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The Scent of *Himantoglossum* Species Found in Basilicata (Southern Italy)

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Abstract: The SPME (Solid Phase Microextraction) analysis of the scent of *H. hircinum* showed the presence of elemicin in the presence of a relevant amount of eugenol. The scent of the sample of *H. adriaticum* collected in Abruzzo showed the presence 4-amino-5-(4-morpholinylmethyl)-2-oxazolidinone, β-ocimene, decyl decanoate, and 9-tricosene as main components. The sample of *H. adriaticum* collected at Marsico Nuovo has an aroma where the main component was pentadecyl hexanoate, 9-tricosene, methyleugenol, tetradecane, pentadecane, and elemicin. The samples of *H. adriaticum* collected at Viggianello showed some similarities in the scent: the main components were 9-tricosene and methyleugenol.

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

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1. Introduction

The determination of the volatile organic compounds emitted from a natural source is smell and taste are the oldest of our senses. They probably developed in very primitive organisms as means of obtaining information about chemical changes in the organism's environment. Animals use smell and taste to find food and to assess its quality. The smell of food has a powerful effect on animals.

Living organisms use the chemical sense as a means of communications. If the communication is between different parts of the same organism, the messenger is referred to as a hormone. Chemicals used to carry signals from one organism to another are known as semiochemicals. In the case of flowers, the aroma components are mainly devoted to attracting pollinator insects.

In recent years, *Himantoglossum* s.l. has included other taxa of considerable interest and conservation [1,2]. Currently, the expanded genus *Himantoglossum* is composed of the subgenus *Himantoglossum* including all the species of the former genus *Himantoglossum*, the subgenus *Barlia*, consisting of the two species of the genus *Barlia*, and the subgenus *Comperia* consisting only of the former species *Comperia comperiana* (Table 1).

The attraction of the pollinator in orchids generally occurs first through the air diffusion of scents, then through sight, as the pollinator approaches its target inflorescence, and finally through tactile signals and hardly volatile, extractable compounds when it lands on the chosen flower.

H. hircinum and *H. adriaticum* have large, showy flowers with a long, captivating lip, often adorned with showy tufts of colored papillae that provide footholds acting as a guide for pollinators. Vöth (1990) speculates that these papillae could also be the seat of osmophores responsible for most of the volatile organic compounds emitted by the plant [3]. The scent emitted by these two species can be strong, unpleasant, or sweet. They

are allogamous species that do not offer food reward; in fact, their short and sack-like spur does not contain nectar [4].

Teschner (1980) has shown that the spur of *H. hircinum* and *H. adriaticum* may contain small amounts of glucose in some populations [5]. Kropf and Renner (2008) chemically demonstrated the presence of nectar in *H. hircinum* [6]. Little is known about the pollination of the various species of the *H. hircinum* group; pollinators are thought to differ locally, with Teschner (1980) suggesting that solitary bees are the true pollinators [5].

In this work, we have dealt with the only two species present in Basilicata belonging to the old genus *Himantoglossum* Spreng. 1826, *Himantoglossum hircinum* (L.) Sprengel 1826 and *Himantoglossum adriaticum* H. Bauman 1978, while, in previous works we analyzed the perfumes of *Barlia robertiana* [7], a species until a few years ago considered to belong to the monospecific genus *Barlia* and now merged into the new clade *Himantoglossum*.

H. hircinum (Figure 1) has a Mediterranean-Atlantic distribution, from southern Great Britain to northern Africa. Present in Italy, the species is reported to be widespread in Sicily between 150 and 1750 m [8], sporadically in the southern regions, also reported in Tuscany, Liguria, and southern Piedmont [9].

Table 1. Taxonomy of the genus *Himantoglossum* s.l. generated by integrating the results of the present study with those of Sramkó [10].

Genus	Subgenus	Clade	Section	Species
Himantoglossum	*Comperia*	-	-	*comperianum*
	Barlia	-	-	*metlesicsianum*
				robertianum
	Himantoglossum	Hircinum-caprinum	Formosum	*formosum*
			Caprinum	*caprinum*
				montistauri
				calcaratum rumelicum
				calcaratum calcaratum
			Hircinum	*adriaticum*
				hircinum

Figure 1. *Himantoglossum hircinum.* Photo of V. A. Romano.

From the observations of one of the authors of the following work (VAR), we report a wide diffusion for Basilicata of this species, so much so that it is very common in the hilly and mountainous area of the Province of Potenza from 400 to 1500 m where it forms large populations with dozens of plants. It blooms from early May to early June.

