Analysis of Volatile Components in Different *Ophiocordyceps sinensis* and Insect Host Products

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Abstract: The artificial production of *Ophiocordyceps sinensis* mycelia and fruiting bodies and the Chinese cordyceps has been established. However, the volatile components from these *O. sinensis* products are not fully identified. An efficient, convenient, and widely used approach based on headspace solid-phase microextraction (HS-SPME) combined with comprehensive two-dimensional gas chromatography and quadrupole time-of-flight mass spectrometry (GC×GC-QTOFMS) was developed for the extraction and the analysis of volatile compounds from three categories of 16 products, including *O. sinensis* fungus, *Thitarodes* hosts of *O. sinensis*, and the Chinese cordyceps. A total of 120 volatile components including 36 alkanes, 25 terpenes, 17 aromatic hydrocarbons, 10 ketones, 5 olefines, 5 alcohols, 3 phenols, and 19 other compounds were identified. The contents of these components varied greatly among the products but alkanes, especially 2,5,6-trimethyldecane, 2,3-dimethylundecane and 2,2,4,4-tetramethyloctane, are the dominant compounds in general. Three categories of volatile compounds were confirmed by partial least squares-discriminant analysis (PLS-DA). This study provided an ideal method for characterizing and distinguishing different *O. sinensis* and insect hosts-based products.

Keywords: *Ophiocordyceps sinensis*; *Thitarodes* hosts; Chinese cordyceps; volatile compounds; HS-SPME GC×GC-QTOFMS; partial least squares-discriminant analysis

1. Introduction

The Chinese cordyceps, a parasitic *Ophiocordyceps sinensis* fungus-*Thitarodes/Hepialus* caterpillar complex endemic only at an elevation of 3000–5000 m in the Tibetan plateau, is a valuable health food and medicinal herb [1]. Modern pharmacological studies indicate that the Chinese cordyceps is good for human circulatory, immune, hematogenic, cardiovascular, respiratory, and glandular systems [2–4]. Due to the limited distribution, high cost, and over exploitation, the natural Chinese cordyceps is extremely expensive and not satisfactory for market demand [5]. Therefore, artificial cultivation is needed to satisfy the natural resource protection and human comsumption.

The artificial cultivation of *O. sinensis* fruiting bodies on rice media [6] and host caterpillar *Thitarodes* spp. [7,8] has been established. Mycelial products of *O. sinensis* fungus have also been manufactured by fermentation technology [9]. More excitingly, the success of cultivation on a large scale has been achieved recently in China [10,11].

Several main bioactive compounds were detected in natural and cultured Chinese cordyceps and fermented fungal products, such as nucleosides (adenosine and inosine), carbohydrates (mannitol, trehalose, and polysaccharides), sterols (ergosterol), and sphingolipids, etc. [12–15]. Some free fatty acids and sterols in natural and cultured Chinese cordyceps were determined by one-step derivatization and GC/MS [16].

From the mycelia of *O. sinensis* cultured with solid-state media and submerged fermentation, 51 volatile compounds were identified, and there is a great difference in the numbers of compounds in the two mycelia, but phenols, acids, and alkanes were the major classes of compounds, while butylated hydroxytoluene was the most abundant volatile compound in both mycelia [17]. The volatile components in several commercial fermentation products from mycelial strains isolated from natural Chinese cordyceps were also analyzed, and 5,6-Dihydro-6-pentyl-2*H*-pyran-2-one (massoia lactone) was discovered as the dominant component in the essential oils of Jinshuibao capsule volatiles, and fatty acids including palmitic acid (C16:0) and linoleic acid (C18:2) were also found to be major volatile compositions of the fermentation products [18]. It seems that products from different cultivation methods may exhibit different volatile components. However, a comprehensive volatile profiling from natural and artificially cultivated Chinese cordyceps is unknown.

The Green Analytical Chemistry technique of headspace solid-phase microextraction (HS-SPME) coupled with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS) proves to be a sensitive, accurate, efficient, and convenient approach for volatile compounds analysis [19–24]. HS-SPME can extract chemicals directly from sample headspace for volatile compounds analysis. It is well suitable for volatile sampling by the advantages of ease of automation, solvent-free procedure, high preconcentration capacity, little manipulation of the sample, and high cost-efficiency [19–21,24,25]. GC×GC is a powerful technique with high resolution and enhanced sensitivity for separating and analyzing complex samples [19–21,24–27]. In addition, with the strengths in accurate mass measurements and good sensitivity in full-scan acquisition mode of TOFMS, GC×GC-TOFMS becomes an increasingly popular analytical technique for characterization of the chemical compositions of biological samples [19–21,24,27]. Recently, the combination of HS-SPME and GC×GC-TOFMS has been applied to volatile analysis in many fields [19–21,24]. However, there is no report of the simultaneous analysis of volatile components in different *O. sinensis* and insect hosts-based products by this method.

In the present work, HS-SPME and GC×GC-QTOFMS were employed to analyze volatile compounds from three categories of samples, including *O. sinensis* fungus, insect hosts of *O. sinensis*, and the Chinese cordyceps. Qualitative analysis was performed by comparing the mass spectra with the library and confirmed by their retention indices and fragmentation patterns. In addition, the three categories (*O. sinensis* fungus, *Thitarodes* hosts of *O. sinensis*, and the Chinese cordyceps) of samples were comparatively analyzed and differentiated using this method combined with multivariate partial least squares-discriminant analysis (PLS-DA).

2. Results and Discussion

2.1. Comparison of 1-DGC and GC×GC

Using the technique of HS-SPME combined with GC×GC-QTOFMS, unknown analytes can be identified and quantified in one GC injection. To compare the techniques of GC-MS and GC×GC-MS, a quality control sample (mix of each collected sample) was analyzed by both techniques under the same chromatographic conditions as described by Xiang et al. [19]. The result shows that the number of detected peaks as well as the chromatographic response in the GC×GC-MS chromatogram significantly increase compared to that of the GC-MS (Figure 1). With the high chromatographic resolution, many overlapped peaks by 1-DGC were resolved by GC×GC. In the quality control sample, the higher resolving power of GC×GC is visibly demonstrated by the constituent D-limonene (peak 3), which appears as a single overlapped peak from 1D-GC (Figure 1A) and can be separated into seven individual peaks by GC×GC (Figure 1B), namely 2,5,9-trimethyldecane, 2,2,4,4-tetramethyloctane, o-cymene, 2,4-dimethyl-2,3-heptadien-5-yne, 3-octene-2-one, and 2,3-dihydropyran-6-one (peaks 1, 2, 4, 5, 6, 7, respectively); these compounds are separated only in the second dimension. The results revealed that the volatile components of *O. sinensis* and insect host products were complex and required GC×GC for complete characterization; GC×GC-MS has a superior sensitivity and resolution, providing an

efficient and convenient approach for studying the volatile compounds of these products. Furthermore, the present method is automated and meets the requirement of the principles of green analytical chemistry, such as solvent-free sampling, small amounts of reagents, hermetic sealing of analytical process, reduced waste, and less time consumption [22,23].
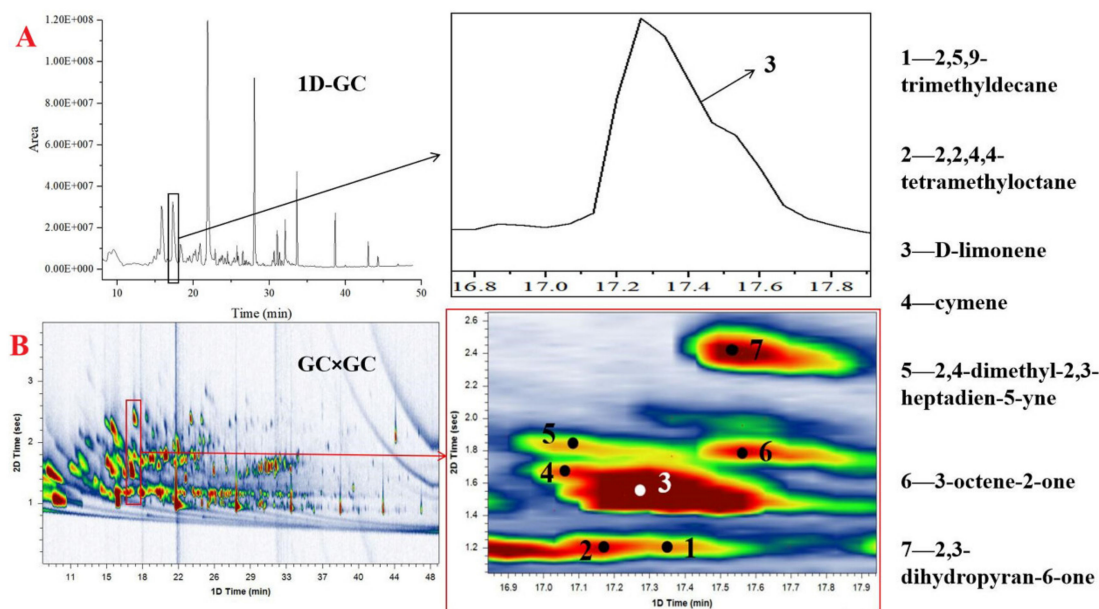

