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Comparison of salep orchid species based on the composition of the volatile compounds of their tubers

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Abstract – The objective of this work was to compare salep orchid species based on the composition of the volatile compounds of their tubers. Tuber samples extracted by solid-phase microextraction were analyzed by gas chromatography-mass spectrometry. In all studied species, p-cresol was the main volatile compound detected. The highest p-cresol proportion was found in *Anacamptis laxiflora* (76.31%), followed by *Ophrys sphegodes* subsp. *mammosa* (62.72%), *Orchis coriophora* (55.95%), and *Anacamptis pyramidalis* (21.19%). The proportions of the other volatile compounds also varied significantly between the evaluated species. The clustering analysis of the species according to their volatile compounds showed that *A. laxiflora* and *O. coriophora* were the closest, while *A. laxiflora* and *A. pyramidalis* were the most distant.

Index terms: aroma profile, food products, SPME, tubers, volatile compounds.

Comparação de espécies de orquídeas salepo com base na composição dos compostos voláteis de seus tubérculos

Resumo – O objetivo deste trabalho foi comparar espécies de orquídeas salepo com base na composição dos compostos voláteis de seus tubérculos. Amostras de tubérculos extraídas por microextração em fase sólida foram analisadas por cromatografia gasosa-espectrometria de massa. Em todas as espécies estudadas, o p-cresol foi o principal composto volátil detectado. A maior proporção de p-cresol foi encontrada em *Anacamptis laxiflora* (76,31%), seguida de *Ophrys sphegodes* subsp. *mammosa* (62,72%), *Orchis coriophora* (55,95%) e *Anacamptis piramidalis* (21,19%). As proporções dos outros compostos voláteis também variaram significativamente entre as espécies avaliadas. A análise de agrupamento das espécies de acordo com os seus compostos voláteis mostrou que *A. laxiflora* e *O. coriophora* foram as mais próximas, enquanto *A. laxiflora* e *A. piramidalis* foram as mais distantes.

Termos para indexação: perfil aromático, produtos alimentícios, família orquidaceae, SPEM, tubérculos, compostos voláteis.

Introduction

The Orchidaceae family is one of the richest in terms of species worldwide (Dressler, 1981), including 850 genera (Castillo-Pérez et al., 2019). In the Mediterranean region, Turkey has one of the richest orchid flora (Molnár et al., 2022), with a total of 80 species (Turkmen, 2021).

In the country, tuber-producing species, also known as salep plants, are distributed in the ten following genera: *Aceras*, *Anacamptis*, *Barlia*, *Comperia*, *Dactylorhiza*, *Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis*, and *Serapias*.

The salep orchid stands out in its use for various food and medicinal purposes (Bazzicalupo et al., 2023). The tubers of these plants are dried and ground into powder and used to prepare a hot drink, also called salep (Tamer et al., 2019), as well as high-quality ice creams due to their glucomannan content (Kurt, 2021). Salep powder also plays an important role in the treatment of some diseases, such as: paralysis, stomach ailments, asthma, inflammation, tuberculosis, rheumatism, and chest pain Hossain (2011); and Alzheimer's and Parkinson's disease, depression, cancer, dysentery, diarrhea, cough, cold, and anemia (Dalar et al., 2015; Jahromi et al., 2018; Singh et al., 2022). In addition, the jelly obtained from salep tubers can be used to treat irritations of the gastrointestinal tract (Véla, 2018).

Currently, there has been an increase in the number of consumers who selectively purchase food products based on their health-promoting and quality features (Short et al., 2021). Therefore, approaches that offer alternatives based on consumer preferences are gaining importance. For consumers, flavor is food's most important quality feature, which is why its stability in the complex structure of the food system plays an important role in food quality (Weerawatanakorn et al., 2015). Another primary factor for the consumption or preference of foods is their aroma, whose profile is formed through the combined effect of several different volatile compounds, at different levels (Reineccius, 1994). Studies that investigate the composition of volatile compounds in foods can present new opportunities to obtain different tastes and flavors for consumers who prefer food products with desired aromas. Therefore, researches on the volatile compounds present in various agricultural products are key for developing quality products with customer focus.

In the case of the tubers of salep plants, there are no known previous studies on their volatile compounds. The researches conducted so far have mostly focused on the volatile compounds in the flowers of these species (Nielsen & Møller, 2015; Baek et al., 2019). However, since tubers are the most used component of salep plants, conducting studies on their tubers is a more appropriate approach.