H. adriaticum (Figure 2) has a Euro-Mediterranean distribution, present in southern Italy up to the Alpine regions, Slovenia, and Croatia; its northeastern limit also touches Austria, Hungary, and Slovakia [9].

Figure 2. *Himantoglossum adriaticum.* Photo of V. A. Romano.

In Basilicata it was reported by Gölz and Reinhard (1982), Conti et al. (2005), Fascetti et al. (2008), Romano et al. (2013) [11–14]. It blooms from mid-June to mid-July from 1300 to 1600 m. Many plants have never been observed on the same site, maximum 10 plants on an area of 0.5 ha. Generally, there are 1–3 isolated plants distant from other single specimens even a few km. The richest area is that of the Pollino National Park between Basilicata and Calabria [15].

For the perfume tests, *H. hircinum* plants from Basilicata and *H. adriaticum* plants from Basilicata and Abruzzo were used. All the plants were collected before anthesis and planted in the gardens of the University of Basilicata and tested, many days later, when they were in full bloom.

The scent of a flower can be an important factor determining the pollination of a plant. The study of the scent of the orchids was the object of several works in the past [16]. Unfortunately, several different approaches have been used in order to determine the composition of the aroma of an orchid. Extraction, headspace analysis, and SPME have used. Often, different chemical procedures allowed to obtain different results. On the basis of these considerations, a research project started devoted to the determination of the scent of the orchids found in Basilicata using the same procedure, solid phase microextraction coupled with gas chromatography and mass spectrometry. This way, the composition of

the scent of *Platanthera bifolia* subsp. *osca* [17,18], *Platanthera chlorantha* [18], *Cephalanthera* orchids [19], *Serapias* orchids [20], *Gymnadenia* orchids [21], *Barlia robertiana* [7], *Neotinea* orchids [22], and *Orchis* species [23] has been investigated.

Some studies report some data on the scent of *H. hircinum*. (*E*)-Ocimene, elemicin, (*E*)-3-methyl-4-decenoic acid, (*Z*)-4-decenoic acid, and lauric acid were considered as the main components of the scent after absorption on charcoal [24]. (*E*,*Z*)-2,6-dimethyl-3,5,7-octatrien-2-ol and the (*E*,*E*) isomer were claimed as major constituents of the aroma [25,26]. Finally, hexane extraction of the labella showed the presence of high molecular weight alkanes such as pentacosane, heptacosane, and nonacosane [27].

2. Experimental Section

2.1. Plant Material

The samples of *H. adriaticum* were collected at Comune di Cocullo, Prov dell'Aquila (Abruzzo), 1070 m. a.s.l., on 20 May 2017 (Sample 1), at Fontana delle Brecce, Marsico Nuovo (Pz), 1439 m. a.s.l., on 10 June 2017 (Sample 2), at Piano Visitone, Viggianello (Pz), 1500 m. a.s.l., on 12 June 2018 (samples 3 and 4). The sample of *H. hircinum* was collected at Contrada Manta, Potenza, 1000 m. a.s.l., on 1 May 2017. The plants were collected by Vito Antonio Romano.

The plants were harvested about two weeks before flowering by taking all the clod of earth, taking care not to damage the root system, planted in special pots in the gardens of the University of Basilicata (Potenza 650 m. a.s.l.), in waiting for their full bloom. Two days before the tests the plants were transferred to an air-conditioned room at 22 °C. The plants were tested, whole without being damaged, under a cylindrical glass bell (12 cm × 45 cm) in which only the inflorescence and the SPME probe are inserted (Figure 3).

Figure 3. The apparatus used to collect the scent of the plants used in this study.

To avoid contamination, the interior of the bell was isolated from the external environment with appropriate closing and sealing systems during the 24 h 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.

The plants were successively used for further studies on pollination, fertility, and germination of the plants. After these studies, the plants were not in condition to be collected in an herbarium. However, these species can be recognized without ambiguities on the basis of their properties, well documented by the Figures 2 and 3. 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 [4] analysis of five different samples of *Himantoglossum* 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 1 to 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

The SPME analysis of scent of *H. hircinum* showed the presence of elemicin (spicy, floral scent) as the main component (61.71%) in the presence of a relevant amount of eugenol (4.50%) (Table 2). Minor products observed in the scent were 3,4,5-trimethoxybenzaldehyde, benzyl benzoate, 3-(4,8,12-trimethyltridecyl)furan, and 9-tricosene. All the values are based on per cent of the TIC area.

