Analysis of Volatile Substances in *Stropharia rugosoannulata* Farlow Cultivated Under Forest Canopy with Four Different Culture Substrates by Electronic Nose and GC-IMS

Hui Wang,[b,¶] Ying Rao,[a,¶] Juanjuan Chen,[a,&] Fei Zhou,[a,&] Jinping Zhang,[a,*] Hongxia Xu,[b,&] and Jianbin Xu [b,&]

* *Corresponding author: jinpingzhang@126.com*

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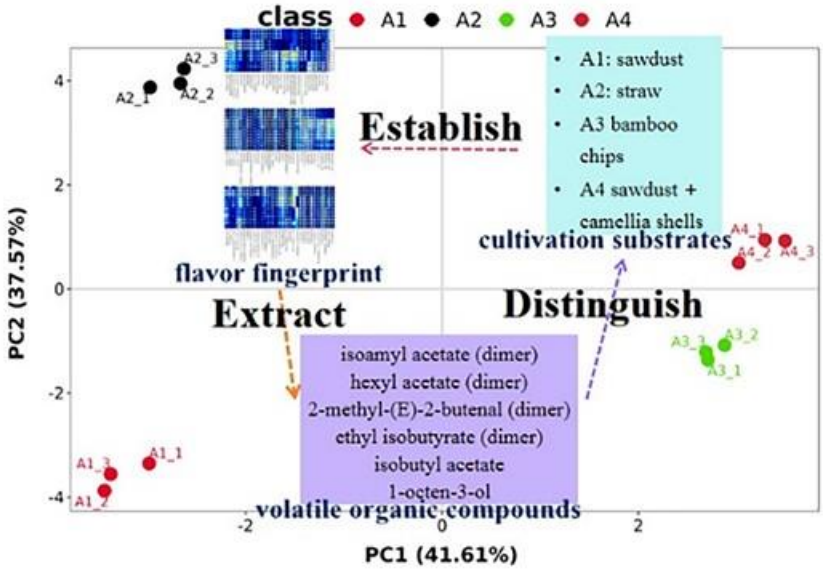
GRAPHICAL ABSTRACT



Analysis of Volatile Substances in *Stropharia rugosoannulata* Farlow Cultivated Under Forest Canopy with Four Different Culture Substrates by Electronic Nose and GC-IMS

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This study examined the volatile organic compounds (VOCs) in *Stropharia rugosoannulata* Farlow that were cultivated on four substrates formulated with agricultural and forestry wastes. The VOCs were analyzed by an electronic nose (E-nose), gas chromatography-ion mobility spectroscopy (GC-IMS), principal component analysis (PCA), and an orthogonal partial least squares discriminant analysis (OPLS-DA). A4(40% sawdust, 30% camellia shells, 20% rice husk, 8% bran, and 2% lime) was the most effective overall at determining the quality of flavor. The E-nose showed that there were similar profiles of aromas for A2(100% *Eleusine coracana* (L.) Gaertn straw) and A3(70% bamboo chips, 20% rice husk, 8% bran, and 2% lime). A total of 91 VOCs, including 82 known compounds, such as formaldehyde, alcohols, esters, and ketones, and 9 unknown compounds, were detected in each sample by GC-IMS. The relative contents of formaldehyde, ketones, alcohols, and esters in the samples was more than 80%. Among the 29 VOCs with variable importance in projection (VIP) values > 1 and P < 0.05, formaldehyde, heptagonal(dimer), 2-methyl-E-2-butenal-M", 3-methyl-2-butenal-M(dimer), 1-octen-3-ol, butyl acetate(dimer), ethyl 3-methylbutanoate, and 2-pentylfuran were the markers that distinguished the volatiles in *S. rugosoannulata* cultivated with different groups of raw substrate materials.

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Keywords: Stropharia rugosoannulata; Volatile organic compounds; Electronic nose; Gas Chromatography-ion mobility spectroscopy

Contact information: a: Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou, Fuyang, China; b: Chun 'an County Thousand-island Lake Forestry Farm, Hangzhou, Zhejiang, China;
¶: These authors contributed equally to this work; &: These authors also contributed equally to this work.
** Corresponding author: jinpingzhang@126.com*

INTRODUCTION

Edible mushrooms are a class of nutritious and healthy foods that have high amounts of protein, dietary fiber, and trace minerals (Roupas *et al.* 2012; Roncero-Ramos and Delgado-Andrade 2017). *Stropharia rugosoannulata* Farlow, commonly known as wine cap stropharia, burgundy mushroom and king stropharia, is a rare edible fungus in the Strophariaceae family (Wu *et al.* 2013; Chen *et al.* 2020). This mushroom is brightly colored and has a smooth cap. In addition, the meat and stems are crispy. *S. rugosoannulata* contains abundant proteins and various mineral elements that are healthy to humans. It is also a good source of various biologically active substances. Therefore, *S. rugosoannulata*

is one of the top 10 traded mushrooms in the international edible fungus market (Hu *et al.* 2020). It is highly promoted in China as a high-quality and rare edible fungus that is rich in nutrients, an antioxidant and has anti-tumor effects (Liu *et al.* 2020). It is recommended by the Food and Agriculture Organization (FAO) of the United Nations as one of the characteristic varieties for cultivation in developing countries (Yang *et al*. 2021). *S. rugosoannulata* is highly resistant to stress and bacterial contamination. It is strongly adaptable, and it has a substantial potential for degrading environmental pollutants (Castellet-Rovira *et al.* 2018).

Its volatile components are among the key factors that determine its quality and consumer perception; thus, they can be used to evaluate the nutritional value and freshness of food to some extent (Fang *et al.* 2017). Volatile flavor compounds are usually qualified by headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS), gas chromatography-olfactometry-mass spectrometry (GC-O-MS) and headspace-gas-chromatography ion-mobility spectrometry (HS-GC-IMS), and an electronic nose (E-nose) is usually used as an auxiliary tool for the qualitative analysis (Chen *et al.* 2020; Adelina *et al.* 2021; Shen *et al.* 2021). Gas chromatography-ion mobility spectroscopy (GC-IMS), an emerging analytical technique of volatile organic compounds (VOCs). It does not require the concentration of VOCs from the sample by SPME, but rather, it directly extracts and analyzes some volume of gas from the sample (Zhang *et al.* 2020). This type of analysis not only can reflect the true aroma components of a sample more accurately, but is also quicker, more sensitive, and cheaper than gas chromatography-mass spectrometry (GC-MS) (Gerhardt *et al.* 2018; Wang *et al.* 2019). The high moisture content and thin skin of fresh *S. rugosoannulata* makes it likely to rot soon after harvest, which seriously affects its value as a commodity (Mahajan *et al.* 2008). Drying is the most common convenient method to control the moisture and effectively prolong the shelf life of these fresh mushrooms. However, high-temperature (≥ 100 ℃) drying tends to cause losses of flavor of the fresh mushrooms (Yang *et al.* 2021). Therefore, *S. rugosoannulata* samples were usually pre-dried at 40 °C for 20 h to reduce the moisture content from 98% to 7%, but the flavor remains unchanged to ensure that the flavor remains as fresh as possible.

