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Identification of volatile organic compounds (VOC) emitted from three European orchid species with different pollination strategies: two deceptive orchids (*Himantoglossum robertianum* and *Ophrys apifera*) and a rewarding orchid (*Gymnadenia conopsea*)

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Abstract

Volatile organic compounds (VOC) emission was evaluated in the inflorescences of three species of the family Orchidaceae: *Himantoglossum robertianum*, *Ophrys apifera* and *Gymnadenia conopsea*, that comprise three different pollination strategies: non-rewarding food deceptive, non-rewarding sexually deceptive and nectar rewarding, respectively. VOC were dynamically sampled in custom packed glass multi-sorbent cartridge tubes (Carbotrap, Carbopack X and Carboxen 569). A modified Tedlar® gas sampling bag was placed in vivo covering the inflorescence of the studied orchid, a design that prevents the dilution of the VOC mixture emitted by the flower. Multi-sorbent bed tubes were analysed through automatic thermal desorption coupled with a capillary gas chromatography/mass spectrometry detector. A total of 106 different VOC were found in the scents emitted by the three different studied orchids. A 54% of these compounds had already been identified in floral scents. Generally, only 3 compounds were highly abundant in each species: α-pinene, β-pinene and limonene in *Himantoglossum robertianum*; 1-butanol, butyl ether and caryophyllene in *Ophrys apifera*; and phenethyl acetate, eugenol and benzaldehyde in *Gymnadenia conopsea*. The employment of the presented methodology for the retention of emitted VOC has proven to be suitable for the identification of a wide range of floral released compounds.

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Introduction

Live organisms generate and emit a multitude of chemical compounds, a "chemodiversity" representative of life on Earth (Gershenzon and Dudareva, 2007). Volatile organic compounds (VOC) emitted by plants are not only aesthetic and odorous benefits for humans, their main functions play a key role in the survival and reproduction of these organisms, such as defence against herbivores and pathogens, and attraction of mating partners for pollination (Kaiser, 1993; Dudareva and Pichersky, 2000; Gershernzon and Dudareva, 2007; Dunkel *et al.,* 2009; Kessler and Halitschke, 2009; Raguso, 2009; Willmer *et al.,* 2009; Jürgens and Viljoen, 2010; Schiestl, 2010; Soler *et al.,* 2011). Plants emit a wide range of low molecular weight (30-300 g mol^{-1}) VOC (Knudsen *et al.,* 2006) through various organs, such as leaves, flowers and fruits (Willmer *et al.,* 2009; Stashenko and Martinez, 2008), as well as nectar (Raguso, 2004); usually biosynthesising them in the epidermal cells, which allows a facile release of these compounds into ambient air (Pichersky *et al.,* 2006). More than 1700 VOC with different chemical structures and characteristics, mainly fatty acid derivates, benzenoids, phenylpropanoids, isoprenoids, nitrogen- and sulphur-containing compounds, have been identified in a wide range of floral fragrances (Knudsen *et al.,* 1993, 2006; Pichersky *et al.,* 2006; Raguso, 2009). Orchids present an important diversity of pollination strategies (Cozzolino and Widmer, 2005; Johnson and Hobbhahn, 2010; Van der Niet *et al.,* 2010; Ayasse *et al.,* 2011), however, rewarding (nectar) and non-rewarding (sexually deceptive and food deceptive) orchids use VOC emission to communicate with their potential pollinators (Kaiser, 1993; Dudareva and Pichersky, 2000). Several studies have observed patterns of commonness that suggest that plants and insects coincide in the variety of VOC produced, both for attraction and defence (Borg-Karlson, 1990; Shiestl, 2010).

The evaluation of VOC emitted from flowers needs appropriate methodologies for their sampling and enrichment, as usually the volatiles are released in very low quantities and can change its profile depending on several circumstances (e.g. day/night, temperature, hydric stress, readiness for pollination, post-pollination, flower age) (Borg-Karlson, 1990; Kaiser 1993; Dudareva and Pichersky, 2000; Huber *et al.,* 2005; Knudsen *et al.,* 2006; Tholl *et al.,* 2006; Stashenko and Martinez, 2008; Willmer *et al.,* 2009; Jürgens and Viljoen, 2010). Hence, the sampling step would be a critical part of airborne VOC analysis. The sample must be representative, and qualitative and quantitative alterations during sampling, storage and analysis must be prevented (Gallego *et al.,* 2009a). Additionally, the sampling strategy must allow sample collection during a specific period of time, and must be easy and simple enough to enable facile field sampling (Dettmer and Engewald, 2002). In the same line, the analytical methodology employed has to assure a high sensitivity to identify VOC in a very complex chemical matrix (Knudsen *et al.,* 1993; Raguso, 2004; Gershenzon and Dudareva, 2007; Jürgens and Viljoen, 2010; Soler *et al.,* 2011).