Table 2. SPME-GC-MS analysis of *Himantoglossum* species.

Compound	r.t. [min.]	KI	Area [%] ± 0.03				
			Species				
			H. hircinum	*H. adriaticum*			
				Sample 1	Sample 2	Sample 3	Sample 4
β-Myrcene	8.99	991		0.31			
2,2,4,6,6-pentamethyl-3-heptene	9.31	1020		0.31			
1-Methoxy-4-methylbenzene	9.63	1030		1.63			
Limonene	9.92	1039			1.62		0.81
β-Ocimene	10.18	1050	0.17	8.23	2.85	10.28	
Methyl benzoate	11.16	1091	0.10				
2-Ethylhexyl acetate	12.18	1159	0.22				
Dodecane	13.09	1200		0.29			
Verbenone	13.53	1209			1.95		0.75
3,4-Dimethoxytoluene	13.86	1230		0.33			
Carvone	14.02	1240			0.33		
Geraniol	14.45	1260		1.49			
Tridecane	14.88	1300			0.93		
2,3-Dimethylhydroquinone	15.70	1348		1.01			0.99
Eugenol	16.05	1374	4.50				
Decanoic acid	16.08	1380			1.23		
Geranyl acetate	16.31	1388	0.35				
Tetradecane	16.64	1400		1.43	3.93	4.49	0.86
Methyleugenol	16.80	1406		2.34	5.93	18.06	4.73
Caryophyllene	17.09	1420	0.72				
2,6-Di-*t*-butylbenzoquinone	17.88	1472		0.74			1.23
Pentadecane	18.22	1500	0.31	2.98	3.41	4.19	1.16
α-Farnesene	18.33	1511	0.10				
2,5-bis(1,1-Dimethylethyl)phenol	18.41	1514		0.53			
Elemicin	19.07	1550	61.71	2.95	3.80		
Octyl hexanoate	19.42	1570	0.14		0.93		
Decyl butanoate	19.48	1590			1.59		
Hexadecane	19.72	1600		2.70	1.95		1.08
3,4,5-Trimethoxybenzaldehyde	19.84	1608	1.59				
Tetradecanal	19.86	1611		0.78			
1-Methylethyl dodecanoate	20.06	1618	0.31				
Isoelemicin	20.47	1644	0.13	0.74	0.87		

Table 2. *Cont.*

Compound	r.t. [min.]	KI	Area [%] ± 0.03				
			Species				
			H. hircinum	*H. adriaticum*			
				Sample 1	Sample 2	Sample 3	Sample 4
Tridecan-1-ol	20.88	1665					1.02
Heptadecane	21.14	1700	0.38	2.11	2.42	3.68	2.12
Pristane	21.18	1705		0.98	1.22		
Benzyl benzoate	22.16	1751	1.59	2.17			
Pentadecyl hexanoate	22.20	1770			22.73		
Pentadecyl isobutanoate	22.27	1776			0.86		
Octadecane	22.49	1800	0.12	0.90	1.22		1.32
Hexadecanal	22.68	1822		0.67	1.02		
Isopropyl myristate	22.84	1830	0.43		2.81		0.74
6,10,14-trimethyl-2-pentadecanone	23.11	1836		1.83	1.29		1.56
3-Amino-5-(4-morpholinylmethyl)-2-oxazolidinone	23.56	1844		17.37			
Nonadecane	23.78	1900		0.92	1.43		3.47
β-Springene	24.31	1918	0.36				
3-(4,8,12-Trimethyltridecyl)furan	24.60	1941	1.05				
Decyl decanoate	24.66	1950		5.36			
Octadecyl hexanoate	24.73	1965			2.48		
11-Hexadecen-1-ol acetate	24.94	1973		2.42			
Eicosane	25.00	2000			0.89		0.87
Octadecanal	25.23	2021		0.58			
Heneicosane	26.18	2100		0.28	2.39		4.26
Nonadecanol	26.40	2151	0.88	0.74	2.31	5.48	
Docosane	28.31	2200		0.28	0.56		
9-Tricosene	28.43	2270	1.31	6.41	5.06	31.21	40.22
Unidentified components			23.52	28.19	19.99	22.61	33.83