Figure 1. Comparison of an expanded region of the 1D GC (**A**) and the same region from GC × GC (**B**) chromatograms of volatile components from a quality control sample. Compounds identification: (1) 2,5,9-trimethyldecane; (2) 2,2,4,4-tetramethyloctane; (3) ᴅ-limonene; (4) o-cymene; (5) 2,4-dimethyl-2,3-heptadien-5-yne; (6) 3-octene-2-one; (7) 2,3-dihydropyran-6-one.

2.2. Identification of Volatile Components

Component identification was achieved by matching the QTOFMS spectral with a commercial mass spectral library (NIST 17), with a minimum match factor of 800. The current quantitative method was consistent with similarly reported references [25,26], and the qualitative data of volatile components in different analyzed products (Table 1) with their peak area percentages are presented in Table 2. A total of 120 volatile compounds were detected in all samples with various concentration levels. A total of 107, 101, 71, 70, 89, 89, 113, 103, 105, 107, 102, 99, 40, 45, 82, and 69 compounds (Figure 2A) were identified in the products of W, A, A0, A1, A2, A3, FB, FC20d, FC40d, FC60d, FC80d, FC100d, Tx-LU, Tx-LI, Tx-PU, and Tx-PI (Table 1), accounting for 95.70%, 88.14%, 91.96%, 89.50%, 90.59%, 90.73%, 93.42%, 91.08%, 89.84%, 87.04%, 91.54%, 91.45%, 87.10%, 90.15%, 87.63%, and 91.81% of the total peaks areas, respectively. The identified compounds included 36 alkanes, 25 terpenes, 17 aromatic hydrocarbons, 10 ketones, 5 olefines, 5 alcohols, 3 phenols, and 19 other compounds. The volatile compound amounts showed great variation in different samples and ranged from 40 compounds in Tx-LU to 113 compounds in FB. In general, natural and mature artificial Chinese cordyceps (fungus–insect complexes), fruiting bodies, and fermented products had more volatile compounds than insect larvae, insect pupae and immature artificial Chinese cordyceps.

Table 1. Information.

Sample Code	Sample Description
W	Natural Chinese cordyceps from Kangding, Sichuan, China
A	Artificial cultured Chinese cordyceps by Liyuan Co., Ltd., Guangzhou
A0	The mummified *Thitarodes* larvae before stroma development
A1	Artificial cultivated Chinese cordyceps with fruiting bodies (lengths about 1 cm)
A2	Artificial cultivated Chinese cordyceps with fruiting bodies (lengths about 2–3 cm)
A3	Artificial cultivated Chinese cordyceps with fruiting bodies (lengths about 4–5 cm)
FB	Fruiting bodies of *Ophiocordyceps sinensis* on rice–wheat medium
FC20d	Fermented culture in PM medium (200 g potato extract, 20 g maltose, 10 g peptone, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 20 mg vitamin B1, and 1000 mL distilled water) [11] supplemented with 0.5% *Galleria mellonella* larvae for 20 days, at 11 ± 2 °C
FC40d	Fermented culture of *Ophiocordyceps sinensis* in PM medium [11] supplemented with 0.5% *Galleria mellonella* larvae for 40 days, at 11 ± 2 °C
FC60d	Fermented culture of *Ophiocordyceps sinensis* in PM medium [11] supplemented with 0.5% *Galleria mellonella* larvae for 60 days, at 11 ± 2 °C
FC80d	Fermented culture of *Ophiocordyceps sinensis* in PM medium [11] supplemented with 0.5% *Galleria mellonella* larvae for 80 days, at 11 ± 2 °C
FC100d	Fermented culture of *Ophiocordyceps sinensis* in PM medium [11] supplemented with 0.5% *Galleria mellonella* larvae for 100 days, at 11 ± 2 °C
Tx-LU	Uninfected L5-L6 instar larvae of *Thitarodes xiaojinensisi*
Tx-LI	Infected L5-L6 instar larvae of *Thitarodes xiaojinensisi* with blastospores of *Ophiocordyceps sinensis* fungus
Tx-PU	Uninfected pupae of *Thitarodes xiaojinensisi*
Tx-PI	Infected pupae of *Thitarodes xiaojinensisi* with blastospores of *Ophiocordyceps sinensis* fungus

Twenty-four volatile compounds were identified in all the 16 samples, including 4-carene (C10), 3-carene (C18), 2,2,4,4-tetramethyloctane (24), 2-propyltoluene (C29), 2,4,6-trimethyldecane (C31), 2,6-dimethyl-6-trifluoroacetoxyoctane (C34), linalool (C41), 2-nonen-1-ol (C42), 6-ethyl-2-methyloctane (C44), (+)-α-terpineol (C61), 2,6-dimethylundecane (C65), 2,8-dimethylundecane (C68), 4-methyldodecane (C73), 4,7-dimethylindan (C75), 2,3-dimethylundecane (C76), 2,4-dimethyldodecane (C77), 2,6,11-trimethyldodecane(C78), 4,6-dimethyldodecane (C84), 2,3,5,8-tetramethyldecane (C86), farnesane (C94), tetradecane (C97), seychellene (C108), 2,6-di-tert-butyl-4-methyl-p-quinol (C109), and hexadecane (C118). The mutual volatile compounds accounted for 40.19%, 41.24%, 51.44%, 48.59%, 50.58%, 41.86%, 38.44%, 48.04%, 48.31%, 47.03%, 44.37%, 48.49%, 64.94%, 68.73%, 49.13%, and 40.87% of the total volatile compounds in W, A, A0, A1, A2, A3, FB, FC20d, FC40d, FC60d, FC80d, FC100d, Tx-LU, Tx-LI, Tx-PU, and Tx-PI, respectively. With the method of simultaneous distillation-extraction (SDE) and GC-MS, 17 and 42 volatile compounds were identified in the mycelia of *O. sinensis* from solid-state media and submerged fermentation, respectively [17]; in Bailing capsule and Zhiling capsule, the commercial fermentation products of *O. sinensis* mycelia, 39 and 56 volatile compounds were identified, respectively [18]. While by the technique of HS-SPME combined with GC×GC-QTOFMS in this study, 99–107 volatile compounds were identified from the fermentation cultures of *O. sinensis* mycelia, indicating the superior sensitivity and resolution of the present method.

Table 2. Volatile components identified in different products.