Orchids (mainly the phytochemical compounds present in their roots, tubers, pseudobulbs, rhizomes and leaves) have been used as a provision of medicine for a wide range of diseases during thousands of years all over the world (Cai *et al.,* 2006; Adams *et al.,* 2011; Hossain, 2011). Nevertheless, the study and evaluation of the volatiles emitted by orchids and their possible medical applications is an extensive and emergent field to be investigated, as volatiles and fragrances are widely used in pharmaceutical, food, flavour and fragrance, and cosmetic industries (Gershenzon and Dudareva, 2007; Dunkel *et al.,* 2009; Jürgens and Viljoen, 2010).

In the present paper the identification, through a non-destructive active sorbent-based methodology, of the VOC emitted from the three European orchid species *Himantoglossum robertianum*, *Ophrys apifera* and *Gymnadenia conopsea* is presented, providing satisfying results.

Materials and Methods

Studied orchids

VOC emission was evaluated in the inflorescences of three species of the family Orchidaceae: *Himantoglossum robertianum*, *Ophrys apifera* and *Gymnadenia conopsea*.

H. robertianum is a non-rewarding food deceptive orchid, mimicking the floral structure of certain food providing species. It is distributed through the South-Mediterranean Region, from the Iberian Peninsula to the Turkish coasts, including the Northern of Africa, from 0-1100 m a.s.l. The plant blooms from February to April, producing a spike that comprises several violaceous sessile flowers, generally between 11 and 45 in number (Castroviejo, 1986).

O. apifera is a non-rewarding sexually deceptive orchid (Borg-Karlson, 1990). It is distributed through the Mediterranean Region, from the Atlantic to the Black Sea, from 0-1650 m a.s.l. The plant blooms from mid-April to July, producing a spike that comprises several purpureal or pale pink flowers, generally between 4 and 10 in number (Castroviejo, 1986). It emits an aroma composed of more than 100 VOC, which resembles very much to the emitted by the virgin female individuals of the bees that pollinate this species (Kaiser, 1993; Borg-Karlson, 1990), and at the same time, mimics visually these females (Dudareva and Pichersky, 2000; Ayasse et al., 2011). In the Ophrys species, the male bees attempt to copulate with the flowers contributes to the pollination (Ayasse *et al.,* 2003), however, it has to be taken into account that *O. apifera* is mainly a self pollinating species (Tashev *et al.,* 2006; Fenster *et al.,* 2007; Chaturvedi, 2009).

G. conopsea R. Br ssp. densiflora is a nectar rewarding orchid. It is widely distributed through Europe and Asia, from 0-2200 m a.s.l. The plant blooms in the summer, from June-July, producing a spike that comprises several pale pink to violet sessile flowers, generally between 17 and 82 in number. The floral nectar is abundant, and strongly scented (Castroviejo, 1986; Huber *et al.,* 2005; Jersáková *et al.,* 2010).

Sampling

Orchids *H. robertianum*, *O. apifera* and *G. conopsea* were sampled in Catalonia (Spain) in Berguedà (31T0419132-UTM4672686), Espai Natural El Remolar: Baix Llobregat (31T0420537-UTM4570097) and Pirineu lleidetà: Bordes de Conflent (31T0419214-UTM4672914) in the months of March, May and July 2010, respectively. A modified Tedlar® gas sampling bag was placed covering the inflorescence of the orchid studied (Fig. 1). This design prevents the dilution of the VOC mixture emitted by the flower (Kaiser, 1993). VOC were dynamically sampled by connecting custom packed glass multi-sorbent cartridge tubes (Carbotrap 20/40, 70 mg; Carbopack X 40/60, 100 mg and Carboxen 569 20/45, 90 mg) (Ribes *et al.,* 2007) to a pump AirChek 2000 SKC. Sampling flows were of 100 ml min^{-1}, with total sample volumes of approximately 15-20 litres. At the same time, an outdoor air sample was taken to determine the VOC present in the vicinity of the orchid that would be used to determine exactly which compounds were orchid-exclusive.

Collected volatile samples were analysed by thermal desorption and gas chromatography-mass spectrometry (TD-GC/MSD) (Ribes *et al.,* 2007). This methodology has been used in previous studies to identify and determine a wide range of VOC in ambient air (Gallego *et al.,* 2009b).