S. rugosoannulata is a straw-rot fungus, and it can be cultivated on a variety of substrates. With the development of the under-canopy economy in China, the under-canopy cultivation technique of *S. rugosoannulata* has become increasingly mature, and various materials, such as straw, awn stalk, bamboo, sawdust and agricultural and forestry wastes, have been used as substrates to cultivate this fungus. Currently, the studies on *S. rugosoannulata* have primarily focused on the pretreatment of raw cultivation materials, formulation of cultivation substrates, cultivation pattern, antioxidant capacity, protoplasts, and breeding among others (Qin *et al.* 2022). In this study, the volatile flavor compounds in *S. rugosoannulata* were analyzed by the E-nose and GC-IMS cultivated with four different formulas of substrates which were primarily composed of wood chips, straw, bamboo chips, and camellia shells. The obtained GC-IMS data were subjected to an orthogonal partial least squares discriminant analysis (OLPS-DA) using SIMCAP14 and a principal component analysis (PCA) to determine the effects of the substrates on the volatile flavor compounds. The VOC fingerprints and evaluation criteria of *S. rugosoannulata* cultivated with four types of substrates were established to provide a scientific basis for improving substrate processing and formulating and screening substrates capable of cultivating *Agaricus blazei* mushrooms with better flavors.

MATERIALS AND METHODS

The study was conducted in Chun'an County, Zhejiang Province, China, (29° 11'-30° 02' N and 118° 20 '-119° 20' E). The area has a mid-subtropical monsoon warm and humid climate with mean annual precipitation of 1,430±309.00 mm and a mean annual temperature of 17 ℃±4.80 ℃. The mushrooms were cultivated under a canopy of a mixed broad-needle forest with a canopy density of 0.7. The study plot is flat and drained well. The soil pH was 5 to 7. The cultivation substrate formulas are shown in Table 1. There were 12 mm particles of sawdust, bamboo chips and camellia shells.

Table 1. The Cultivation Substrate Formulas

Groups	Formula
A1	70% sawdust (*Quercus*), 20% rice husk, 8% bran, and 2% lime
A2	100% *Eleusine coracana* (L.) Gaertn straw
A3	70% bamboo chips, 20% rice husk, 8% bran, and 2% lime
A4	40% sawdust (same composition as in A1 sawdust), 30% camellia shells, 20% rice husk, 8% bran, and lime 2%

The ingredients in formula A1, A3, and A4 were added with 2% quicklime, moisturized to 70 to 75%, and fermented in piles for 1 month. The piles were turned once in the middle. A total of 20% rice husk and 8% bran were added sequentially, moisturized to a content of 65 to 75%, and piled in trapezoidal heaps for fermentation. The pile was first turned 3 days after the temperature reached 50 °C. The pile was turned again approximately 2 to 3 days after the temperature had reached 65 ºC, and water was added to rebuild the heap of the same size with a moisture content of 75%. The pile was turned a third time after 3 to 4 days. The fungus was only sown on the substrate when the temperature dropped below 28 °C. No fermentation was conducted on formula A2. Finger millet (*Eleusine coracana*) straw was air-dried and used directly as the substrate. The strain was first sown on Nov 1, 2022. An 8-10 cm thick substrate layer was first spread on a cultivation bed that was 50 to 60 cm wide. The strain was broken into sizes of approximately 2.5 cm, sown on the substrate with spaces of 10 cm, and gently pressed tight. The second layer of substrate was spread 10 to 12 cm thick, and the strain was sown by the same method. Finally, the strain was covered with a third layer of substrate that was 3 to 5 cm thick. The cultivation piles were gently compacted, and the pile surfaces were shaped to resemble the back of a turtle.

On March 15, 2023, 3 kg of well-mixed samples were removed from each cultivation formulation. *S. rugosoannulata* specimens were observed to have a height of 1 to 2 centimeters, stem cap diameter of 4 to 5 centimeters, stem diameter of 1 to 2 centimeters, and stem length of 3 to 4 centimeters. The test samples of three triplicate were dried for 20 h at 40 °C after the surface dirt had been removed and stored at room temperature for the E-nose and GC-IMS analyses (Sun *et al*. 2023).

E-nose Sensing

The PEN3 E-nose (Airsense Analytics Co. Ltd., Schwerin, Germany) contains 10 different metal oxide sensors. The individual sensors of the electronic nose exhibit different selectivities for classes of compounds, such as W1C (aromatic components and benzenes), W5S (nitrogen oxides), W3C (ammonia and aromatic components), W6S (hydrogen), W5C (alkane aromatic components), W1S (short chain alkanes, such as methane), W1W

(inorganic sulfides), W2S (alcohol, ethers, aldehydes and ketones), W2W (aromatic components and organic sulfides) and W3S (long-chain alkanes) (Shen *et al.* 2021). The preparation for the E-nose to detect the aroma profiles included placing each 0.5 g sample in a 15 mL headspace bottle and incubating it in a 26 °C water bath for 30 min before the profiles of the aroma were measured using the following conditions: cleaning time, 120 s; reset time, 5 s; pre-injection time, 5 s; flow rate of carrier gas, 400 mL/min; and measurement time, 60 s. The data were analyzed by a linear discriminant analysis (LDA) using the E-nose software. Three replicates were established for each group of the *S. rugosoannulata* samples.

GC-IMS Analysis

The GC-IMS analysis was conducted on a Flavour Spec® flavor analyzer [FlavourSpec®, GAS (Adelina *et al*. 2021). Gesellschaft fuir analytische Sensorsysteme GmbH, Dortmund, Germany] that consisted of a syringe and an automatic headspace sampling unit. The headspace was sampled by transferring 3.0 g of *S. rugosoannulata* powder to a 20 mL headspace vial and incubating it at 80 °C at 500 rpm for 15 min. A syringe at 85 °C was used to inject a 200 μL headspace sample into a MXT-WAX gas chromatography column (30 m, 0.53 mm ID, 1.0 μm df) (Restek Corporation, Bellefonte, PA, USA) for pre-separation followed by IMS detection (45 °C). The column temperature was 60 °C, and 99.999% N_2 was used as the carrier gas. The initial flow rate (E1) of the carrier gas was 150 mL/min. The E2 was held at 2 mL/min for 5 min, increased to 10 mL/min, held at 10 mL/min for 20 min, increased to 100 mL/min, and then held at 100 mL/min until 30 min (Xi *et al*. 2024). There were three replicates of each group of the *S. rugosoannulata* samples.

Calculation of the ROAV

The contribution of each compound to the flavor of *S. rugosoannulata* was evaluated by the relative odor activity value (ROAV). The OAV_i and $ROAV_i$ were calculated as described by Eqs. 1 and 2,

$$OAV_i = C_i / OT_i \quad (1)$$

$$ROVA_i = OAV_i / OAV_{max} \times 100\% \quad (2)$$

where C_i is the content of a compound /(mg/kg) in the sample; OT_i is the odor threshold (mg/kg) of the compound, and OAV_{max} is the maximum value of OAV of all the compounds in each sample. The compounds of ROAV 0.1 contributed to the overall flavor (Wei *et al.* 2019), while the other compounds contributed less. A larger ROVA represents a greater contribution of the flavor compounds to the overall flavor in the sample (Fan *et al.* 2019).

Statistical Analysis

The data were processed using Microsoft Excel 2019 (Redmond, WA, USA) and SPSS 21.0 (IBM, Inc., Armonk, NY, USA). The LSD analysis method was used for multiple comparison analysis. The OPLS-DA was conducted in the SIMCAP14.1 software. VOCal was used to examine the spectra and qualitatively and quantitatively analyze the data. The compounds detected were identified by searches against the built-in NIST and IMS databases. The spectral differences among the four samples were determined using the Reporter plug-in and visualized as a three-dimensional (3-D) graph, two-dimensional

(2-D) topography and a difference graph. The flavor fingerprints were compared using the Gallery Plot plug-in. The dynamic PCA was conducted using the Dynamic PCA plug-in. The contents of volatile substances were determined as the corresponding normalized relative peak areas (%).