The scent of the sample of *H. adriaticum* collected in Abruzzo (Sample 1) showed the presence 4-amino-5-(4-morpholinylmethyl)-2-oxazolidinone, β-ocimene (green tropical woody floral vegetable scent), decyl decanoate, and 9-tricosene as main components (17.37, 8.23, 5.36, and 6.41%, respectively) (Table 2). Furthermore, several compounds are presents in relevant amounts: 1-methoxy-4-methylbenzene (1.63%), geraniol (1.49%), geranyl acetate (1.43%), methyleugenol (2.34%), pentadecane (2.98%), elemicin (2.95%), hexadecane (2.70%), heptadecane (2.11%), benzyl benzoate (2.17%), 6,10,14-trimethyl-2-pentadecanone (1.83%), and 11-hexadecen-1-ol acetate (2.42%). The sample of *H. adriaticum* collected at Marsico Nuovo (Sample 2) has an aroma where the main component was pentadecyl hexanoate (22.73%), 9-tricosene (5.06%), methyleugenol (5.93%), tetradecane (3.93%), pentadecane (3.41%), and elemicin (3.80%) (Table 2). The samples of *H. adriaticum* collected at Viggianello (samples 3 and 4) showed some similarities in the scent: the main components were 9-tricosene (31.21% in sample 3 and 40.22% in sample 4) and methyleugenol (18.06% in sample 3 and 4.73% in sample 4) (Table 2). Furthermore, β-ocimene was found in relevant amount (10.28%) only in sample 3.

4. Discussion

It is interesting to note the large differences between our reported results and those reported in the introduction section for *H. hircinum*. We did not find a correlation with the results reported by Schiestl and Cozzolino [27]. While they determined the presence of high molecular weight alkanes, we did not find these compounds in the scent. We think that the observed different results depend on the different procedures used in the determination of the scent. Schiestl and Cozzolino used an extraction of labella. Probably they determined the presence of waxy compounds present in the surface of labella but not involved in the composition of the scent. Furthermore, the components determined in that article [27] are not volatile compounds. Furthermore, in a previous work, (*E*)-ocimene, elemicin, (*E*)-3-methyl-4-decenoic acid, (*Z*)-4-decenoic acid, and lauric acid were determined as the main components of the aroma [24]. While elemicin was the main component of the scent in the sample we analyzed, we did not find the other compounds determined in the work of Kaiser [24]. Finally, we did not find (*E*,*Z*)- and (*E*,*E*)-2,6-dimethyl-3,5,7-octatrien-2-ol whose presence has been claimed in [25,26].

The scent of *H. adriaticum* has not been studied until now. It is interesting to note that all the samples we analyzed have common components, although in different amounts. Thus, methyleugenol and 9-tricosene were found in all the samples. Furthermore, some linear hydrocarbons (tetradecane, pentadecane, and hexadecane) were present. Nevertheless, sample 1 of *H. adriaticum* showed the presence of an oxazolidinone as an important component, while in sample 2 we found pentadecyl hexanoate, and in sample 3 β-ocimene was one the main components.

Based on this work, we can assume that the scent of *H. hircinum* found in Basilicata has a different composition from those described elsewhere. We can assume also that *H. hircinum* has a scent showing a different composition in comparison with the compounds found in *H. adriaticum*. Finally, we can assume that the samples of *H. adriaticum* we analyzed have some common characters, with some differences depending on the place where the samples have been collected.

5. Conclusions

This work shows the analysis of samples from Basilicata and Abruzzo of *H. hircinum* and *H. adriaticum*. The analyses have been performed by using the same procedure and the same fiber in SPME-GC-MS, allowing to have a homogenous data set. The analysis of *H. hircinum* showed a peculiar composition that differs from those observed in *H. adriaticum*. In fact, the analysis of the scent of *H. hircinum* showed the presence of elemicin in the presence of a relevant amount of eugenol. The scent of the sample of *H. adriaticum* showed the presence 4-amino-5-(4-morpholinylmethyl)-2-oxazolidinone, β-ocimene, decyl decanoate, 9-tricosene, pentadecyl hexanoate, methyleugenol, tetradecane, pentadecane, and elemicin.

The observed differences, when other head-space techniques are used, can depend both on different absorption rates of the analytes on the fiber and on variation of the scent due to natural adaptation of the plant to different environmental conditions. Furthermore, the observed differences can be due to different pollination insects. The analysis of the scent of *H. adriaticum* showed the presence of some common components (i.e., 9-tricosene and methyleugenol) that are not depending on the origin of the flowers.

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