Analytical instrumentation

The analysis of VOC was performed by TD-GC/MS, using a Perkin Elmer ATD 400 (Perkin Elmer, Boston, Massachusetts, USA) and a Thermo Quest Trace 2000 GC (ThermoQuest, San Jose, California, USA) interfaced with a Thermo Quest Trace Finnigan MSD.

The methodology is described in the literature (Ribes et al., 2007; Gallego et al., 2009b). Thermal primary desorption of the sampling tubes was carried out at 300ºC, with a Helium flow rate of 50 ml min^{-1} for 10 minutes. The double-split applied to the TD system (cold trap inlet and outlet splits of 4 ml min^{-1} and split 7 ml min^{-1}, respectively) allowed 12% of the tube analytes to reach the MS detector. The cold trap (15 mg Tenax TA and 15 mg Carbotrap), was maintained at -30ºC. After primary desorption, the cold trap was rapidly heated from -30ºC to 300ºC (secondary desorption), and maintained at this temperature for 10 minutes. Analytes were then injected onto the capillary column (DB-624, 60 m x 0.25 mm x 1.4 µm) via a transfer line heated at 200˚C. The column oven temperature started at 40˚C for 1 min, increased to 230˚C at a rate of 6˚C min^{-1} and then was maintained at 230˚C for 5 min. Helium (99.999%) carrier gas flow in the analytical column was approximately 1 ml min^{-1} (1.4 bar).

Mass spectral data were acquired over a mass range of 20-300 amu. The qualitative identification of VOC was based on the match of the ion ratios of the target qualifier ions (Ribes et al., 2007) using Xcalibur 1.2 validated software package with the NIST05 mass spectral library (NIST/EPA/NIH, Nist MS Search version 2.0 d, April 2005). VOC were verified, when possible, using retention times of authentic standards of VOC. On the other hand, several compounds were confirmed by comparison of estimated and published Kovat's Retention Index (RI) values with the NIST library that included manifold literature citations, and with reported data (Jersáková *et al.,* 2010; Soler *et al.,* 2011) (Table 1).

Chemicals and materials

Standards of VOC with a purity of no less than 98% were obtained from Aldrich (Milwaukee, WI, USA), Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Methanol for gas chromatography (SupraSolv®) with a purity ≥ 99.8% was obtained from Merck (Darmstadt, Germany). Perkin Elmer glass tubes (Pyrex, 6 mm external diameter, 90 mm long), unsilanised wool, Carbotrap (20/40 mesh), Carbopack X (40/60 mesh) and Carboxen 569 (20/45 mesh) adsorbents were obtained from Supelco (Bellefonte, PA, USA).

Results and discussion

Sampling strategy

Monitoring VOC emitted by flowers requires a highly efficient and sensitive technique, as the volatile emission is not constant and homogeneous through time (Stashenko and Martinez, 2008; Jürgens and Viljoen, 2010). The sampling methodology used in the present article to determine VOC is a highly selective procedure, as it has been developed to retain a wide range of target compounds present in ambient air (Ribes et al., 2007). The analysis of the sampling tubes with a TD-GC/MS system allows for a good chromatographic separation and a reliable identification of the target compounds through their characteristic mass spectra. Co-elution problems are solved using VOC-characteristic ions in the qualification/quantification step (Ribes et al., 2007). Thermal desorption is a precise methodology, and all trapped pollutants are cromatographed and directed to the detector in only one run, avoiding sample manipulation and dilution. In addition, solvent use is eliminated, preventing the potential interferences of the solvent with the target VOC in the chromatographic analysis (Gallego *et al.,* 2009a). Direct extraction with organic solvents is a basic methodology to obtain volatile concentrates; however, it leads to dirtier samples with large quantities of lipids, pigments and waxes (Stashenko and Martinez, 2008). In the present case, only the VOC emitted by the flowers are sampled, through an in-vivo strategy and avoiding the excision of the inflorescences, as their most properly collection is in situ and from the whole plant (Tholl *et al.,* 2006), obtaining a realistic and representative visualization of the VOC emitted by orchids, as the sampling of the air in the surroundings of the flower alive is a more sensible approach than the extraction of the VOC present in the flower tissues.

Table 1. VOC identified qualitatively and the relative abundance (%) of each scent compound in the three studied orchid species. Calculated Kovats retention index (R.I.), and Estimated and Confidence intervals (95%) of the Kovats retention indexes (NIST/EPA/NIH, Nist MS Search version 2.0 d, April 2005) are also shown. (*) Indicates the identification of the compound both in the sample from the orchid flower and in the outdoor blank sample.