RESULTS AND DISCUSSION

E-nose Detection

A linear discriminant analysis (LDA) maximizes the interclass variance and minimizes the intraclass variance, *i.e.*, it reduces the differences within the class and enhances the differences between different classes (Gerhardt *et al.* 2018; Xu *et al.* 2021). Therefore, an LDA was used to distinguish the four groups of the *S. rugosoannulata* samples. As shown in Fig. 1I, the variance contribution rates of LD1 and LD2 were 92.27% and 6.26%, respectively, and there were relatively large distances among the four samples. In particular, the longest distances were observed between A1 and the other three groups. A2 and A3 were the closest. This may be related to the fact that substrate A1 was primarily composed of sawdust that originated from woody trees, while substrate A4 contained 30% *Camellia oleifera* shells and 40% sawdust. Substrates A2 and A3 were composed of finger millet (*Eleusine coracana*) and bamboo, respectively, which are both members of the Gramineae family. Therefore, the smallest difference was observed between A2 and A3.

As shown in Fig. 1II, W1W was more sensitive to A4 than to A2, A3, and A1, and its responses to A4, A2, and A3 were 2.52-, 2.08-, and 1.65-fold higher than that of A1, respectively. The significantly differential responses suggest that the sensor can clearly distinguish the substrates used to culture *S. rugosoannulata* samples by their odors. The W1W, W5S and W2W sensors showed stronger responses, which indicated that the *S. rugosoannulata* samples, particularly A4, may contain high amounts of organosulfur compounds and nitrogen oxides. A1 had the lowest responses, which suggested that there was a low content of organic sulfide in A1. This was consistent with the low amount of dimethyl trisulfide detected by the GC-IMS (Table 1). There were weak responses from the other sensors, such as the W2S sensor, which is sensitive to alcohols, ethers, aldehydes and ketones. This suggested that the E-nose is only sensitive to a limited number of volatiles in *S. rugosoannulata*, and it can be used as a tool to supplement the GC-IMS analysis.

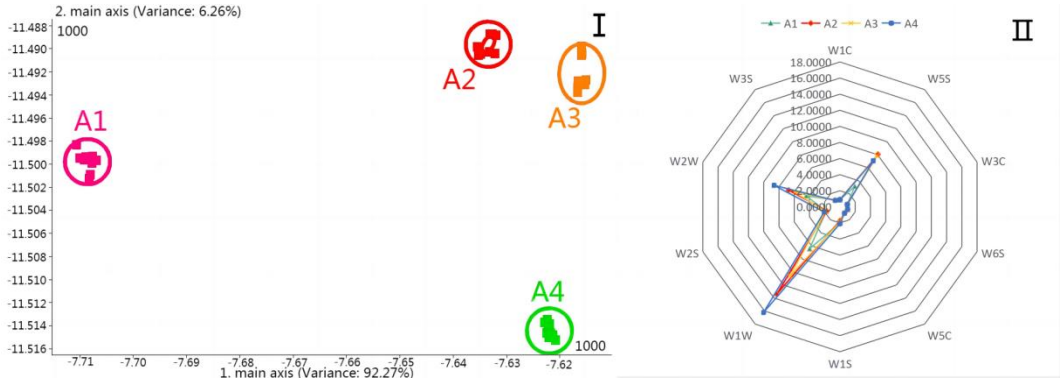

Fig. 1. Linear discriminant analysis (LDA) (I) and E-nose response radar map (II)
Note: The horizontal coordinate in I is the first principal component contribution, and the vertical coordinate is the second principal component contribution.

Characterization of VOCs

Qualitative analysis

The VOCs in the four groups of *S. rugosoannulata* samples were analyzed by GC-IMS. Figure 2 shows the 3-D graph, 2-D topography and difference graph of the ion mobility mass spectra. The qualitative analysis was based on the 2-D separation. Topography that consists of many 2-D maps can serve as the VOC fingerprint of a sample (Gerhardt *et al.* 2018).

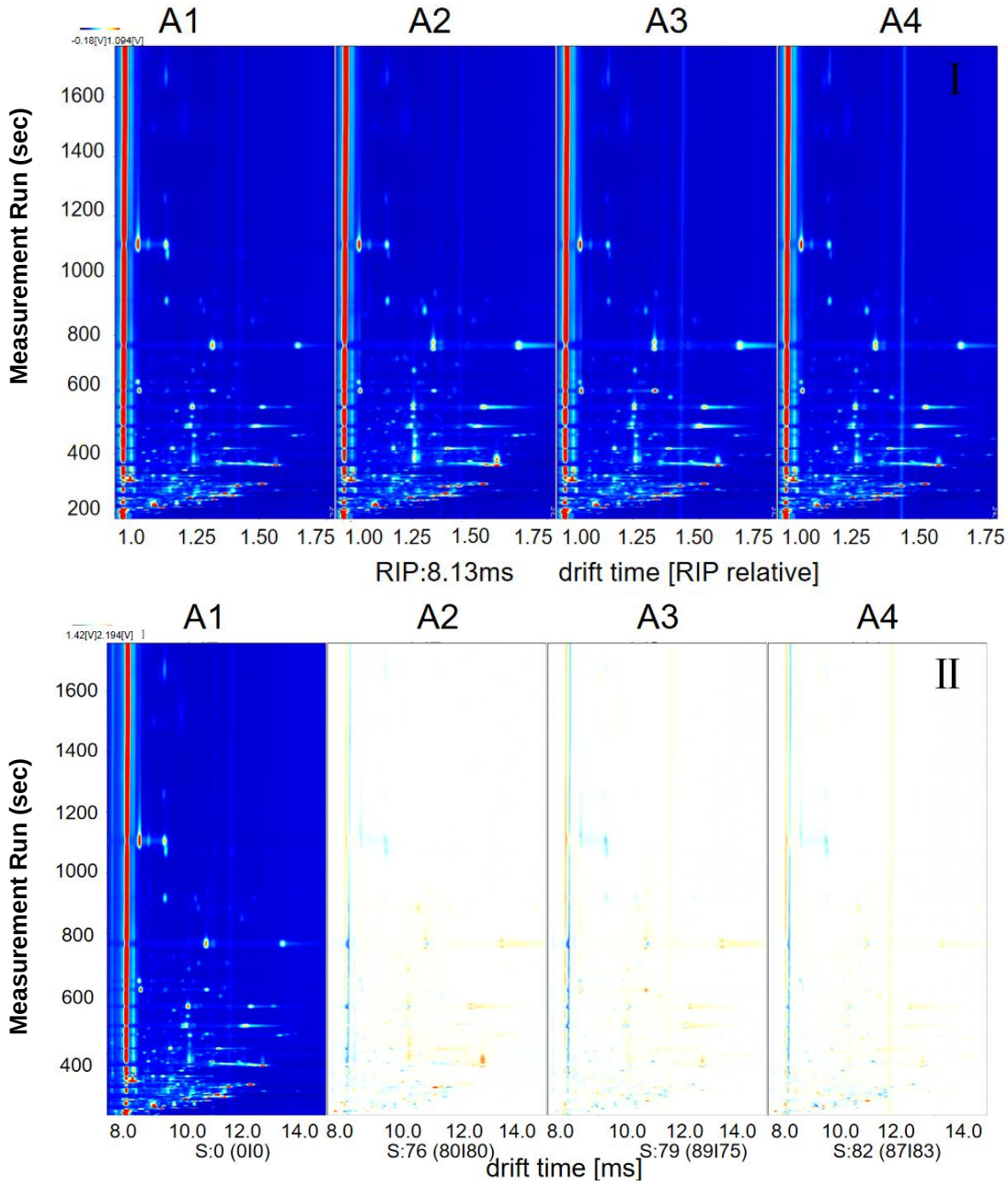

Fig. 2. GC-IMS analysis of volatile flavor substances in the four groups of *S. rugosoannulata* samples. Note: I: Top view of the map; Ⅱ: Difference map. For Ⅰ/Ⅱ, the ordinate, abscissa and vertical lines on the abscissa represent the retention time RT (s), ion migration time Dt (ms), and RIP, respectively.