The objective of this work was to compare salep orchid species based on the composition of the volatile compounds of their tubers.

Materials and Methods

For the study, the four following salep species were used: *Anacamptis laxiflora* (Lam.) R.M.Bateman, Pridgeon & M.W.Chase; *Ophrys sphegodes* Mill. subsp. *mammosa*; *Orchis coriophora* L.; and *Anacamptis pyramidalis* (L.) Rich. On September 10, 2021, tubers of these species were planted at a 20×10 cm distance from each other and at a 5 cm depth in small plots, containing a 2:1 mixture of field soil and stream sand, in an area located in Rize, Turkey (41°10.667'N, 40°54.018'E). Before planting, a NPK (15-15-15) compound fertilizer was applied, with 50 kg N, 50 kg P, and 50 kg K per hectare.

In the blooming period, ten plants of each species were uprooted from the soil, and new tubers were harvested. The harvested tubers were washed with water until completely cleaned from dust and, then, were left to dry for 20 days at 25±1°C and 65–70% relative humidity. The dried tubers, with a water content of 18–20%, were completely pulverized using a grinder to obtain a powder that could pass through a 1.0 mm sieve. The ground samples were kept in airtight ziplock bags and stored in a refrigerator, at 4°C, until their analysis.

The study was conducted in two replicates. First, the samples were extracted from the tubers by solid-phase microextraction (SPME). Immediately after the extraction, the volatile compounds of the extracted samples were identified by gas chromatography-mass spectrometry (GC-MS) using the QP2010 Ultra chromatograph (Shimadzu, Tokyo, Japan). The method of Mus et al. (2020) was used for the SPME/GC-MS analysis, with some modifications.

A fiber (divinylbenzene/carboxen/polydimethylsiloxane) with a thickness of 50/30 μm and a length of 2.0 cm was used to extract the volatile compounds. After the samples were placed in vials, the volatile compounds were retained on the SPME holder (Supelco, Merck KGaA, Darmstadt, Germany) at the end of a 5 min waiting period, at 65°C. Subsequently, the volatile compounds retained on the SPME fiber were analyzed by GC-MS. The column furnace temperature was adjusted to 250°C, with a gradual increment starting from 40°C. The volatile compounds were separated using the Rxi-5Sil MS silica capillary column (Resteck, Centre County, PA, USA) with a 30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The GC-MS interface and ionization source temperatures were 210 and 250°C, respectively. Ionization voltage was 70 eV. Retention index values were obtained from the GC-MS records of the analyzed samples. Volatile compound measurements were performed based on peak-area normalization, and the relative density of each compound was expressed as percentage (Kafkas et al., 2006). All compounds were identified by the NIST (1992) library.

Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) was used to create graphs and determine standard deviation values. Before the statistical analysis, all data were subjected to logarithmic transformation (log+1). The hierarchical clustering analysis and principal component analysis were conducted using the SPSS 20.0 software (IMB, Armonk, NY, USA). The Ward method was used in the clustering analysis, considering square Euclidean distances (Carter et al., 1989).

Results and Discussion

The studied salep orchid species differed according to their chemical compounds (Table 1). The main volatile compound detected in the evaluated species was p-cresol, mostly found in animal products such as cheese (Kilcawley et al., 2018) and yoghurt (Cheng et al., 2022). The highest proportion of this compound was found in *A. laxiflora* (76.31%), followed by

Table 1. Retention index (RI), area average (Area), and standard deviation (SD) values of the volatile compounds detected by the solid-phase microextraction/gas chromatography-mass spectrometry analysis in the tubers of the studied salep species[(1)].