Compound	CAS#	Calc. R.I. (i.u.)	Est. R.I. (i.u.)	Conf. int. 95% (i.u.)	Scent characteristics[a]	March 2010 *Himantoglossum robertianum*		May 2010 *Ophrys apifera*		July 2010 *Gymnadenia conopsea*	
						Presence	%	Presence	%	Presence	%
Alkanes											
2,4-dimethylheptane‡	2213-23-2	783	788	167		-		-		x*	0.2
ethylidene-cyclopentane‡	18631-83-9	546	544	167		x	3.8	x	0.8	x*	0.2
isohexane‡	107-83-5	611	554	167		x	0.2	-		-	
isopentane‡	78-78-4	490	454	167		x*	0.1	x*	0.3	x*	0.1
n-decane†	124-18-5					-		x*	0.3	x	0.1
n-dodecane†	112-40-3					-		x	0.1	x*	0.3
n-hexane†	110-54-3					-		x*	0.3	x*	0.1
n-octane†	111-65-9					-		x	0.3	-	
n-nonane†	111-84-2					-		x	0.2	-	
n-pentane†	109-66-0					x*	0.2	x*	0.3	x*	0.05
n-tetradecane†	629-59-4					-		-		x*	0.6
n-undecane†	1120-21-4					-		x	0.3	x	0.1
Alkenes											
2,6-dimethyl-1,3,5,7-octatetraene‡	460-01-5	1012	966	167		x	0.8	-		-	
2-methylpropene‡	115-11-7	389	386	167		x	0.4	x	1.5	-	
Alkynes											
methylphenylacetylene‡	673-32-5	1233	1010	238		-		x	0.3	-	
Alcohols											
1-butanol†	71-36-3				medicinal, fermented	-		x	26.4	x*	1.4
2,6-dimethyl-3,5-heptadien-2-ol‡	77411-76-8	1090	1001	176		x	0.5	-		-	
2-methyl-2-propanol‡	75-65-0	591	511	176		-		x	3.3	-	
2-nonen-1-ol‡	22104-79-6	1289	1167	176	melon, waxy, green fatty	x	0.3	-		-	
2-propyl-1-pentanol‡	58175-57-8	1039	995	176		-		x	0.8	-	
5,8,10-undecatrien-3-ol‡	56255-81-3	1161	1283	176		x	0.1	-		-	
benzeneethanol‡	60-12-8	1115	1136	176		-		-		x	0.9
cis-4-decen-1-ol‡	57074-37-0	1172	1266	176	waxy, fatty, fruity	x	0.1	-		-	
ethanol†	64-17-5					x*	0.5	x*	0.2	-	
isopropanol†	67-63-0					-		x*	0.1	-	
methylbutenol‡	115-18-4	680	600	176	herbaceous, earthy, oily	x	0.3	-		-	
Ketones											
acetone†	67-64-1					x*	2.8	x*	3.9	x*	0.9
Aldehydes											
2-butenal‡	4170-30-3	640	615	196		-		x	1.0	-	
benzaldehyde†	100-52-7				bitter almond	-		x	0.4	x*	5.2
benzeneacetaldehyde‡	122-78-1	1088	1081	196	green floral, honey, sweet, fruity	-		-		x	0.4
butanal†	123-72-8				fruity, ethereal, meat	-		x	1.5	x	0.1
decanal†	112-31-2				alcoholic citrus, orange peel	-		x	0.8	x*	0.2
dodecanal†	112-54-9				intense, woody, fresh, waxy, floral, sweet	-		x	0.5	-	
heptanal†	111-71-7				harsh, pungent, sweet, rancid	-		x	1.0	x*	0.1
hexanal†	66-25-1				fatty, fruity, green	x	0.2	x	1.1	x*	1.4
methacrylaldehyde‡	78-85-3	624	574	196		x	0.1	-		x*	0.04
nonanal†	124-19-6				citrus, floral, green, orange, fatty	-		x	3.5	x*	0.3
octanal†	124-13-0				citrus, fatty, earthy, green			x	0.8	x*	0.1
pentanal†	110-62-3				fermented, bready, fruity	-		x	0.3	x	0.3
undecanal†	112-44-7				citrus peel, fatty, rose, waxy	-		x	0.3	-	