The four groups showed a similar composition of the VOCs with the differences only observed in the peak intensities of a few components. Their topographies were compared to facilitate the convenience of their observations (Fig. 2I). The ordinate and abscissa axes in Fig. 2I represent the retention time (RT, s) of the GC and ion migration time (Dt, ms). The red line on the abscissa axis is the reaction ion peak (RIP). Each dot on the two sides of the RIP represents a VOC, and a darker dot indicates a higher concentration. The retention and ion migration times of most of the VOCs ranged from 200 to 800 s and 1.00 to 1.75 ms for all the groups, respectively. The differences in their spectra were visualized more directly using the spectrum of A1 as a reference, and the spectra of the other groups were subtracted from the reference to obtain the difference graph as shown in Fig. 2II. Identical contents of a VOC in the two groups resulted in the appearance of the subtraction results in the white background. A red dot indicated that the concentration of VOC was higher than that in the reference, and a blue dot indicated that the VOC concentration was lower than that of the reference. Overall, the composition of the four groups of VOCs in *S. rugosoannulata* were generally similar, and only a few compounds had different peak intensities. A1 differed significantly from the other three groups, and A2 and A 3 were close, which was consistent with the results of the E-nose.

Quantitative Analysis

The VOCs in the headspace samples of the four groups were analyzed by GC-IMS and matched against the built-in NIST and IMS databases. A total of 91 VOCs are detected in each group, which included 82 known VOCs (3 acids, 25 aldehydes, 17 alcohols, 17 esters, 17 ketones, 3 hydrocarbons, 1 furan, and 2 organosulfurs), and 9 unknown compounds (Table 2).

The VOCs in the samples of the four groups were primarily aldehydes, ketones, alcohols and esters, such as propanal[#], 2-methyl propanal, 2-methylbutanal, hexanal[#], acetone, butan-2-one, 2-pentanone, butan-1-ol[#], 1-propanol[#], methyl 3-methylbutanoate[#], and ethyl acetate among others. These compounds were followed by unknown ones, acids, hydrocarbons, furans, and organosulfurs. A1 contained the highest contents of alcohols, acids, hydrocarbons and organosulfurs and the lowest amounts of unknown compounds. A2 had the highest contents of aldehydes, ketones and furans and the lowest contents of alcohols, esters, and hydrocarbons. A3 had the highest contents of esters, while the lowest contents of organosulfurs. A4 had the highest contents of unknown compounds and the lowest contents of aldehydes, ketones, acids and furans. The cellulose content of oil tea husk is 18.6%, hemicellulose content is 49.3%, lignin is 29.7%, saponin content is 4.8%, and tannin content is 2.3% (Zhang *et al*. 2018). The content of its main components and secondary metabolites differed greatly from that of oak (46.0% cellulose, 25.4% hemicellulose, 17.3% lignin) (Li *et al.* 2023) and bamboo (42.5% cellulose, 27.0% hemicellulose, 23.1% lignin) (Zhang *et al.* 2011), and there were few secondary metabolites of saponin and tannin in oak and bamboo. It can be seen that the addition of an appropriate proportion of oil tea husk to the cultivation substrate can affect the growth and metabolism of *S. rugosoannulata* by influencing the production and release of VOCs.

Table 2. GC-IMS Identification Results of VOCs in Four *S. rugosoannulata* Groups

Number	Compounds	CAS	RI	RT（s）	Dt(a.u)	Contents(%)			
						A1	A2	A3	A4
	Acids				Total	3.35±0.00a	3.06±0.00b	3.06±0.00b	2.73±0.00c
1	Butanoic acid	107-92-6	1622.00	1670.97	1.16	0.20±0.04a	0.15±0.02a	0.15±0.02a	0.16±0.04a
2	Acetic acid*	64-19-7	1486.90	1103.76	1.06	2.03±0.07a	2.02±0.03a	2.02±0.03a	1.94±0.08a
3	Acetic acid#	64-19-7	1488.10	1107.79	1.16	1.12±0.10	0.89±0.06	0.89±0.06	0.63±0.15
	Aldehydes				Total	33.82±0.00a	33.89±0.00a	33.89±0.02a	31.46±0.00b
4	Benzaldehyde	100-52-7	1530.30	1261.02	1.16	0.14±0.01a	0.13±0.01b	0.13±0.01b	0.08±0.01c
5	(E)-2-Heptenal*	18829-55-5	1330.50	683.13	1.26	0.19±0.01c	0.52±0.02a	0.52±0.02a	0.34±0.04b
6	Heptanal*	111-71-7	1194.20	467.79	1.33	0.52±0.03	0.64±0.02	0.64±0.02	0.37±0.02
7	Heptanal#	111-71-7	1194.60	468.31	1.70	0.12±0.01b	0.25±0.01a	0.25±0.01a	0.06±0.00c
8	Hexanal*	66-25-1	1100.20	366.72	1.26	1.08±0.06a	1.04±0.04a	1.04±0.04a	1.04±0.05a
9	Hexanal#	66-25-1	1096.70	363.48	1.56	3.59±0.01b	3.77±0.06a	3.77±0.06a	3.75±0.11a
10	2-Methyl-(E)-2-butenal*	497-03-0	1111.10	377.26	1.09	0.89±0.01a	0.38±0.01c	0.38±0.01c	0.47±0.00b
11	2-Methyl-(E)-2-butenal#	497-03-0	1110.80	376.99	1.35	1.74±0.03a	1.63±0.05a	1.63±0.05a	0.57±0.09b
12	Pentanal*	110-62-3	996.70	292.87	1.18	1.25±0.03a	1.22±0.01a	1.22±0.01a	1.00±0.03b
13	Pentanal#	110-62-3	997.80	293.53	1.42	3.50±0.06ab	3.53±0.09a	3.53±0.09a	3.34±0.11b
14	3-Methyl butanal*	590-86-3	958.20	274.93	1.17	0.52±0.03a	0.52±0.1a	0.52±0.1a	0.52±0.01a
15	3-Methyl butanal#	590-86-3	956.10	273.98	1.40	0.73±0.05a	0.38±0.05b	0.38±0.05b	0.30±0.02b
16	2-Methylbutanal	96-17-3	920.70	258.62	1.40	4.01±0.02a	3.70±0.04b	3.70±0.04b	3.99±0.13a
17	Butanal*	123-72-8	885.20	244.07	1.11	0.70±0.02b	0.64±0.03b	0.64±0.03b	1.02±0.07a
18	Butanal#	123-72-8	887.20	244.88	1.28	2.97±0.02c	3.86±0.06a	3.86±0.06a	3.34±0.01b
19	2-Methyl propanal	78-84-2	831.70	223.67	1.28	4.73±0.05a	3.90±0.13c	3.90±0.13c	4.26±0.03b
20	Propanal*	123-38-6	824.60	221.09	1.06	1.21±0.01b	1.24±0.07b	1.24±0.07b	1.45±0.08a
21	Propanal#	123-38-6	823.00	220.55	1.14	5.27±0.02a	5.12±0.19a	5.12±0.19a	4.83±0.03b
22	Nonanal	124-19-6	1401.80	850.22	1.48	0.13±0.02a	0.12±0.01ab	0.12±0.01ab	0.10±0.02b
23	(E)-2-Octenal	2548-87-0	1435.50	942.90	1.33	0.06±0.01b	0.13±0.01a	0.13±0.01a	0.07±0.02b
24	(E)-2-Heptenal#	18829-55-5	1330.20	682.48	1.67	0.03±0.01b	0.10±0.01a	0.10±0.01a	0.04±0.01b

