	25.40	2013	0.96	5.06	2.24	29.62	5.56			2.73	2.26	14.73
Heinecosane	26.25	2100		5.12	3.26	4.85	3.95	4.13	1.38			1.50
Ethyl oleate	26.98	2169	0.92							1.81		
Docosane	27.38	2200	1.52			1.30		0.87		1.81	9.94	0.71
1-Heneicosyl formate	28.10	2250						0.77				
9-Tricosene	28.14	2270		4.26								
Tricosane	28.47	2300	1.04	2.97				1.44		2.45	0.62	1.74

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The analysis of the scent of *O. crabronifera* subsp. *biscutella* showed that the aroma is mainly due to the presence hydrocarbon compounds, as in several species as reported above. However, we found the presence of pentadecene (6.43%), pentadecane (8.06%), heptadecane (8.37%), nonadecane (8.18%), and heinecosane (5,12%). Furthermore, *i*-propyl palmitate was detected in a relevant amoun (5.06%) (Table 2). In other *Ophrys* species, the scent analysis performed through the labella extraction gave high molecular weight hydrocarbons (higher than thirty carbon atoms), while, in our determination, the main component of the scent has seventeen carbon atoms. Unfortunately, other analyses of the same species are not available.

The scent of *O. bertolonii* subsp. *bertolonii* gave a similar result. The main component was pentadecane (28.62%), while other significant compounds were heptadecane (7.23%) and nonadecane (6.30%). Also in this case, a significant difference has been observed considering the results obtained in the SPME analysis of *O. bertolonii* subsp. *benacensis* [28]. The observed differences can be due to the different subspecies, to the different harvesting places (Basilicata and Lecco), or to different pollinators.

When a sample of *O. passionis* subsp. *garganica* was analyzed the main component of the scent was *i*-propyl palmitate (29.62%), while other components were caryophyllene (8.34%), pentadecane (8.69%), and heptadecane (9.22%). In this case, this is the first reported analysis of this species. In the case of *O. holosericea* subsp. *apulica* the main component were a-copaene (11.30%) and pentadecane (11.48%), while other compound found in the scent were caryophyllene (7.90%), and heptadecane (8.15%). The analysis of labella extracts gave some alkenes as main components of the scent [26]. In this case the different analytical procedure is responsible for the observed differences.

The same trend was observed in the analysis of *O. lacaitae*: the main components were a-copaene (12.08%), pentadecane (13.40%), and heptadecane (14.43%), while caryophyllene was found in relevant amount (6.68%).

The scent of *O. bombyliflora* showed the presence of cyclosativene (10.09%), pentadecane (10.33%), and ethyl dodecanoate (9.46%). The analysis of the scent of *O. insectifera* gave the following results: the main component was 8-heptadecene (18.88%) followed by pentadecane (13.73%), and heptadecane (7.98%). Previous results, obtained on labella extracts, showed the presence of high molecular weight compounds [17,18]. On the contrary, the compounds detected through absorption on Porapak Q are quite similar, with the difference of caryophyllene, to the results presented here [19]. We have to note, finally, that all the high molecular weight hydrocarbons detected in the labella are solid and it is very difficult that they could be present in the scent.

Nonanal was the compound detected in a previous work in *O. lutea* [27]. SPME analysis showed the presence of heptadecane (39.37%), 8-heptadecene (7.25%), nonadecane (8.87%), and docosane (9.94%). This result is consistent with the trend of SPME analysis on *Ophrys* orchids, where, with some differences for different orchid species, the compounds we detected were very similar. The difference with previous results can depend on the analytical procedure. Finally, the scent of *O. tenthredinifera* subsp. *neglecta* has as components a-copaene (11.61%), caryophyllene (11.73%), and *i*-propyl palmitate (14.73%), showing that is another case, beyond *O. apifera* where hydrocarbons are not present in relevant amount in the scent.

4. Conclusions

In this article we have determined the composition of the aroma of some orchids belonging to the *Ophrys* genus. This result was obtained using SPME coupled with GC-MS as an analysis technique. In the ten samples analyzed, 62 compounds were found; however, the compounds present in greater quantities are almost always the same, with variations (sometimes substantial) between species. It is important to note that there is never any correspondence between our analyzes and those obtained through chemical extraction of plant labels. In this case, high molecular weight hydrocarbons are always recovered, which, however, can hardly be constituents of any aroma, being solid compounds with a low vapor pressure.

Author Contributions: Conceptualization, M.D. and V.A.R.; investigation, M.M. and R.R.; data curation, R.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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