	RI	Compound	A		B		C		D	
			Area (%)	SD	Area (%)	SD	Area (%)	SD	Area (%)	SD
		Aldehydes								
1	806	Capronaldehyde	4.36	1.32	-	-	-	-	9.69	3.62
2	920	2,4-Dimethylbenzaldehyde	2.22	1.56	-	-	3.18	2.24	17.16	4.60
3	1111	Pelargonaldehyde	4.13	1.10	4.68	0.62	7.52	3.50	7.58	2.06
4	1214	Capraldehyde	3.51	0.86	3.46	0.25	6.42	3.95	9.73	2.40
		Total	14.22		8.14		17.12		44.16	
		Alkanes								
5	1208	Dodecane	0.98	0.69	0.75	0.52	3.25	1.90	2.67	1.88
6	1410	Tetradecane	3.05	0.42	3.13	0.69	7.82	5.83	6.29	2.40
7	1582	Eicosane	0.99	1.40	1.04	0.74	-	-	2.67	1.88
8	1612	Hexadecane	1.60	1.13	2.91	1.03	5.68	1.73	5.83	1.55
		Total	6.62		7.83		16.75		17.46	
		Alcohols								
9	1038	2-Ethyl hexanol	3.27	0.39	3.70	0.25	3.48	0.85	7.24	2.13
10	1043	Phenylmethanol	3.75	3.04	2.35	0.21	0.92	0.64	-	-
		Total	7.02		6.05		4.40		7.24	
		Terpenes								
11	1033	Limonene	3.91	0.26	1.71	1.20	4.33	2.21	7.60	0.15
12	1379	Cyclosativene	3.04	1.75	-	-	-	-	1.19	0.84
		Total	6.95		1.71		4.33		8.79	
		Esters								
13	1464	Isopulegyl acetate	0.81	0.57	-		1.01	0.71	-	-
14	1608	Diethyl phthalate	1.69	1.20	-		0.48	0.33	-	-
		Total	2.50				1.49			
		Phenols								
15	1091	p-cresol	62.72	6.63	76.31	4.05	55.95	12.10	21.19	7.33

[(1)]A, *Ophrys sphegodes* subsp. *mammosa*; B, *Anacamptis laxiflora*; C, *Orchis coriophora*; and D, *Anacamptis pyramidalis*.

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O. sphegodes subsp. *mammosa* (62.72%), *O. coriophora* (55.95%), and *A. pyramidalis* (21.19%). However, this volatile compound, belonging to the phenol group, was the only one considered undesirable due to its animal-like, ink, and fecal odor (Beauchamp & Zardin, 2017). Therefore, the much lower proportion of p-cresol in *A. pyramidalis* allowed of achieving a more balanced distribution of the remaining compounds.

In the literature, previous researches on salep species have focused on the volatile compounds present in their flowers and not on their tubers (Nielsen & Møller, 2015; Baek et al., 2019). Robustelli della Cuna et al. (2022) found that the p-cresol content in the essential oils obtained from salep flowers was 38.10% in *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase; 15.28% in *Himantoglossum robertianum* (Loisel.) P.Delforge; 12.75% in *O. sphegodes*; and 12.99% in *Orchis purpurea* Huds. These p-cresol proportions are much lower than those obtained in the present study, a difference that could be due to the different species and plant parts evaluated (flowers vs. tubers). However, there are no known works explaining this variation in volatile compounds between the flowers and tubers of different orchid species.

Regarding volatile-compound groups, phenol was the most dominant in all species, except in *A. pyramidalis*, followed by aldehydes, alkanes, and other chemical groups. A proportional distribution of the chemical groups was observed according to the salep species (Figure 1). Moreover, the ester group was not detected in *A. laxiflora* and *A. pyramidalis*. Manzo et al. (2014) also found significant differences among three salep species based on the volatile compounds present in their flowers, i.e., on the content of aldehydes, esters, phenols, and terpenes.

In terms of the number of volatile compounds, the richest species was *O. sphegodes* subsp. *mammosa*, with 15 compounds, whereas the poorest was *A. laxiflora*, with 10 compounds. Considering compounds with an area value over 5.0%, both *O. sphegodes* subsp. *mammosa* and *A. laxiflora* contained only one, while *O. coriophora* and *A. pyramidalis* had five and nine, respectively.

There was a wide variation between the evaluated species with regard to the presence and proportion of volatile compounds. For example, capronaldehyde, which has a sharp green-grass smell (NLM, 2023b), was not detected in the tubers of *A. laxiflora* and *O. coriophora*, but was present in 36 and 9.69% in *O. sphegodes* subsp. *mammosa* and *A. pyramidalis*, respectively. 2,4-dimethylbenzaldehyde, which has a mild sweet and bitter almond odor (NLM, 2023a), was detected in all species, except in *A. laxiflora*. Although the proportion of this volatile compound reached a high value of 17.16% in *A. pyramidalis*, it remained below 5.0% in the other salep species. Furthermore, cyclosativene was not detected in *A. laxiflora* and *O. coriophora*, but was present in the proportions of 3.04 and 1.19% in *O. sphegodes* subsp. *mammosa* and *A. pyramidalis*, respectively. According to Manzo et al. (2014) and Zito et al. (2019), cyclosativene, which has a terpene-like aroma (Chung et al., 2020), is a floral fragrance compound specific to the genus *Ophrys*. Isopulegyl acetate (NLM, 2023c) and diethyl phthalate, which have a mint-like, sweet odor (Paiva et al., 2021), were not detected in *A. laxiflora* and *A. pyramidalis*. Eicosane, an alkane with a fruity, sweet, and woody odor (Ngoenchai et al., 2022), was only not found in *O. coriophora*, whereas phenylmethanol, with a sweet-spicy odor (Rapior et al., 2002), was only not identified in *A. pyramidalis*. Therefore, the present study revealed significant differences between the investigated species based on their volatile compounds. Similarly, in a research conducted in southern Italy using five different species of the genus *Anacamptis* [*Anacamptis coriophora* subsp. *fragrans* (Pollini) R.M.Bateman, Pridgeon & M.W.Chase; *A. pyramidalis*; *A. morio*; *A. laxiflora*; and *A. papilionacea* (L.) R.M.Bateman, Pridgeon & M.W.Chase] from the natural flora, D'Auria