25	(E)-2-Hexenal*	6728-26-3	1226.90	510.58	1.18	0.14±0.01c	0.45±0.01a	0.45±0.01a	0.27±0.03b
26	(E)-2-Hexenal#	6728-26-3	1226.10	509.46	1.52	0.02±0.01b	0.07±0.01a	0.07±0.01a	0.03±0.01b
27	3-Methyl-2-butenal*	107-86-8	1208.90	486.58	1.09	0.25±0.01b	0.45±0.01a	0.45±0.01a	0.16±0.02c
28	3-Methyl-2-butenal#	107-86-8	1209.40	487.14	1.36	0.05±0.01b	0.11±0.01a	0.11±0.01a	0.05±0.01b
	Alcohols				Total	15.40±0.00a	13.76±0.00b	13.76±.002b	14.91±0.00a
29	1-Octen-3-ol	3391-86-4	1477.60	1072.84	1.16	0.51±0.01a	0.21±0.02b	0.21±0.02b	0.16±0.03c
30	(E)-2-Hexen-1-ol*	928-95-0	1426.30	916.57	1.16	0.56±0.05a	0.57±0.03a	0.57±0.03a	0.45±0.07b
31	(E)-2-Hexen-1-ol#	928-95-0	1426.60	917.25	1.53	0.05±0.00ab	0.06±0.01a	0.06±0.01	0.04±0.01b
32	1-Hexanol*	111-27-3	1369.00	768.68	1.33	1.44±0.03a	1.30±0.03b	1.30±0.03b	1.41±0.06a
33	1-Hexanol#	111-27-3	1368.40	767.33	1.64	0.88±0.02b	0.85±0.04b	0.85±0.04b	0.96±0.02a
34	1-Pentanol*	71-41-0	1261.50	560.08	1.26	1.45±0.04a	1.34±0.03b	1.34±0.03b	1.43±0.07ab
35	1-Pentanol#	71-41-0	1260.80	559.06	1.51	1.13±0.01b	1.2±0.03b	1.2±0.03b	1.32±0.07a
36	3-Methyl-1-butanol*	123-51-3	1215.70	495.48	1.24	1.10±0.02a	1.15±0.01b	1.15±0.01b	1.18±0.03a
37	3-Methyl-1-butanol#	123-51-3	1215.30	494.97	1.49	1.33±0.02b	1.11±0.01a	1.11±0.01a	1.26±0.06a
38	Butan-1-ol*	71-36-3	1152.60	419.97	1.18	1.48±0.03a	1.41±0.03b	1.41±0.03b	1.45±0.06a
39	Butan-1-ol#	71-36-3	1152.60	419.97	1.38	0.57±0.02a	0.13±0.01a	0.13±0.01a	0.42±0.02a
40	2-Methyl-1-propanol*	78-83-1	1105.80	372.13	1.17	0.38±0.01a	0.05±0.01c	0.05±0.01c	0.19±0.01b
41	2-Methyl-1-propanol#	78-83-1	1104.70	371.05	1.37	0.73±0.03a	0.69±0.05c	0.69±0.05c	0.75±0.06b
42	1-Propanol*	71-23-8	1049.70	328.42	1.11	1.34±0.03a	1.23±0.02a	1.23±0.02a	1.37±0.06a
43	1-Propanol#	71-23-8	1049.70	328.42	1.25	1±0.04a	1.05±0.02b	1.05±0.02b	1.05±0.06a
44	Butan-2-ol*	78-92-2	1049.90	328.53	1.15	0.31±0.01a	0.34±0.01a	0.34±0.01a	0.31±0.01a
45	Butan-2-ol#	78-92-2	1049.90	328.53	1.35	0.19±0.01b	0.18±0.01a	0.18±0.01a	0.10±0.02b
	Esters				Total	10.51±0.00b	10.3±0.00b	10.3±0.00b	12.78±0.00a
46	(Z)-3-Hexenyl acetate	3681-71-8	1339.40	702.10	1.30	0.25±0.01a	0.19±0.01a	0.19±0.01a	0.52±0.03b
47	Pentyl acetate	628-63-7	1181.60	452.67	1.31	0.27±0.02c	0.06±0.01c	0.06±0.01b	0.38±0.01a
48	Isoamyl acetate*	123-92-2	1132.40	398.61	1.30	0.06±0.01b	0.03±0.01c	0.03±0.01c	0.19±0.03a
49	Isoamyl acetate#	123-92-2	1131.90	398.07	1.75	0.31±0.02b	0.22±0.01c	0.22±0.01c	0.51±0.02a
50	Butyl acetate*	123-86-4	1086.20	355.37	1.24	0.04±0.01b	0.04±0.01c	0.04±0.01c	0.13±0.01a
51	Butyl acetate#	123-86-4	1086.90	355.91	1.62	0.67±0.01b	0.65±0.04b	0.65±0.04b	0.68±0.04a
52	Methyl 3-methylbutanoate*	556-24-1	1010.10	301.46	1.21	3.74±0.04a	4.04±0.10a	4.04±0.1a	3.64±0.06a

53	Methyl 3-methylbutanoate#	556-24-1	1009.30	300.96	1.51	3.46±0.12b	2.68±0.08a	2.68±0.08	4.88±0.10b
54	Ethyl acetate	141-78-6	894.40	247.74	1.34	0.33±0.03a	0.24±0.03b	0.24±0.03b	0.30±0.02a
55	Ethyl isobutyrate	97-62-1	967.40	279.07	1.57	0.25±0.02a	1.23±0.03b	1.23±0.03b	0.16±0.02a
56	Isobutyl acetate	110-19-0	1022.20	309.44	1.23	0.30±0.01b	0.21±0.01a	0.21±0.01a	0.52±0.06c
57	Hexyl acetate*	142-92-7	1283.80	594.54	1.39	0.03±0.00b	0.03±0.00c	0.03±0.00c	0.08±0.02a
58	Hexyl acetate#	142-92-7	1282.60	592.52	1.90	0.13±0.04b	0.08±0.02b	0.08±0.02b	0.14±0.01a
59	Ethyl 3-methylbutanoate*	108-64-5	1080.10	350.73	1.26	0.03±0.01a	0.02±0.00	0.02±0.00b	0.03±0.01a
60	Ethyl 3-methylbutanoate#	108-64-5	1078.80	349.73	1.66	0.12±0.01a	0.13±0.01a	0.13±0.01a	0.32±0.01a
61	Ethyl isobutyrate*	97-62-1	976.30	283.15	1.20	0.33±0.03c	0.27±0.05b	0.27±0.05b	0.19±0.02a
62	Ethyl isobutyrate#	97-62-1	974.00	282.09	1.56	1.03±0.19a	0.82±0.05a	0.82±0.05a	0.52±0.08b
	Ketones				Total	24.57±0.00a	25.12±0.00b	25.12±0.00b	23.29±0.00b
63	1-Hydroxypropan-2-one*	116-09-6	1310.90	643.14	1.06	0.18±0.08a	0.11±0.01a	0.11±0.01a	0.06±0.01b
64	1-Hydroxypropan-2-one#	116-09-6	1311.10	643.65	1.23	1.67±0.03aa	1.62±0.03ab	1.62±0.03ab	1.63±0.05b
65	3-Hydroxybutan-2-one (acetoin)*	513-86-0	1295.70	613.91	1.06	1.02±0.22a	1.01±0.08a	1.01±0.08a	0.88±0.20a
66	3-Hydroxybutan-2-one (acetoin)#	513-86-0	1295.40	613.40	1.33	0.91±0.02a	0.88±0.03a	0.88±0.03a	0.93±0.01a
67	Heptan-2-one*	110-43-0	1190.90	463.69	1.26	0.69±0.02ab	1.11±0.02b	1.11±0.02b	0.61±0.09a
68	Heptan-2-one#	110-43-0	1189.60	462.15	1.63	0.60±0.02b	0.32±0.02a	0.32±0.02a	0.51±0.03b
69	3-Penten-2-one, 4-methyl*	141-79-7	1145.00	411.86	1.12	0.15±0.00a	0.09±0.01c	0.09±0.01c	0.13±0.03b
70	3-Penten-2-one, 4-methyl#	141-79-7	1144.00	410.78	1.45	0.37±0.01a	0.41±0.02b	0.41±0.02b	0.66±0.02a
71	Hexan-2-one*	591-78-6	1139.90	406.45	1.19	0.06±0.00c	0.28±0.01b	0.28±0.01b	0.17±0.01a
72	Hexan-2-one#	591-78-6	1138.90	405.37	1.49	1.24±0.16c	0.59±0.02a	0.59±0.02a	0.53±0.03b
73	2,3-Pentanedione	600-14-6	1075.50	347.26	1.23	4.68±0.05a	5.08±0.03b	5.08±0.03b	4.70±0.06b
74	2-Pentanone	107-87-9	942.20	267.86	1.38	5.34±0.07b	5.80±0.02a	5.80±0.02a	4.99±0.02b
75	Butan-2-one	78-93-3	910.30	254.27	1.25	6.63±0.07b	6.99±0.03a	6.99±0.03a	6.96±0.16c
76	Acetone	67-64-1	839.80	226.66	1.11	0.49±0.03b	0.34±0.01a	0.34±0.01a	0.47±0.04a
	Hydrocarbons				Total	1.71±0.00a	0.86±0.00c	0.86±0.00c	1.54±0.00b
77	(E)-Beta-ocimene	3779-61-1	1252.50	546.75	1.20	0.57±0.02a	0.35±0.01b	0.35±0.01b	0.24±0.00a
78	P-xylene	106-42-3	1139.20	405.64	1.08	0.65±0.02a	0.17±0.02b	0.17±0.02b	0.84±0.05c
79	Alpha-Pinene	80-56-8	1025.30	311.53	1.30	0.54±0.01b	0.71±0.01c	0.71±0.01c	0.32±0.05a