Ethers											
butyl ether‡	142-96-1	870	892	293	ethereal	x	0.2	x	14.9	x*	0.1
eugenol‡	97-53-0	1419	1392	382	spicy, clove leaf	-		-		x	11.3
Carboxylic acids											
acetic acid†	64-19-7					x	0.1	x	0.1	-	
Esters											
amyl acetate‡	628-63-7	906	884	201	banana, earthy, pear, fruity	-		-		x	0.3
benzyl acetate‡	140-11-4	1139	1160	201	mild jasmine, floral, fruity, apple, banana, apricot	-		-		x	0.4
butyl acetate†	123-86-4				sweet fruity, pungent	-		x*	1.1	x*	1.4
butyl acrylate‡	141-32-2	890	874	201		-		x	0.6	-	
butyl butyrate‡	109-21-7	977	984	201	fruity, pineapple, sweet	-		x	1.8	x	0.1
butyl propionate‡	590-01-2	899	884	201	sweet, rum-like toasty taste, banana	-		x	1.7	-	
decyl acetate‡	112-17-4	1399	1381	201	orange, pineapple, rose	-		-		x	0.2
ethyl acetate†	141-78-6				fruity, musty	-		x*	0.2	x	0.3
formyl acetate‡	2258-42-6	389	675	382		x	0.8	-		-	
linalyl isobutyrate‡	78-35-3	1275	1407	201	fruity, lavender, woody, bergamot, floral	-		x	0.8	-	
methyl acetate†	79-20-9				ethereal, sweet	-		-		x	1.3
phenethyl acetate‡	103-45-7	1220	1259	201	sweet, rose, honey	-		-		x*	69.2
Aromatic hydrocarbons											
α-ionene‡	475-03-6	1386	1414	238		-		x	0.3	-	
1-methylnaphthalene†	90-12-0					-		-		x*	0.1
1,2,4-trimethylbenzene†	95-63-6					x*	0.03	x*	0.6	-	
1,3,5-trimethylbenzene†	108-67-8					-		x	0.3	-	
2-methylnaphthalene†	91-57-6					-		-		x*	0.1
benzene†	71-43-2					x*	0.04	x*	0.1	x*	0.04
cyclopropylbenzene‡	873-49-4	947	994	238		-		x	0.3	-	
ethylbenzene†	100-41-4					x*	0.02	x*	0.3	x*	0.1
m-xylene†	108-38-3					x*	0.02	x*	0.02	x*	0.05
naphthalene†	91-20-3					-		x	0.9	-	
o-ethyltoluene†	611-14-3					-		x	0.3	-	
n-propylbenzene†	103-65-1					-		x	0.1	-	
o-xylene†	95-47-6					x*	0.01	x*	0.1	x*	0.1
p-cymenene†	1195-32-0				burnt wood, roasted coffee, spicy	x	0.1	-		-	
p-xylene†	106-42-3					x*	0.02	x*	0.01	x*	0.05
toluene†	108-88-3					x*	0.03	x*	0.4	x*	0.1
Halocarbons											
carbon tetrachloride†	56-23-5					-		x	0.1	-	
chloroform†	67-66-3					-		x	0.04	-	
dichloromethane†	75-09-2					x*	0.2	x*	0.5	x*	0.1
m-dichlorobenzene†	541-73-1					-		x	0.1	-	
p-dichlorobenzene†	106-46-7					-		x	0.2	-	
tetrachloroethylene[b]†	127-18-4					-		x*	0.1	-	
trichlorotrifluoroethane[b]‡	76-13-1	497	442	382		x*	0.1	x*	0.6	x*	0.1
Terpenoids											
Monoterpene hydrocarbons											
α-pinene†	80-56-8				pine, resinous	x	42.9	x	0.4	x*	0.1
β-phellandrene‡	555-10-2	1042	964	167	citrus, black pepper	x	1.0	-		-	
β-pinene†	127-91-3				turpentine, dry woody or resinous aroma	x	12.4	-		x*	0.1
γ-terpinene‡	99-85-4	1058	998	167	fatty, lemon-citrusy, lime	x	0.2	-		-	
2,4-thujadiene‡	36262-09-6	964	879	167		x	0.6	-		-	
camphene‡	79-92-5	968	943	167	coniferous, harsh, fresh	-		-		x	0.1
limonene†	7705-14-8				lemon, turpentine	x*	26.9	x	0.3	x*	0.1
thujene‡	58037-87-9	927	873	167		x	0.4	-		-	
ocimene†	502-99-8				fruit, wet cloth	x	0.1	-		-	
sabinene‡	3387-41-5	990	897	167	woody, spicy, citrus, pine-like, green	x	0.2	-		-	
terpinolene‡	586-62-9	1023	1052	167	pine, sweet citrus	x	0.2	-		-	
tetracyclo[3.3.1.0(2,8).0(4,	N/A[c]	1003	673	752		-		x	0.3	-	