NO.	Compound	Class	Retention Time (min)	Formula	RI	Peak Area Percentage (%)															
						W	A	A0	A1	A2	A3	FB	FC20d	FC40d	FC60d	FC80d	FC100d	Tx-LU	Tx-LI	Tx-PU	Tx-PI
C1	3-Prop-2-enylidenecyclobutene	Olefine	8.50	C_7H_8	804	0.00	0.52	0.57	1.04	0.53	0.35	0.53	3.84	0.32	1.39	0.13	0.43	0.14	1.22	0.00	0.42
C2	2-Methyl cyclopentanol	Alcohol	8.90	C_6H12O	815	1.00	2.14	2.20	1.21	1.82	1.83	10.80	0.00	1.16	1.21	0.00	0.54	0.41	0.57	0.82	0.91
C3	Methyl carbamate	Others	9.23	$C_2H_5NO_2$	824	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00
C4	4-Methyl pyrimidine	Others	9.83	$C_5H_6N_2$	842	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.15	0.25	0.00	0.07	0.00	0.00	0.17	0.00
C5	o-Xylene	Aromatic hydrocarbon	11.23	C_8H_{10}	881	2.15	2.65	1.57	2.05	1.51	2.86	3.68	0.00	1.04	0.47	0.00	0.35	1.57	3.72	3.93	5.09
C6	2,2-Dimethoxy ethanol	Alcohol	11.57	$C_4H_{10}O_3$	890	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C7	N-methoxy formamide	Others	12.03	$C_2H_5NO_2$	903	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00
C8	2-Methyl cyclopentanone	Ketone	12.03	$C_6H_{10}O$	904	0.07	0.04	0.14	0.02	0.32	0.11	0.05	0.65	1.00	0.95	0.83	0.74	0.00	0.00	0.18	0.00
C9	4,6-Dimethyl pyrimidine,	Others	12.97	$C_6H_8N_2$	928	0.05	0.04	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.19
C10	4-Carene	Terpenes	13.57	$C_{10}H_{16}$	943	4.52	0.02	0.14	0.08	0.13	0.04	0.03	0.04	0.15	0.08	0.06	0.05	1.13	1.09	0.61	0.85
C11	(2,5-Dimethyloxan-2-yl) methanol	Alcohol	14.83	$C_8H_{16}O_2$	976	0.50	0.53	0.25	0.28	0.27	0.18	0.57	0.44	0.72	0.87	0.40	0.47	0.00	0.00	0.90	0.16
C12	1-Phenyl-1,2-propanedione	Ketone	14.77	$C_9H_8O_2$	975	0.94	0.76	1.27	0.13	0.10	1.55	1.52	0.23	0.29	0.34	0.01	0.20	0.00	0.00	1.71	1.85
C13	Sabinene	Terpenes	15.03	$C_{10}H_{16}$	981	0.00	0.02	0.08	0.09	0.02	0.08	0.02	0.56	0.63	1.10	0.02	0.36	0.00	0.00	0.00	0.00
C14	6-Ethenyl-2,2,6-trimethyloxan-3-one	Ketone	15.30	$C_{10}H_{16}O_2$	988	0.45	0.51	0.46	0.18	0.34	0.76	5.29	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C15	(4-Hydroxyphenyl) phosphonic acid	Others	15.70	$C_6H_7O_4P$	999	0.49	0.32	0.38	0.03	0.00	0.24	0.03	4.06	0.00	0.19	0.00	0.00	1.04	0.93	0.00	0.00
C16	(−)-β-Pinene	Terpenes	15.70	$C_{10}H_{16}$	998	0.00	0.02	0.00	0.00	0.00	0.00	0.82	0.17	0.01	0.01	0.00	0.00	0.00	0.00	0.03	0.00
C17	1,2,3-Trimethyl benzene	Aromatic hydrocarbon	15.90	C_9H_{12}	1004	0.27	0.36	0.16	0.09	0.09	0.24	0.11	0.22	0.39	0.28	0.29	0.25	0.00	0.05	0.04	0.67
C18	3-Carene	Terpenes	16.50	$C_{10}H_{16}$	1019	4.78	1.19	1.11	1.31	1.05	0.60	1.17	0.80	0.75	1.43	0.21	0.50	0.29	0.17	1.60	1.14
C19	2,5,6-Trimethyl decane	Alkane	16.57	$C_{13}H_{28}$	1020	8.24	21.12	13.94	20.95	17.49	17.56	8.18	0.00	9.40	0.00	7.63	0.00	1.29	1.09	14.60	29.16
C20	2,6,7-Trimethyl decane	Alkane	16.83	$C_{13}H_{28}$	1027	2.91	2.12	10.77	8.39	9.75	6.41	3.03	5.63	0.00	9.35	0.00	7.61	0.39	0.29	3.69	3.82
C21	o-Cymene	Aromatic hydrocarbon	17.10	$C_{10}H_{14}$	1034	0.19	0.32	1.72	0.00	0.10	7.31	1.41	0.12	0.16	0.21	7.69	5.17	0.99	0.61	0.04	0.21
C22	2,4-Dimethyl-2,3-heptadien-5-yne	Olefine	17.10	C_9H_{12}	1034	0.49	0.08	0.00	0.02	0.05	0.02	0.12	0.03	0.03	0.03	0.10	0.00	0.00	0.00	0.00	0.00
C23	D-Limonene	Terpenes	17.17	$C_{12}H_{26}$	1036	0.32	2.38	0.07	0.00	0.08	0.00	0.00	0.21	0.38	0.36	0.31	0.30	3.22	2.55	0.12	0.71
C24	2,2,4,4-Tetramethyl octane	Alkane	17.23	$C_{10}H_{16}$	1037	11.42	1.19	3.24	6.32	2.61	4.13	2.55	6.73	9.70	8.54	7.56	5.38	56.02	61.87	0.68	1.50
C25	2,5,9-Trimethyl decane	Alkane	17.37	$C_{13}H_{28}$	1041	0.76	4.31	1.17	1.58	1.42	1.13	0.70	0.00	0.00	0.00	0.08	0.00	0.33	0.00	1.83	1.37
C26	2,3-Dihydro pyran-6-one	Ketone	17.57	$C_5H_6O_2$	1046	0.25	0.15	0.04	0.05	0.09	0.25	2.36	0.17	0.18	0.09	0.02	0.07	0.00	0.00	0.00	0.00
C27	3-Octene-2-one	Ketone	17.57	$C_8H_{14}O$	1046	1.39	0.30	0.34	0.19	0.19	0.33	2.50	0.24	0.01	0.19	0.00	0.00	0.00	0.00	0.00	0.00
C28	2,7,10-Trimethyl dodecane	Alkane	18.23	$C_{15}H_{32}$	1063	0.15	0.04	0.00	0.00	0.07	0.11	0.51	0.10	0.04	0.11	0.10	0.07	0.00	0.00	0.00	0.00
C29	2-Propyl toluene	Aromatic hydrocarbon	18.10	$C_{10}H_{14}$	1060	2.42	6.47	9.82	8.11	10.91	5.65	2.94	4.54	5.25	7.38	4.70	4.78	0.63	0.85	11.44	5.72
C30	2-Ethyl-1,4-dimethyl benzene	Aromatic hydrocarbon	18.37	$C_{10}H_{14}$	1066	0.47	0.06	0.00	0.00	0.01	0.06	0.09	0.01	0.01	0.09	0.06	0.06	0.00	0.00	0.00	0.00
C31	2,4,6-Trimethyl decane	Alkane	18.43	$C_{13}H_{28}$	1068	2.09	4.14	8.78	5.02	5.90	3.98	2.79	0.45	0.63	0.58	0.54	0.51	0.25	0.30	5.91	3.64
C32	Phenylglyoxyl monohydrate	Others	18.77	$C_8H_8O_3$	1077	0.18	0.11	0.03	0.00	0.07	0.14	0.52	0.46	0.16	0.02	0.16	0.14	0.00	0.00	0.24	0.00
C33	3,5-Octadien-2-one	Ketone	18.83	$C_8H_{12}O$	1078	0.16	0.06	0.00	0.00	0.00	0.04	0.11	0.01	0.09	0.05	0.09	0.03	0.00	0.00	0.00	0.00
C34	2,6-Dimethyl-6-trifluoroacetoxyoctane	Others	19.10	$C_{12}H_{21}F_3O_2$	1085	3.29	8.16	4.93	5.34	5.61	5.30	4.23	0.73	0.75	0.79	0.63	0.56	1.48	0.33	5.71	7.31
C35	1,3-Dimethyl-3-ethyl benzene	Aromatic hydrocarbon	19.17	$C_{10}H_{14}$	1087	0.39	0.02	0.00	0.00	0.04	0.10	0.02	0.03	0.05	0.04	0.03	0.02	0.00	0.13	0.28	0.24
C36	Piperityl acetate	Others	19.57	$C_{12}H_{20}O_2$	1097	0.50	0.10	0.00	0.00	0.04	0.10	0.31	0.03	0.04	0.07	0.02	0.04	0.00	0.00	0.09	0.00