	Furans				Total	0.54±0.00b	0.71±0.00a	0.71±0.00a	0.32±0.00c
80	2-Pentylfuran	3777-69-3	1238.90	527.27	1.25	0.12±0.01b	0.39±0.03a	0.39±0.03a	0.30±0.04c
	Organosulfurs				Total	0.41±0.00a	0.45±0.00a	0.45±0.00a	0.38±0.00a
81	Dimethyl trisulfide	3658-80-8	1414.70	884.52	1.30	0.28±0.06c	0.05±0.02a	0.05±0.02a	0.08±0.01b
82	2,5-Dimethyl thiophene	638-02-8	1185.90	457.74	1.08	1.36±0.04a	1.65±0.08b	1.65±0.08b	1.64±0.01b
	Unknowns				Total	9.69±0.00c	11.86±0.00b	11.86±0.00b	12.59±0.00a
83	Unknown1					1.90±0.22b	0.70±0.02a	0.70±0.02a	3.12±0.10a
84	Unknown2					2.02±0.01b	2.08±0.08c	2.08±0.08c	1.45±0.10a
85	Unknown3					1.61±1.05a	2.02±0.01a	2.08±0.08a	2.08±0.08b
86	Unknown4					0.80±0.04c	3.43±0.06a	3.43±0.06a	1.56±0.09b
87	Unknown5					0.83±0.02b	0.83±0.02b	0.83±0.02b	1.10±0.02a
88	Unknown6					0.22±0.01b	0.41±0.01a	0.41±0.01a	0.17±0.02c
89	Unknown7					1.26±0.04b	1.43±0.03a	1.43±0.03a	1.37±0.06a
90	Unknown8					1.02±0.03b	1.05±0.03b	1.05±0.03b	1.70±0.02a
91	Unknown9					0.26±0.03b	0.28±0.06b	0.28±0.06b	0.46±0.09a
	Total					100.00	100.00	100.00	100.00

Note: "*" is for monomer; "#" is for dimer. Different lowercase letters in the same column indicate intergroup $P<0.05$.

Figure 3 shows how the VOCs fingerprint visually presents the differences in the VOCs of the four groups. The background of the figure was blue. Each row contained all the signal peaks of the selected sample, and each column represented the signal peaks of the same VOC in the different samples. The depths of color and areas of the dots reflected the contents of VOCs. A darker color and larger dot area represented a higher content of the VOC. The relative contents of VOCs in the four groups were consistent with those listed in Table 1.

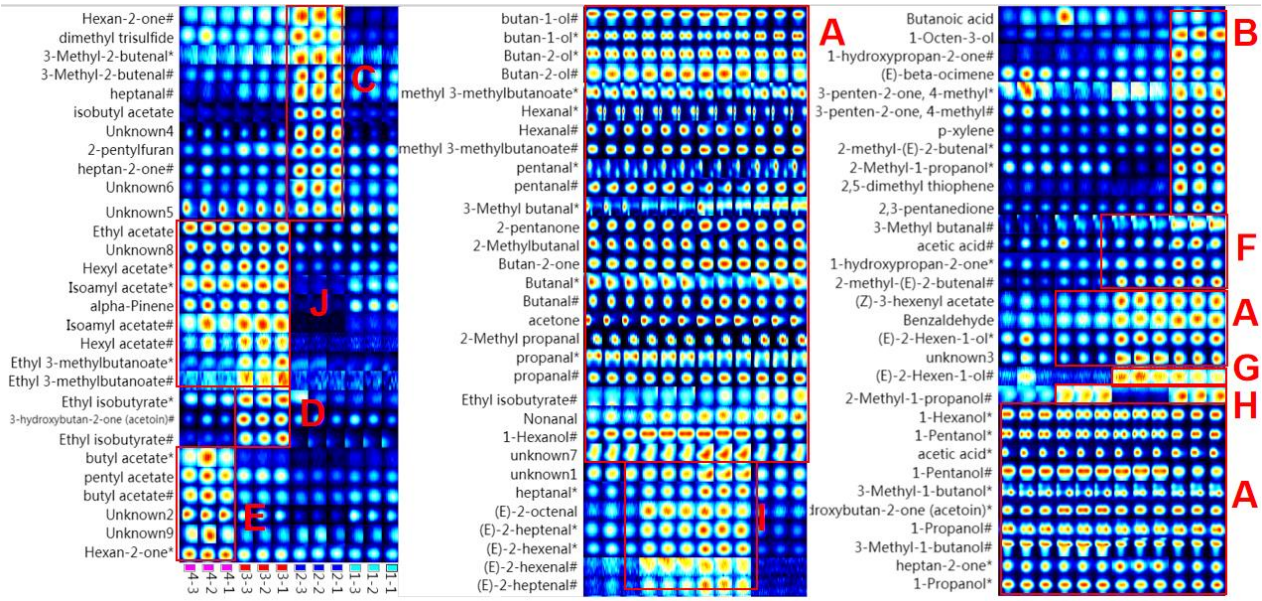

Fig. 3. Gallery plot of VOCs (fingerprint); Note: Each row represents all the signal peaks selected in a sample; each column represents the difference of the signal peaks of the same substance in different samples. The darker the colour indicates the higher concentration of the substance and the stronger the signal peaks.