6)]-non-2-ene‡										
Monoterpene alcohols										
α-terpineol[d‡]	98-55-5	1180	1143	176	lilac, citrus, lime, apple blossom, earthy character	x	0.1	-	-	
4-terpineol‡	562-74-3	1164	1137	176	pepper, woody, earth, musty, sweet	x	0.2	-	-	
6-camphenol‡	3570-04-5	1292	1131	176		x	0.5	-	-	
carveol‡	99-48-9	1139	1206	176	spearmint-like odour	x	0.3	-	-	
cis-geraniol‡	106-25-2	1076	1228	176	rose like	x	0.6	-	-	
Monoterpene ketones										
1b,5,5,6a-tetramethyl-octahydro-1-oxa-cyclopropa[a]inden-6-one‡	N/A	1213	1445	382		x	0.5	-	-	
carvol‡	99-49-0	1129	1190	246	minty, liquorice	x	0.1	-	-	
verbenone[d‡]	80-57-9	1119	1119	246	camphor, menthol, celery	x	0.3	-	-	
Monoterpene ethers										
eucalyptol‡	470-82-6	1048	1059	293	mint, turpentine	x	0.2	-	-	
Sesquiterpene hydrocarbons										
(-)-alloisolongifolene[d‡]	87064-18-4	1429	1390	167		-		x	3.9	-
caryophyllene‡	13877-93-5	1455	1494	167	spicy, pepper-like, woody, sweet, citrus background	-		x	12.1	-
(+)-cycloisosativene[d‡]	N/A	1310	1125	752		-		x	1.7	-
longicyclene[d‡]	1137-12-8	1350	1184	752	sweet woody rose	-		x	0.6	-
neoisolongifolene[d‡]	N/A	1393	1416	167		-		x	2.8	-
Organosulfurs										
benzothiazole†	95-16-9					-		-	x*	0.4
Organonitrogenates										
dimethyl-diazene‡	503-28-6	420	390	356		x	0.2	-	-	

[a]Source: The Good Scent Company (http://www.thegoodscentscompany.com)

[b]The identification of these compounds should be secure with their isotope signatures

[c]Not available

[d]The identification of these compounds need to be confirmed using a chirospecific chromatographic technique

†Compounds identified and verified using authentic standards of VOC

‡Compounds identified and verified using Kovats Retention Index (RI)

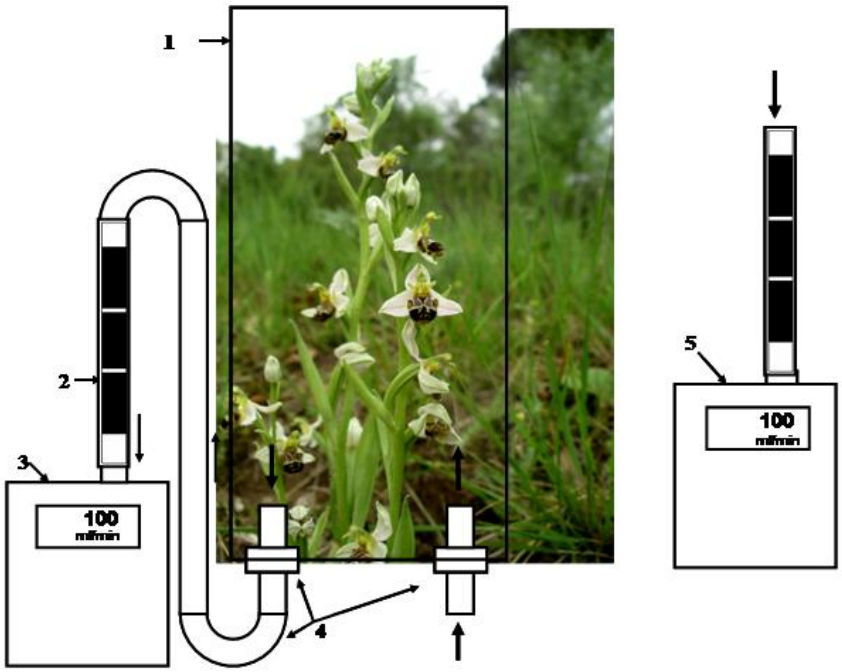

Fig. 1. Sampling strategy. 1) Tedlar bag; 2)Multi-sorbent bed tube; 3) Air sampler; 4) Teflon tube connections; 5) Outdoor air sampler.