Table 2. *Cont.*

NO.	Compound	Class	Retention Time (min)	Formula	RI	Peak Area Percentage (%)															
						W	A	A0	A1	A2	A3	FB	FC20d	FC40d	FC60d	FC80d	FC100d	Tx-LU	Tx-LI	Tx-PU	Tx-PI
C37	1,2,4,5-Tetramethyl benzene	Aromatic hydrocarbon	19.43	$C_{10}H_{14}$	1093	0.30	0.05	0.00	0.00	0.01	0.07	0.21	0.02	0.01	0.03	0.04	0.01	0.00	0.00	1.05	0.00
C38	1,2-Dimethoxyethyl benzene	Aromatic hydrocarbon	19.43	$C_{10}H_{14}O_2$	1094	0.00	0.00	0.00	0.00	0.03	0.02	0.00	6.28	8.33	1.12	6.72	7.70	0.00	0.00	0.29	0.03
C39	Dodecane,2,6,11-trimethyl	Alkane	19.50	$C_{15}H_{32}$	1095	0.49	0.07	0.00	0.00	0.00	0.04	0.01	0.02	0.10	0.10	0.04	0.06	0.07	0.02	0.16	0.00
C40	4,5-Dimethylnonane	Alkane	19.97	$C_{11}H_{24}$	1107	0.46	0.04	0.59	0.25	0.57	0.81	0.03	1.64	0.70	0.00	0.02	0.77	0.36	0.24	0.74	0.74
C41	Linalool	Terpenes	19.90	$C_{10}H_{18}O$	1105	1.60	3.78	4.48	3.76	4.60	3.44	2.27	2.34	2.50	3.99	2.12	2.21	0.67	0.70	4.13	2.33
C42	2-Nonen-1-ol	Alcohol	20.03	$C_9H_{18}O$	1109	1.20	0.02	0.59	0.52	0.57	0.81	0.30	1.03	0.70	0.98	0.73	0.77	0.36	0.24	0.74	0.74
C43	1,2,3,5-Tetramethyl benzene	Aromatic hydrocarbon	20.30	$C_{10}H_{14}$	1116	0.23	0.06	0.00	0.00	0.04	0.08	0.14	0.06	0.04	0.05	0.01	0.05	0.00	0.00	0.05	0.08
C44	6-Ethyl-2-methyl octane	Alkane	20.43	$C_{11}H_{24}$	1119	0.53	2.20	0.97	0.63	0.73	0.72	0.47	0.96	1.13	0.41	1.16	1.04	0.68	0.75	1.16	3.42
C45	1,5,6,7-Tetramethyl bicyclo[3.2.0]hepta-2,6-diene	Olefine	20.57	$C_{11}H_{16}$	1123	0.14	0.11	0.42	0.10	0.21	0.34	0.21	0.03	0.02	0.02	0.02	0.02	0.00	0.00	0.31	0.00
C46	1,2,3,4-Tetramethyl benzene	Aromatic hydrocarbon	20.83	$C_{10}H_{14}$	1130	1.29	0.31	0.18	0.26	0.23	0.26	0.28	0.04	0.03	0.04	0.03	0.03	0.00	0.00	0.25	0.16
C47	3,5-Diethyl-1-methyl benzene	Aromatic hydrocarbon	21.43	$C_{11}H_{16}$	1146	0.12	0.12	0.00	0.00	0.00	0.00	0.07	0.04	0.03	0.04	0.00	0.01	0.00	0.00	0.00	0.00
C48	4-Methyl indane	Others	21.57	$C_{10}H_{12}$	1149	0.10	0.03	0.00	0.00	0.01	0.01	0.03	0.01	0.00	0.01	0.05	0.01	0.00	0.00	0.00	0.00
C49	1,4-Diethyl-2-methyl benzene	Aromatic hydrocarbon	21.70	$C_{11}H_{16}$	1153	0.27	0.07	0.00	0.01	0.03	0.06	0.14	0.23	0.01	0.12	0.02	0.06	0.00	0.00	0.00	0.00
C50	1,2,3,4-Tetramethyl fulvene	Others	22.03	$C_{10}H_{14}$	1162	0.08	0.16	0.04	0.07	0.04	0.12	0.07	0.10	0.01	0.03	0.04	0.00	0.00	0.00	0.13	0.03
C51	[but-2-en-2-yl]Benzene	Aromatic hydrocarbon	21.97	$C_{10}H_{12}$	1160	0.40	0.19	0.07	0.13	0.15	0.16	0.12	0.10	0.01	0.03	0.04	0.02	0.00	0.00	0.13	0.03
C52	2,3-Dimethyl decane	Alkane	22.10	$C_{12}H_{26}$	1163	0.12	0.98	0.37	0.22	0.25	0.19	0.18	0.77	0.76	2.27	2.47	0.82	0.00	0.02	0.26	0.34
C53	4′-Methyl propiophenone	Ketone	22.50	$C_{10}H_{12}O$	1174	0.23	0.52	0.56	0.51	0.61	0.41	0.42	2.34	2.37	0.53	3.39	2.26	0.00	0.02	0.56	0.24
C54	5-Butan-2-ylnonane	Alkane	22.30	$C_{13}H_{28}$	1168	0.14	0.78	0.18	0.18	0.17	0.17	0.19	0.07	2.08	0.00	2.20	0.09	0.00	0.00	0.22	0.20
C55	9-Methyl heptadecane	Alkane	22.50	$C_{18}H_{38}$	1174	0.16	0.09	0.02	0.00	0.02	0.02	0.12	0.11	0.04	0.04	0.02	0.00	0.00	0.00	0.00	0.00
C56	3-Methyl undecane	Alkane	22.70	$C_{12}H_{26}$	1179	0.09	0.08	0.02	0.00	0.02	0.05	0.12	0.12	0.09	0.04	0.02	0.03	0.00	0.00	0.00	0.00
C57	1-Ethyl-2,4,5-trimethyl benzene	Aromatic hydrocarbon	22.63	$C_{11}H_{16}$	1177	0.20	0.77	0.56	0.43	0.55	0.39	0.45	0.00	2.24	2.60	0.07	2.26	0.00	0.00	0.68	0.25
C58	1-Methyl-cyclododecene	Olefine	22.83	$C_{13}H_{24}$	1183	0.23	0.17	0.08	0.05	0.00	0.07	1.91	0.02	0.05	0.05	0.04	0.02	0.00	0.22	0.93	0.73
C59	3,7,11-Trimethyl dodecan-1-ol	Alcohol	23.30	$C_{15}H_{32}O$	1195	0.11	0.25	0.01	0.01	0.07	0.09	0.10	0.20	0.22	5.81	5.15	0.17	0.00	0.00	0.11	0.00
C60	Dodecane	Alkane	23.57	$C_{12}H_{26}$	1202	0.02	0.00	0.00	0.00	0.01	0.05	0.42	0.05	0.04	0.04	0.02	0.03	0.00	0.00	0.00	0.00
C61	(+)-α-Terpineol	Terpenes	23.50	$C_{10}H_{18}O$	1200	1.26	1.23	2.87	2.49	3.06	2.47	2.14	2.35	5.94	2.38	5.64	6.01	0.29	0.28	2.82	1.93
C62	3,4-Dimethyl cumene	Aromatic hydrocarbon	23.70	$C_{11}H_{16}$	1206	0.18	0.04	0.00	0.00	0.05	0.05	0.11	0.02	0.09	0.01	0.02	0.02	0.00	0.00	0.00	0.00
C63	4,7-Dimethyl undecane	Alkane	23.77	$C_{13}H_{28}$	1208	0.25	1.13	0.76	0.55	0.55	0.52	0.64	1.06	1.67	1.06	1.63	1.10	0.00	0.06	0.56	1.29
C64	Cyclodecanol	Others	23.83	$C_{10}H_{20}O$	1210	0.12	0.14	0.04	0.08	0.07	0.09	0.15	0.33	0.08	0.02	0.08	0.07	0.00	0.00	0.03	0.04
C65	2,6-Dimethyl undecane	Alkane	24.10	$C_{13}H_{28}$	1217	0.61	0.95	1.24	1.06	1.28	1.02	1.47	3.87	2.49	2.32	2.32	3.43	0.08	0.08	1.13	0.46
C66	2,4-Dimethyl acetophenone	Ketone	24.17	$C_{10}H_{12}O$	1219	0.06	0.03	0.02	0.00	0.02	0.03	0.06	0.04	0.28	0.02	0.02	0.01	0.00	0.00	0.00	0.00
C67	2-Hydroxicineole	Others	24.23	$C_{10}H_{18}O_2$	1221	0.00	0.01	0.00	0.00	0.01	0.00	0.47	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00
C68	2,8-Dimethyl undecane	Alkane	24.37	$C_{13}H_{28}$	1225	0.11	0.12	0.39	0.33	0.40	0.28	0.38	0.28	0.20	0.23	0.21	0.26	0.06	0.04	0.42	0.48
C69	2,6-Dimethyl benzaldehyde	Others	24.43	$C_9H_{10}O$	1227	0.01	0.00	0.00	0.00	0.05	0.06	0.01	0.42	0.21	0.29	0.20	0.23	0.00	0.00	0.00	0.00