The VOCs primarily included alcohols, acids, ketones, and esters among others (Zhang *et al.* 2016; Nn *et al*. 2021; Zhang *et al.* 2022). There was no significant difference in the contents of VOCs of the four groups in region A, including 1-pentanol[#,*], 1-propanol[#,*], butan-1-ol[#,*], and butan-2-ol[#,*], which are then considered as the primary VOCs of *S. rugosoannulata.* The VOCs in region B included butanoic acid, 1-octen-3-ol, 1-hydroxypropan-2-one[#], (E)-beta-ocimene, 3-penten-2-one, 4-methyl[#,*] and *p*-xylene, which were the highest components in A1, and there were very low contents in the other three groups. The contents of the VOCs in region C, including hexan-2-one[#], dimethyl trisulfide, 3-methyl-2-butenal[#,*], heptanal[#], isobutyl acetate, 2-pentylfuran, and heptan-2-one, were found to be the highest in A2 and present at very low concentrations in the other three groups. A3 shows the highest contents of the ethyl isobutyrate[#,*] and 3-hydroxybutan-2-one (acetoin)[#] in region D, and they were found at very low levels in the other three groups. Region E had the highest contents of the VOCs, including butyl acetate[#,*], pentyl acetate and hexan-2-one*, that were found at the highest levels in A4 and at very low levels in the other three groups. The contents of the VOCs in region F, including 3-methyl butanal[#], acetic acid[#], 1-hydroxypropan-2-one*, and 2-methyl-(E)-2-butenal[#], were higher in A1, A2 and A3 than in A4. (E)-2-Hexen-1-ol[#] was present at much higher contents in region G in both A1 and A2 than in A3 and A4, while there was a much higher content of 2-methyl-1-propanol[#] in region H in A1 and A3 than in the other two groups.

The contents of the VOCs in region 1, including (E)-2-octenal, (E)-2-heptenal[#,*] and (E)-2-hexenal[#,*], were higher in A2 and A3 than in A1 and A4, while the contents of those in region J, including ethyl acetate, hexyl acetate[#,*], isoamyl acetate[#,*], alpha-pinene, and ethyl 3-methylbutanoate[#,*], were higher in A3 and A4. Hexanal[#,*], pentanal[#,*], 3-methyl butanal[#,*], butanal[#,*], 1-hexanol[#,*], 1-pentanol[#,*], 2-methyl-1-propanol[#,*], 1-propanol[#,*] and heptan-2-one*, are also found in wild matsutake (*Tricholoma matsutake*) (Li *et al.* 2023), and similar levels of these chemicals were found in the four groups of *S. rugosoannulata* cultivated in this study.

ROVA (Relative Odor Activity Value) Analysis

The contribution of the volatile components to the overall flavor depends on their concentration and threshold. It is not accurate to describe the contribution of the volatile components. Therefore, to clarify the contribution of each component of volatile flavor to the flavor overall, the composition of flavor in the giant bulb mushroom was analyzed by ROAV (Zhou *et al.* 2018). ROAV is a method based on the sensory thresholds and relative odour activity values of compounds, which is used to quantitatively evaluate the extent to which different compounds contribute to the overall flavour of a food product, and thus to identify the key flavour compounds. Components with a ROAV value >1 are important modifiers of flavour. There were 25 volatile compounds in four formulations with ROAV> 1, and their detailed results are shown in Table 3.

Multivariate Statistical Analysis of the VOCs

To further analyze the differences in volatile flavor of the different matrix mushrooms, the electronic nose data were subjected to a PCA. As shown in Fig. 4I, the contribution of PC1 was 43%, PC2 35%, and the cumulative contribution of the two principal components was 78%, which indicated that the analytical results can effectively reflect the overall information of the flavor of the four groups of samples. The intensities of the VOCs peaks shared by all four groups were processed in the SIMCA-P 14.1 software for the OPLS-DA analysis. Figure 4II and 4III shows the corresponding cross-validation plot, OPLS-DA results and variable importance in projection (VIP) values. The cross-validation analysis of OPLS-DA model produced R^2_Y=0.96, which suggested that the model effectively explained the VOCs. Q^2=0.92 and the Q^2 intercept of PCA < 0, which indicated that the model was accurate; there was no overfitting, and the PCA was highly reliable.

Table 3. The ROAV of Four Substrates

	Compound	Threshold (μg/kg)	ROVA				Odor description
			A1	A2	A3	A4	
1	Benzaldehyde	0.015	8.90	7.97	6.43	5.02	Almond, fruity
2	(E)-2-Heptenal	0.05	3.91	10.37	9.20	6.83	Fatty taste
3	Heptanal	0.0648	1.75	3.84	1.76	0.93	Green incense, fruital
4	Hexanal	0.08	13.49	12.99	13.41	13.01	Green, fat and apple flavour
5	2-Methyl-(E)-2-butenal	0.4	2.23	0.95	1.07	1.18	Green incense
6	Pentanal	0.24	5.19	5.08	4.41	4.18	Wood and fruit aromas
7	3-Methyl butanal	0.0136	37.98	38.29	26.30	38.28	Green and cocoa aroma
8	2-Methyl butanal	0.0843	47.55	43.97	48.42	47.38	Fruity, green, cocoa notes
9	Butanal	0.028	24.84	22.86	31.26	36.47	Fruital
10	2-Methyl propanal	0.086	54.94	45.32	50.49	49.47	Meaty, fruital
11	Nonanal	0.0025	50.81	49.62	46.94	39.04	Lipstick, citrus, rose
12	(E)-2-Octenal	0.003	20.45	42.82	42.47	22.59	Fatty flavour
13	(E)-2-Hexenal	0.004	4.61	16.38	16.62	8.55	sweet smell of incense
14	3-Methyl-2-butenal	0.007	36.25	64.89	25.63	23.21	Green incense, floral fragrance
15	1-Octen-3-ol	0.031	16.49	6.79	5.93	5.05	Mushroom and green aroma
16	1-Hexanol	0.3807	2.32	2.25	1.95	2.54	Floral, fatty flavour
17	Butan-1-ol	0.038	34.92	29.16	31.37	33.32	fragrance of flowers
18	Butan-2-ol	0.5	2.01	2.10	2.05	2.10	Floral, rose scent
19	Isoamyl acetate	0.0222	2.88	1.20	10.17	8.43	Fruital
20	Butyl acetate	0.183	1.73	1.22	1.48	2.79	Green, sweet and fruity aroma
21	Ethyl isobutyrate	0.0052	63.17	46.13	44.10	58.77	Sweet aroma, fruity flavour
22	Hexyl acetate	0.067	4.52	3.12	8.88	7.76	Fruity, tallowy
23	Ethyl 3-Methyl butanoate	0.0022	13.93	9.25	30.25	10.95	Sweet and fruity
24	Ethyl isobutyrate	0.0052	22.57	26.18	84.10	61.13	fruity flavour
25	3-Penten-2-one, 4-methyl	0.3	2.00	1.06	1.15	1.71	—
26	Acetone	0.832	7.97	8.41	8.23	8.37	*Saussurea costus*
27	Alpha-Pinene	0.274	2.39	0.63	2.66	3.06	Turpentine
28	2-Pentylfuran	0.1	5.42	7.09	4.79	3.21	Botanical aroma, nutty

The four groups were scattered in four quadrants with significant differences between the groups (Fig. 4II). A1 and A4 belong to the first and three quadrants, that is, the *Camellia oleifera* shell in the cultivation material had a great influence on the VOCs content of *S. rugosoannulata*. A2 and A3 belong to the two and fourth quadrants, indicating that *E. coracana* and bamboo chips, as well as Gramineae, had relatively little effect on the odor of *S. rugosoannulata* (Fig. 1II), but they had a great impact on the VOCs of *S. rugosoannulata*. However, the distance between A3 and A4 was similar. That is, the VOCs of the two groups of *S. rugosoannulata* were slightly different, indicating that the influence of the main cultivation material on the VOCs of *S. rugosoannulata* can be reduced by careful formation of cultivation substrate.