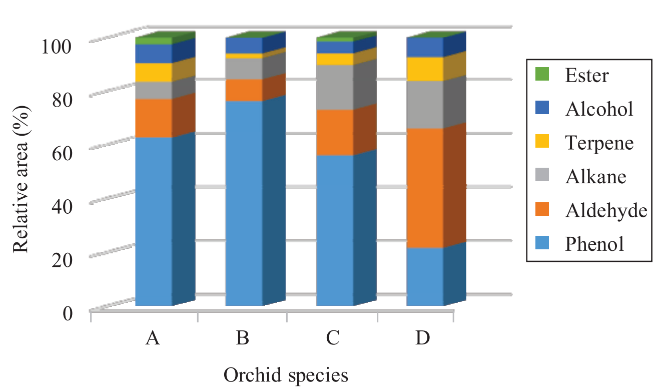

Figure 1. Relative area-based distribution of the compound groups found in the studied salep orchid species. A, *Ophrys sphegodes* subsp. *mammosa*; B, *Anacamptis laxiflora*; C, *Orchis coriophora*; and D, *Anacamptis pyramidalis*.

et al. (2024) observed that the scent compositions of the flowers, determined through the SPME/GC-MS analysis, varied according to the species.

In the hierarchical clustering of the orchid species based on their volatile compounds, two groups were formed (Figure 2). The first included *O. sphegodes* subsp. *mammosa*, *A. laxiflora*, and *O. coriophora*, with a subcluster containing *A. laxiflora* and *O. coriophora* and another, *O. sphegodes* subsp. *mammosa*, which was distant from the other two species. The second group included *A. pyramidalis*, which is completely separated from the other species.

Based on the dendrogram, the salep species were distributed in three different clusters. The values of the proximity matrix obtained by the cluster analysis showed that *A. laxiflora* and *O. coriophora* were the closest species, while *A. laxiflora* and *A. pyramidalis* were the most distant based on the composition of their volatile compounds.

The clustering analysis was performed to determine the relationship between the compound groups (Figure 3). Initially, two groups were formed, which were later separated into two clusters within themselves. Phenols and esters were separated from the other compounds in the same group.

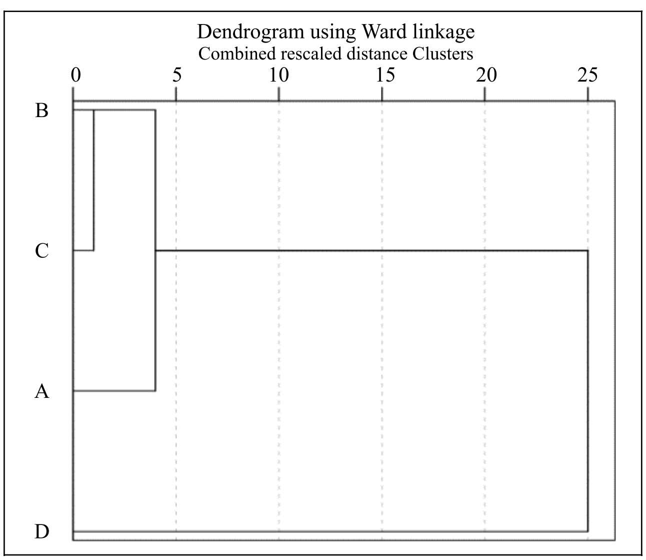

Figure 2. Hierarchical clustering between the studied salep orchid species based on their volatile compounds. A, *Ophrys sphegodes* subsp. *mammosa*; B, *Anacamptis laxiflora*; C, *Orchis coriophora*; and D, *Anacamptis pyramidalis.*

Four clusters were defined by three points on the dendrogram. Aldehydes and alkanes formed a cluster together, and alcohols and terpenes another one. In contrast, phenols and esters were separated into different clusters. According to the proximity matrix values obtained by the clustering analysis, aldehydes and alkanes were the closest groups, while phenol and esters were the most distant. These findings revealed that aldehydes act with alkanes, while alcohols act with terpenes in the studied salep tubers, whereas phenols and esters act independently from the other compounds.