Table 2. *Cont.*

NO.	Compound	Class	Retention Time (min)	Formula	RI	Peak Area Percentage (%)															
						W	A	A0	A1	A2	A3	FB	FC20d	FC40d	FC60d	FC80d	FC100d	Tx-LU	Tx-LI	Tx-PU	Tx-PI
C70	2-Hydroxycineol	Others	24.63	$C_{10}H_{18}O_2$	1232	0.00	0.10	0.00	0.00	0.00	0.00	0.51	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C71	Thieno[2,2,3]pyridine	Others	24.83	C_7H_5NS	1239	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.10	0.05	0.06	0.06	0.05	0.00	0.00	0.00	0.00
C72	3-Hydroxy cineole	Others	25.23	$C_{10}H_{18}O_2$	1249	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.10	0.10	0.06	0.03	0.03	0.00	0.00	0.00	0.00
C73	4-Methyl dodecane	Alkane	25.57	$C_{13}H_{28}$	1258	0.81	1.52	1.43	1.74	1.84	1.68	2.44	2.22	1.23	1.39	1.26	1.40	0.18	0.10	1.00	1.89
C74	1,3-Di-tert-butyl benzene	Aromatic hydrocarbon	25.70	$C_{14}H_{22}$	1262	0.04	0.01	0.00	0.00	0.01	0.00	0.07	0.01	0.02	0.01	0.00	0.03	0.00	0.00	0.00	0.00
C75	4,7-Dimethyl indan	Others	25.63	$C_{11}H_{14}$	1261	0.13	0.89	0.27	0.24	0.28	0.96	0.39	3.83	3.36	3.66	3.34	3.69	0.15	0.12	0.33	0.75
C76	2,3-Dimethyl undecane	Alkane	25.70	$C_{13}H_{28}$	1262	2.97	5.75	6.96	6.91	7.49	6.84	7.11	7.59	6.91	7.50	6.77	7.64	1.25	0.91	7.15	5.78
C77	2,4-Dimethyl dodecane	Alkane	25.90	$C_{14}H_{30}$	1268	0.22	0.25	0.74	0.63	0.28	0.55	0.95	0.21	0.16	0.18	0.20	0.25	0.14	0.12	0.49	0.37
C78	2,6,11-Trimethyl dodecane	Alkane	26.43	$C_{15}H_{32}$	1283	0.49	0.85	1.42	1.19	1.64	1.14	2.27	4.41	4.14	2.16	3.96	4.52	0.19	0.18	1.45	0.57
C79	2,3,4-Trimethyl dodecane	Alkane	26.77	$C_{13}H_{28}$	1292	0.10	0.21	0.44	0.32	0.33	0.25	0.48	2.58	1.61	2.21	1.93	2.43	0.00	0.02	0.31	0.25
C80	1,5,6,7-Tetramethylbicyclo[3.2.0]hepta-2,6-diene	Olefine	26.77	$C_{11}H_{16}$	1293	0.05	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
C81	1,7,7-Trimethylbicyclo[2.2.1]heptan-2-yl acetate	Others	26.90	$C_{12}H_{20}O_2$	1296	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.09	0.01	0.01	0.01	0.00	0.00	0.00	0.00
C82	2-Undecanone	Ketone	26.97	$C_{11}H_{22}O$	1298	0.20	0.09	0.00	0.00	0.06	0.08	1.10	0.13	0.09	0.12	0.09	0.12	0.00	0.00	0.08	0.00
C83	Tridecane	Alkane	27.17	$C_{13}H_{28}$	1304	0.17	0.00	0.11	0.00	0.14	0.15	0.00	2.78	1.44	2.13	1.30	2.34	0.15	0.06	0.32	0.17
C84	4,6-Dimethyl dodecane	Alkane	27.23	$C_{14}H_{30}$	1306	0.12	0.24	0.28	0.41	0.32	0.28	0.63	2.04	0.26	1.61	0.27	1.72	0.20	0.08	0.34	0.13
C85	1-Methyl naphthalene	Aromatic hydrocarbon	27.30	$C_{11}H_{10}$	1308	0.07	0.04	0.00	0.00	0.00	0.00	0.04	0.01	0.01	0.01	0.02	0.01	0.00	0.00	0.00	0.00
C86	2,3,5,8-Tetramethyl decane	Alkane	27.43	$C_{14}H_{30}$	1312	0.68	1.50	0.94	1.51	1.52	1.48	2.42	0.43	0.31	0.29	0.27	0.38	0.22	0.08	1.20	0.93
C87	α-Methyl-1H-indene-1-methanol acetate	Others	27.90	$C_{13}H_{14}O_2$	1326	0.08	0.02	0.00	0.00	0.00	0.00	0.03	0.26	0.01	0.04	0.03	0.01	0.00	0.00	0.00	0.00
C88	2,7,10-Trimethyl dodecane	Alkane	28.37	$C_{15}H_{32}$	1339	0.09	0.29	0.17	0.16	0.18	0.17	0.33	1.57	0.61	0.74	0.67	1.01	0.00	0.00	0.19	0.06
C89	Silphiperfol-5-ene	Terpenes	28.37	$C_{15}H_{24}$	1339	0.17	0.00	0.00	0.00	0.00	0.00	0.07	0.01	0.03	0.02	0.02	0.01	0.00	0.00	0.12	0.00
C90	4-Ethyl undecane	Alkane	28.97	$C_{13}H_{28}$	1357	0.08	0.11	0.06	0.10	0.09	0.09	0.18	0.20	0.00	0.21	0.44	0.68	0.00	0.00	0.13	0.14
C91	Silphinene	Terpenes	29.10	$C_{15}H_{24}$	1361	0.45	0.02	0.00	0.00	0.00	0.00	0.03	0.01	0.00	0.01	0.02	0.00	0.00	0.00	0.05	0.00
C92	2,3-Dimethyl dodecane	Alkane	29.30	$C_{14}H_{30}$	1367	0.05	0.13	0.12	0.16	0.14	0.18	0.11	0.44	0.37	0.48	0.44	0.61	0.00	0.00	0.07	0.08
C93	α-Longipinene	Terpenes	29.30	$C_{15}H_{24}$	1367	0.10	0.02	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.01	0.02	0.02	0.00	0.00	0.10	0.00
C94	farnesane	Alkane	29.70	$C_{15}H_{32}$	1378	0.05	0.08	0.03	0.03	0.05	0.05	0.19	0.21	0.25	0.28	0.41	0.58	0.07	0.04	0.04	0.06
C95	(+)-Cyclosativene	Terpenes	29.83	$C_{15}H_{24}$	1382	0.15	0.01	0.00	0.00	0.00	0.00	0.14	0.01	0.02	0.02	0.02	0.02	0.00	0.00	0.10	0.13
C96	Modephene	Terpenes	30.37	$C_{15}H_{24}$	1398	1.00	0.02	0.00	0.00	0.00	0.00	0.05	0.02	0.06	0.01	0.03	0.03	0.00	0.00	0.20	0.20
C97	Tetradecane	Alkane	30.50	$C_{14}H_{30}$	1402	0.27	0.36	0.44	0.50	0.01	0.02	0.51	1.64	0.68	0.61	0.92	1.77	0.24	0.18	0.38	0.38
C98	α-Isocomene	Terpenes	30.57	$C_{15}H_{24}$	1404	2.56	0.02	0.00	0.00	0.00	0.00	0.05	0.00	0.05	0.01	0.01	0.02	0.00	0.00	0.05	0.03
C99	(−)-α-Gurjunene	Terpenes	30.97	$C_{15}H_{24}$	1417	6.99	0.02	0.07	0.06	0.12	0.60	0.10	0.07	0.03	0.03	0.03	0.04	0.00	0.00	0.06	0.00
C100	2,6-Dimethyl heptadecane	Alkane	31.23	$C_{19}H_{40}$	1425	0.04	0.08	0.08	0.05	0.11	0.08	0.09	0.91	0.02	0.30	0.03	0.92	0.00	0.00	0.09	0.00
C101	β-Maaliene	Terpenes	31.30	$C_{15}H_{24}$	1428	3.45	0.03	0.00	0.00	0.01	0.02	0.05	0.03	0.01	0.06	0.01	0.02	0.00	0.00	0.07	0.07
C102	(−)-Aristolene	Terpenes	31.57	$C_{15}H_{24}$	1436	1.57	0.00	0.00	0.00	0.01	0.02	0.05	0.02	0.01	0.03	0.03	0.00	0.00	0.00	0.00	0.11
C103	2-Isopropyl-5-methyl-9-methylene[4.4.0]dec-1-ene	Terpenes	31.70	$C_{15}H_{24}$	1441	0.29	0.01	0.00	0.00	0.00	0.00	0.05	0.00	0.03	0.03	0.05	0.05	0.00	0.00	0.12	0.12
C104	α-Bergamotene	Terpenes	31.90	$C_{15}H_{24}$	1447	0.32	0.02	0.00	0.08	0.01	0.00	0.06	0.01	0.02	0.06	0.00	0.07	0.00	0.00	0.00	0.00