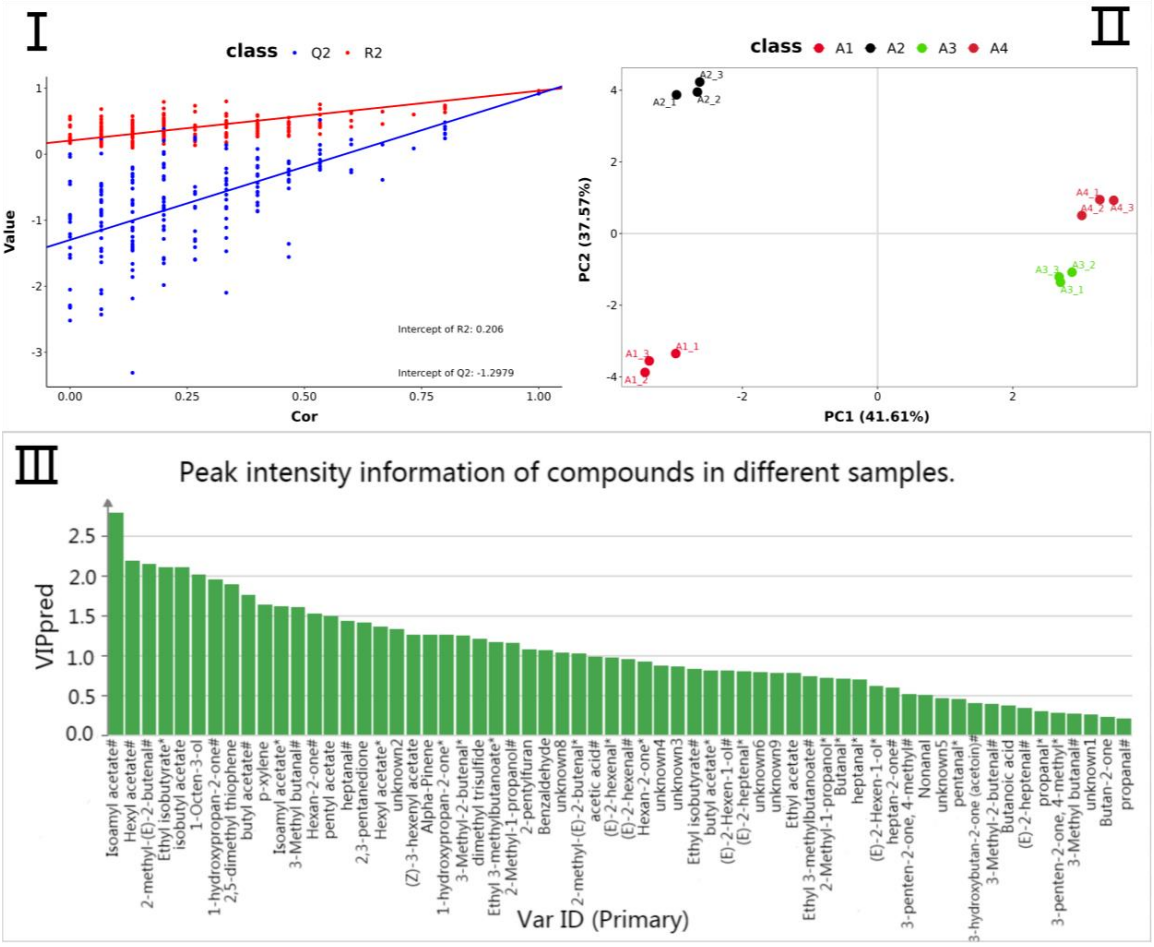

Fig. 4. Multivariate statistical analysis of four groups of *S. rugosoannulata.* Note: Ⅰ: Cross validation of OPLS-DA model of four groups of *S. rugosoannulata*; Ⅱ: Scatter plot of OPLS-DA fraction of four groups of *S. rugosoannulata*; Ⅲ: OPLS-DA VIP value map of four groups of *S. rugosoannulata*.

Based on the criterion of VIP > 1 and the levels of significant differences in the volatile components between the different samples ($P < 0.05$), the key compounds that differentiated the flavors of *S. rugosoannulata* cultivated with different raw materials were screened. Among the 82 VOCs identified by GC-IMS, 29 had a VIP > 1 in *S. rugosoannulata* cultivated with the four different substrates. A total of 28 had statistically significant differences among their contents in the different groups ($P < 0.05$). Among

them, eight VOCs, including benzaldehyde, heptanal#, 2-methyl-E-2_butenal-M#, 3-methyl-2-butenal-M#'', 1-octen-3-ol#, butyl acetate, ethyl 3-methylbutanoate*, and 2-pentylfuran, met both criteria and could thus be used as the key components to distinguish the differences in the flavors of *S. rugosoannulata* cultivated with different raw substrates. The most significant difference among the samples was that of isoamyl acetate# with a VIP of 2.80 (Fig. 4III). Its contents were at significant levels in the order A3 > A4 > A1 > A2. In particular, the contents in A3, A4 and A1 were 8.57-, 6.36- and 2.48-fold those of A2, respectively. Therefore, isoamyl acetate# can be used as an indicator to distinguish the samples of *S. rugosoannulata* cultivated with the four substrates. Hexyl acetate# (VIP=2.20), 2-methyl-(E)-2-butenal# (VIP=2.16), ethyl isobutyrate* (VIP=2.12), isobutyl acetate (VIP=2.11) and 1-octen-3-ol (VIP=2.02) with VIP values > 2 can also serve as indicators to distinguish between these substrates.

In sum, the VOCs in *S. rugosoannulata* cultivated on four substrates showed certain similarities, and thus, they exhibited some similar characteristics of odors. However, the cultivation substrates significantly affected the contents of VOCs, which caused significant differences in the odors and volatile flavor compounds of *S. rugosoannulata*. Therefore, the odors and volatile flavor compounds of *S. rugosoannulata* cultivated in different substrates varied substantially. Further cultivation can be conducted with mixed substrates to verify the influence of the contents of key aroma compounds in the substrate on the characteristics of the odors *S. rugosoannulata*. In future studies, we can investigate important issues such as the potential correlation between volatile organic compounds or alcohols released by *S. rugosoannulata* and the constituent elements of the various substrates utilized.

CONCLUSIONS

1. The volatile organic compounds (VOCs) in the *Stropharia rugosoannulata* cultivated on four different substrates were analyzed using an E-nose and gas chromatograph with ion mobility spectrometry (GC-IMS). Their differences were attributed to the substrates used for their cultivation.
2. The E-nose results showed that the composition of the VOCs of *S. rugosoannulata* differed significantly when the fungus was cultivated on different substrates.
3. The flavor of the A4(40% sawdust, 30% camellia shells, 20% rice husk, 8% bran, and lime 2%) substrate was richer than that of the other substrates. The aroma profile of A1(70% sawdust, 20% rice husk, 8% bran, and 2% lime) differed greatly from those of other groups, and those of A2 (100% *Eleusine coracana* (L.) Gaertn straw) and A3(70% bamboo chips, 20% rice husk, 8% bran, and 2% lime) were similar.
4. GC-IMS analysis suggested that all the groups of *S. rugosoannulata* contained high amounts of aldehydes, ketones, alcohols and esters with a total content > 80%, and the VOC fingerprint can effectively distinguish the *S. rugosoannulata* cultivated on the four substrates.

5. The multivariate statistical analyses, primarily the orthogonal partial least squares discriminant analysis (OPLS-DA), revealed that there were significant differences among the four groups. In all, the VOCs in *S. rugosoannulata* cultivated on four substrates showed certain similarities, and thus, they exhibited some similar characteristics of odors.
6. The results of the study showed that more unknown volatile flavor substances were found in the mushrooms cultivated in the substrate with added oil tea husk, which was related to its high content of secondary metabolites tannins and saponins, so the addition of raw materials with high content of secondary metabolites should be minimized in the substrate for mushroom cultivation.

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