Additionally, high humidity conditions derived from the presence of moisture generated by the plant inside the sampling bag can induce an adsorption of water in the sorbent tube (Stashenko and Martinez, 2008), leading to a competition for the adsorbent active surface area between water and the target compounds to be sampled (Marisová and Škrabáková, 1995), reducing the adsorption capacity of the sorbent (Strandberg *et al.,* 2005; Gallego *et al.,* 2009a). Hence, the use of our highly hydrophobic custom packed multi-sorbent bed tubes ensures a good performance of the measurement (Ribes *et al.,* 2007).

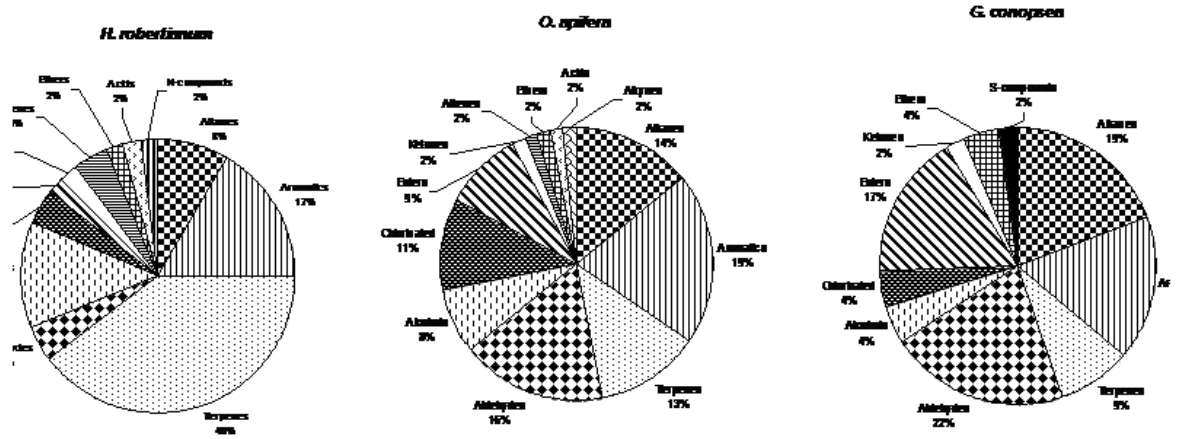

Fig. 2. VOC chemical class distribution for all qualitatively identified compounds for each species. Percentage of compounds of each chemical class in respect to all compounds identified.

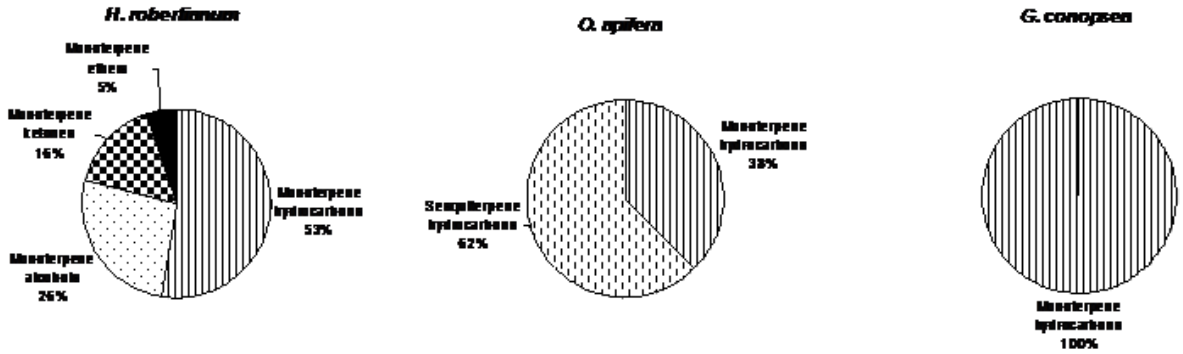

Fig. 3. Terpenes distribution for each species. Percentage of terpene-type in respect to all terpenes identified.

Compounds identified in the three orchid species

A total of 106 different VOC were found in the fragrances emitted by the three different studied orchids. A 54% of these compounds (meaning 57 of the identified compounds in the present study) had already been identified in floral volatile mixtures (Knudsen *et al.,* 1993, 2006). On the other hand, 39 of the identified compounds were generally found both in the floral volatile mixture and in the ambient air blank samples (Table 1). Additionally, an 83% of the coincident compounds (identified in the present study and already identified in previous studies) had been observed to be emitted from Orchidaceae (Knudsen *et al.,* 2006).