Table 2. *Cont.*

NO.	Compound	Class	Retention Time (min)	Formula	RI	Peak Area Percentage (%)															
						W	A	A0	A1	A2	A3	FB	FC20d	FC40d	FC60d	FC80d	FC100d	Tx-LU	Tx-LI	Tx-PU	Tx-PI
C105	Isoledene	Terpenes	32.03	$C_{15}H_{24}$	1451	6.98	0.00	0.00	0.00	0.00	0.00	0.04	0.04	0.03	0.04	0.03	0.02	0.00	0.00	0.12	0.05
C106	Guaia-3,9-diene	Terpenes	32.23	$C_{15}H_{24}$	1458	0.25	0.00	0.00	0.00	0.00	0.00	0.03	0.01	0.01	0.01	0.02	0.04	0.00	0.00	0.00	0.00
C107	2,10-Dimethyl heptadecane	Alkane	32.50	$C_{19}H_{40}$	1466	0.58	0.00	0.00	0.00	0.00	0.00	0.03	0.06	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
C108	Seychellene	Terpenes	32.43	$C_{15}H_{24}$	1464	0.17	0.22	0.21	0.28	0.23	0.24	0.46	1.21	0.77	0.20	0.92	0.89	0.14	0.11	0.20	0.17
C109	2,6-Di-tert-butyl-4-methyl-p-quinol	Ketone	32.97	$C_{15}H_{24}O_2$	1481	0.38	0.04	0.04	0.06	0.01	0.06	0.24	0.02	0.01	0.04	0.04	0.01	0.22	0.12	0.08	0.10
C110	Pentadecane	Alkane	33.30	$C_{15}H_{32}$	1491	0.05	0.11	0.12	0.19	0.16	0.16	0.23	1.18	0.48	0.17	0.57	0.59	0.00	0.00	0.11	0.00
C111	(+)-Valencene	Terpenes	33.30	$C_{15}H_{24}$	1492	0.22	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.05	0.02	0.02	0.03	0.83	0.42	0.03	0.03
C112	β-Sesquiphellandrene	Terpenes	33.57	$C_{15}H_{24}$	1500	0.87	0.09	0.02	0.22	0.07	0.07	0.14	0.01	0.01	0.03	0.01	0.01	0.00	0.00	0.03	0.08
C113	1-Iodo-2-methyl undecane	Alkane	33.90	$C_{12}H_{25}I$	1511	0.04	0.04	0.04	0.05	0.16	0.41	0.11	0.15	0.10	0.11	0.12	0.15	0.00	0.00	0.00	0.00
C114	α-Bulnesene	Terpenes	33.97	$C_{15}H_{24}$	1514	0.19	0.04	0.00	0.00	0.02	0.06	0.02	0.00	0.00	0.00	0.02	0.03	0.07	0.03	0.00	0.04
C115	2,4-Di-tert-butyl phenol	Phenols	34.17	$C_{14}H_{22}O$	1520	0.12	0.04	0.13	0.25	0.16	0.51	0.10	0.75	0.26	0.00	0.00	0.00	0.00	0.00	0.11	0.08
C116	2,4-Di-tert-butyl-6-methyl phenol	Phenols	34.30	$C_{15}H_{24}O$	1525	0.00	0.01	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.02	0.70	0.76	11.27	9.14	0.10	0.21
C117	(+)-Cuparene	Terpenes	34.37	$C_{15}H_{22}$	1527	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.00	0.00	0.00	0.00	0.00	0.00
C118	Hexadecane	Alkane	36.70	$C_{16}H_{34}$	1605	0.06	0.09	0.11	0.12	0.06	0.11	0.08	0.11	0.06	0.02	0.12	0.15	0.02	0.02	0.12	0.24
C119	2,6-Di-tert-butyl-4-sec-butyl phenol	Phenols	37.97	$C_{18}H_{30}O$	1649	0.01	0.03	0.03	0.03	0.02	0.03	0.01	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.03	0.06
C120	Pristane	Alkane	39.70	$C_{19}H_{40}$	1710	0.02	0.02	0.03	0.04	0.03	0.05	0.05	0.03	0.04	0.04	0.04	0.03	0.00	0.00	0.02	0.06