The principal component analysis identified three principal components with an eigenvalue > 1 (Table 2 and Figure 4). The first principal component was mainly represented by the aldehyde, phenol, terpene, and alkane groups, explaining 54.012% of total variance.

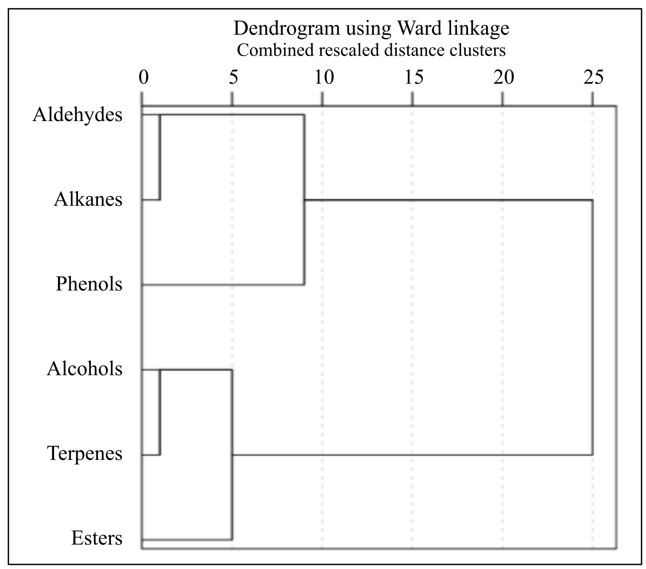

Figure 3. Hierarchical clustering between the groups of volatile compounds found in the studied salep orchid species.

Table 2. Eigenvalues, contribution rates, and cumulative variance contribution of the three principal components.

Principal component	Rotation sums of squared loadings		
	Eigenvalue	Contribution rate (%)	Cumulative variance contribution (%)
1	3.241	54.012	54.012
2	1.408	23.460	77.472
3	1.352	22.528	100.000

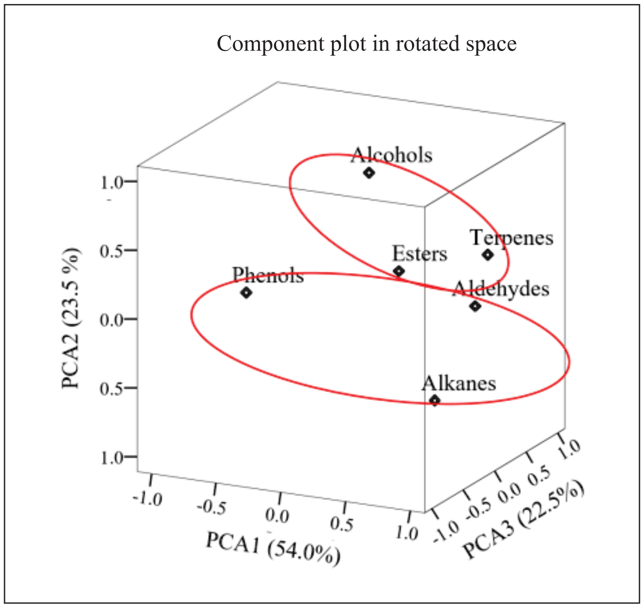

Figure 4. Principal component analysis (PCA) of the volatile compound groups found in the studied salep orchid species.

The second principal component was represented by the alcohol group, and the third principal component, by the ester group, each explaining 23.460 and 22.528% of total variance. The principal component analysis revealed that the variation in the volatile compounds of the examined salep tubers could be explained by the collective contributions of all compound groups.

Conclusion

The evaluated salep species differ significantly in terms of the presence and proportions of volatile compounds, among which p-cresol was the one found in the highest proportion in all studied species, followed by 2,4-dimethylbenzaldehyde, which was only detected in high proportion in *Anacamptis pyramidalis*.

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