Proportional abundance of compounds (relative amounts with respect total peak areas, using the total ion current (TIC) chromatogram) was used to show the great variability of VOC composition among samples (Table 1). It has to be taken into account, however, that TIC chromatograms do not represent a quantity but the ease of ionisation in the given mode.

Nevertheless, as the amount and nature of the emitted volatiles varied greatly among the three studied orchids, the abovementioned proportional abundance of VOC was found to be a useful tool to compare data. Only a few VOC had a relative proportion in the volatile mixture higher than 1%. In *H. robertianum*, *O. apifera* and *G. conopsea*, only 5, 15 and 7 compounds, respectively, exceeded this value. In addition to that, generally rarely more than 3 compounds were relatively abundant (e.g. α-pinene, β-pinene and limonene in *H. robertianum*; 1-butanol, butyl ether and caryophyllene in *O. apifera*; and phenethyl acetate, eugenol and benzaldehyde in *G. conopsea*) (Table 1). The differences observed between the present results and those of previous studies of *O. apifera* (Borg-Karlson 1990) and *G. conopsea* (Kaiser, 1993; Huber *et al.,* 2005; Jersáková *et al.,* 2010) species may be derived from the variations among the studied populations, as well as from differences in sampling methods (Huber et al., 2005), as in preceding studies VOC are generally sampled by headspace sorption (Knudsen *et al.,* 1993, 2006) and in the present study through active multi-sorbent bed adsorption.

VOC families' distributions for all qualitatively identified compounds and for each studied species are shown in Fig. 2. Alkanes, aromatics, ethers, alkenes and ketones are found in similar proportion in the three species, ranging from 8-19%, 17-19%, 2-4%, 0-4% and 2%, respectively. Terpenes and alcohols are the most distinctive emitted compounds in *H. robertianum*, which presents the higher percentage of terpenes (40%) of all studied orchids, as well as a more varied composition of them, including, monoterpene hydrocarbons, monoterpene alcohols, monoterpene ketones and monoterpene ethers (Fig. 3). Terpenes, aldehydes, alcohols, chlorinated compounds and esters are the most characteristic VOC released by *O. apifera*. Finally, aldehydes and esters are the distinctive compounds emitted by *G. conopsea*.

The different pollination strategies (non-rewarding food deceptive, non-rewarding sexually deceptive and nectar rewarding orchids) surely play an important role in the observed differences in the presence/abundance of the identified compounds in each studied orchid species. The huge diversity in the odorous volatile mixtures of orchids may be interpreted as a reproduction of the comparable enormous diversity of pollination procedures (Kaiser, 1993). In previous studies it has been observed that different VOC families have different functions in pollination and/or defence in plants (Raguso *et al.,* 2006; Gershenzon & Dudareva, 2007; Schiestl, 2010). Additionally, it has to be considered that not all flower emitted compounds are physiologically active to pollinators and are capable of triggering a response from them (Huber *et al.,* 2005; Jersáková *et al.,* 2010). At the same time, specific VOC may play multiple roles in the same species. Depending on the situation and the quantity of the emitted chemical compounds may undertake an attractant or defensive role (Shiestl, 2010). In previous studies it has been observed that sometimes odorous emitting species are closely related to non-scented species. On the other hand, in other occasions nearly related species do not show emissions of matching mixtures of VOC (Dudareva and Pichersky, 2000). The evolutive ability of plants in emitting volatiles could be an explanation for the differences found (Dudareva and Pichersky, 2000), as well as a co-evolution between pollinators and flowers in volatile compounds synthesis and release (Shiestl, 2010).

Conclusions

Floral volatile mixtures are usually evaluated using head space techniques. In the present study, however, a highly selective procedure based in the retention of VOC in a custom made multi-sorbent bed tube coupled to TD-GC/MS has proven to be suitable for the identification of a wide range of floral emitted compounds in complex gaseous samples. A total of 106 different VOC were found in the volatile mixtures emitted by the studied orchids, giving valuable information of the kind of compounds and

their relative abundance in the fragrance of the three different species.

The high diversity and abundance of VOC determined in the different examined orchid specimens may respond to an evolutive strategy to attract a maximum variety of pollinators, even in *Ophrys apifera* despite its morphological and self-pollination strategies, as well as a defensive role.

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