Note: For descriptions of W, A, A-0, A-1, A-2, A-3, FB, FC20d, FC40d, FC60d, FC80d, FC100d, Tx-LU, Tx-LI, Tx-PU, and Tx-PI, please refer to Table 1.
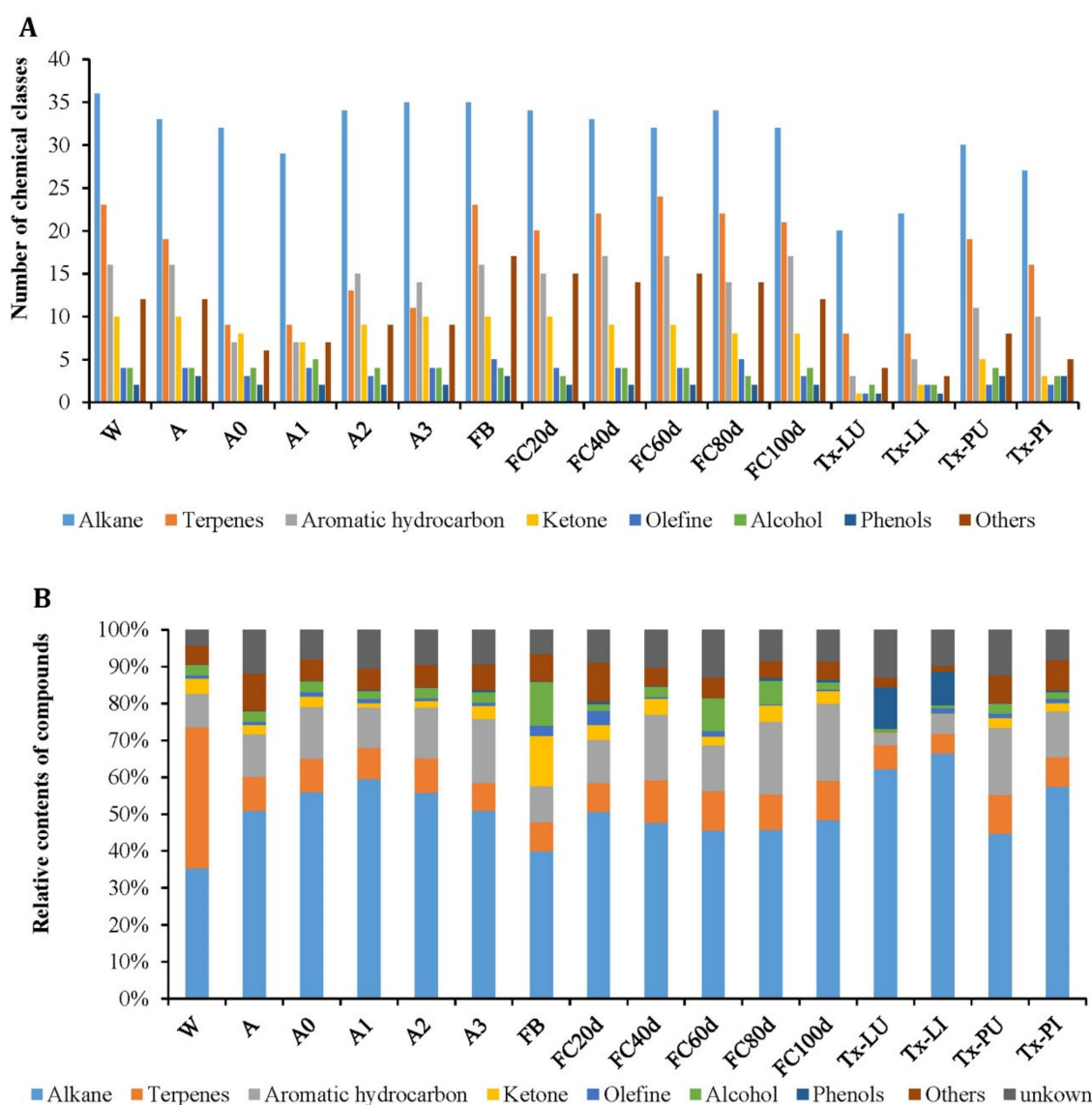
Figure 2. The number (**A**) and relative content (**B**) of volatile compounds of each class in different products. Note: For descriptions of W, A, A0, A1, A2, A3, FB, FC20d, FC40d, FC60d, FC80d, FC100d, Tx-LU, Tx-LI, Tx-PU, and Tx-PI, please refer to Table 1).

2.3. Major Compounds in Different Products

The numbers and percentage contents of volatile compounds in samples are of marked differences. Alkanes are the dominant volatile compounds in all samples. Alkane is also the class with the largest number in all samples (Figure 2). The top five compounds in the concentration of each sample are shown in Table 3.

Table 3. Top five compounds in concentration from different products.

Sample	Top Five Compounds									
	1		2		3		4		5	
	Compound No.	Peak Area Percentage (%)	Compound No.	Peak Area Percentage (%)	Compound No.	Peak Area Percentage (%)	Compound No.	Peak Area Percentage (%)	Compound No.	Peak Area Percentage (%)
W	C24	11.42	C19	8.24	C99	6.99	C105	6.98	C17	4.78
A	C19	21.12	C34	8.16	C29	6.47	C76	5.75	C25	4.31
A0	C19	13.94	C20	10.77	C29	9.82	C31	8.78	C76	6.96
A1	C19	20.95	C20	8.39	C29	8.11	C76	6.91	C24	6.32
A2	C19	17.49	C29	10.91	C20	9.75	C76	7.49	C31	5.90
A3	C19	17.56	C21	7.31	C76	6.84	C20	6.41	C29	5.65
FB	C2	10.80	C19	8.18	C76	7.11	C14	5.29	C34	4.23
FC20d	C76	7.59	C24	6.73	C38	6.28	C20	5.63	C29	4.54
FC40d	C24	9.70	C19	9.40	C38	8.33	C76	6.91	C61	5.94
FC60d	C20	9.35	C24	8.54	C76	7.50	C29	7.38	C59	5.81
FC80d	C21	7.69	C19	7.63	C24	7.56	C76	6.77	C38	6.72
FC100d	C38	7.70	C76	7.64	C20	7.61	C61	6.01	C24	5.38
Tx-LU	C24	56.02	C116	11.27	C23	3.22	C5	1.57	C34	1.48
Tx-LI	C24	61.87	C116	9.14	C5	3.72	C23	2.55	C1	1.22
Tx-PU	C19	14.60	C29	11.44	C76	7.15	C31	5.91	C34	5.71
Tx-PI	C19	29.16	C34	7.31	C76	5.78	C29	5.72	C5	5.09

Note: C1: 3-Prop-2-enylidenecyclobutene; C2: 2-methylcyclopentanol; C5: o-xylene; C14: 6-ethenyl-2,2,6-trimethyloxan-3-one; C17: 1,2,3-trimethylbenzene; C19: 2,5,6-trimethyldecane; C20: 2,6,7-trimethyldecane; C21: o-cymene; C23: D-limonene; C24: 2,2,4,4-tetramethyloctane; C25: 2,5,9-trimethyldecane; C29: 2-propyltoluene; C31: 2,4,6-trimethyldecane; C34: 2,6-dimethyl-6-trifluoroacetoxyoctane; C38: 1,2-dimethoxyethylbenzene; C59: 3,7,11-trimethyldodecan-1-ol; C61: (+)-α-terpineol; C76: 2,3-dimethylundecane; C99: (−)-α-gurjunene; C105: isoledene; C116: 2,4-ditert-butyl-6-methyl phenol. The compounds corresponding to the compound numbers are also shown in Table 2.

2,5,6-trimethyldecane (C19) is the most abundant compound in artificial cultivated Chinese cordyceps (A, A0, A1, A2, A3) and insect pupae (Tx-PU and Tx-PI), and it is also the major compound in W, FB, and FC40d, FC80d, and FC100d (Table 3). This compound is so far detected from beneficial plants such as Irish York cabbage [28], stevia *Stevia rebaudiana* leaves [29], an aquatic perennial herb *Limnophila indica* extract [30], plant-based food such as chestnut and jujube honey [31], and from exhaled breath in both children with allergic asthma and control [32]. 5,6-Dihydro-6-pentyl-2*H*-pyran-2-one (massoia lactone) is discovered as the dominant volatile component in a fermented mycelial product of *Paecilomyces hepiali* fungus [18]. 2,5,6-trimethyldecane is the first reported dominant volatile compound in *O. sinensis*-based products in the present study. Moreover, it seemed interesting that uninfected insect pupae also contained high concentrations of this compound. Its characteristics and possible pharmacological functions need further study.

2,3-dimethylundecane (C76) is another major component presented in 13 samples accounting for >5% of the total peak areas; however, the contents in samples of W, Tx-LU, and Tx-LI were lower, accounting for 2.97%, 1.25%, and 0.91%, respectively (Tables 2 and 3). This compound was found from the essential oil of a small glabrous, perennial herb *Viola serpens* [33] and from the odors emitted from the dung of free-ranging white rhinos for differentiating sex [34].

A high content of 2,2,4,4-tetramethyloctane (C24) was found in the two larval samples of Tx-LU and Tx-LI, accounting for 56.02% and 61.87% of the total peak areas, although it is not reported from other insects. 2,2,4,4-tetramethyloctane is also the major compound of all the liquid fermentation samples (FC20d, FC40d, FC60d, FC80d, and FC100d). It was reported also in aged vinegar as an aroma compound [35], common wasp *Vespula vulgaris* colonies [36], Manchego and Gouda cheeses [37], *Allium macrostemon* flowers and aerial parts [38], the seeds and leaves of *Synsepalum dulcificum* [39], green teas [40], dry-cured meat products [41], and the stem of Guanyin tea [42]. It appears that this volatile mainly acts as an aroma compound from the plants and foods.

2,4-di-tert-butyl-6-methylphenol (C116) is the second principal component in the two larval samples of Tx-LU and Tx-LI, accounting for 11.27% and 9.14% of the total peak areas, but it accounts for little or no proportion in other samples. This volatile is detected from the essential oil in eaglewood [43] and entomopathogenic *Metarhizium anisopliae* fungus cultures [44].

The most abundant volatile compound was butylated hydroxytoluene, and the major classes compounds were phenols, acids, and alkanes in the mycelia of *O. sinensis* cultured by solid-state media and submerged fermentation [17]. 5,6-Dihydro-6-pentyl-2*H*-pyran-2-one (massoia lactone) was the dominant component in Jinshuibao capsule (*Paecilomyces hepiali*) volatiles, and fatty acids including palmitic acid (C16:0) and linoleic acid (C18:2) were also found to be major volatile compositions in the commercial fermentation products of Bailing capsule (*O. sinensis*), Zhiling capsule (*Mortierella* SP), Ningxinbao capsule (*Cephalosporium sinensis*), and Xinganbao capsule (*Gliocladium roseum*) [18]. In the present study, volatile compounds of alkanes are the most abundant all products, although there are differences among the volatile compound profiles of *O. sinensis* fungus, *Thitarodes* hosts of *O. sinensis*, and the natural and aritificial-producing Chinese cordyceps, even between the natural and artificial-producing Chinese cordyceps. It appeared that *O. sinensis*-based products from different culture conditions exhibit quite different metabolites.

The fermented products of *O. sinensis* mycelia are claimed to be used as sustainable substitutes for natural Chinese cordyceps [45]. However, from the view of the differences in volatile compounds, it seems that the fermented products are not the same as the natural and artificial Chinese cordyceps.

2.4. Multivariate PLS-DA Analysis

PLS-DA was performed to evaluate the variations among the volatile compound profiles obtained from GC×GC-QTOFMS data for different products. The PLS-DA scores plot shows clear classification of the three groups: fruiting bodies and fermented cultures of *O. sinenis* fungus, *T. xiaojinensis* insects, and insect–fungus complexes (Figure 3A). PLS (Partial least square) component 1 (PLS 1) and PLS component 2 (PLS 2) explained 21.5% and 19.0% of the variance, respectively, and hence together, they

explained 40.05% of the total variance. The parameters of the cross-validation modeling for the fifth PLS component were $R^2X = 0.73$, $R^2Y = 0.992$, and $Q^2Y = 0.896$, showing high levels of an explained variance and predictability. A permutation test involving 200 iterations was also conducted to validate the model, which yielded $R^2 = 0.770$ and $Q^2 = -0.480$.
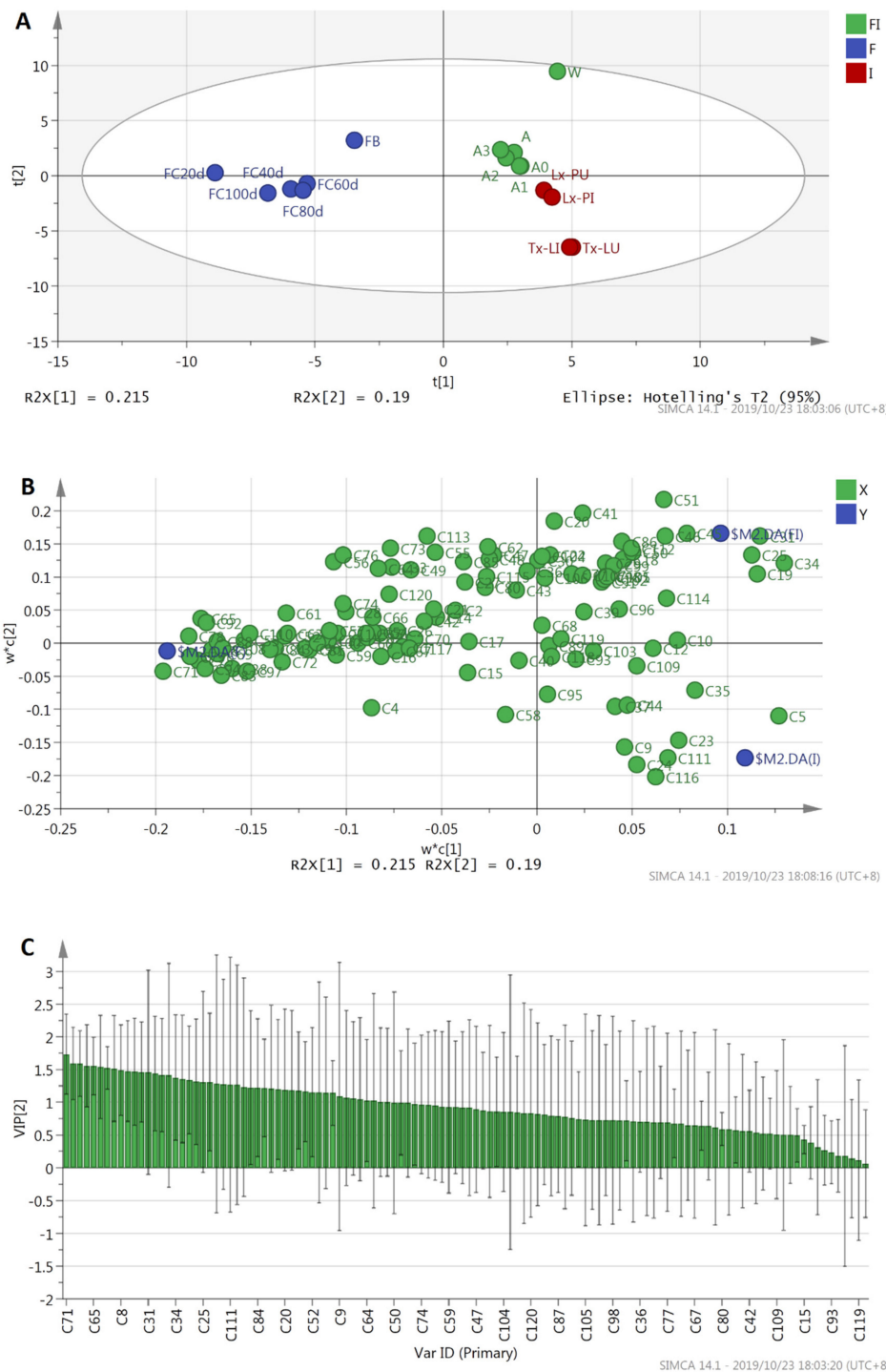

Figure 3. Partial least squares-discriminant analysis (PLS-DA) analysis of 16 products. (**A**): Scores plot, (**B**): variable importance projection (VIP) score plot, (**C**): loadings plot. F: Fungus of *Ophiocordyceps sinensis*; I: Larvae or pupae of *Thitarodes xiaojinensis*; FI: Fungus–insect complexes.

To explain the relationships between variables and products, loading scatter plots were performed (Figure 3B). As shown in the loadings plot PLS-DA model (Figure 3B), X-variables situated in the vicinity of the dummy Y-variables had the highest discriminatory power among the groups and had higher VIP (variable importance projection) values, thus contributing more to the differences of different groups. The VIP values of each compound were calculated (Figure 3C). The compounds with larger VIP values represent higher contributions to the discrimination of different groups. In the study, volatile components with VIP values > 1 and $p < 0.05$ were considered as representative differential compounds. A total of 28 differential volatile compounds were identified, although there were 48 volatile compounds with VIP values > 1. It showed that the majority of variables gave a not significant contribution to the model. The 28 differential volatile compounds included thieno [2,3-c] pyridine (C71), 2,6,11-trimethyldodecane (C78), 2,3,4-trimethyldodecane (C79), 4,7-dimethylindan (C75), 2,6-dimethylundecane (C65), farnesane (C94), 2,3-dimethyldodecane (C92), [but-2-en-2-yl] benzene (C51), 2-methylcyclopentanone (C8), 2-undecanone (C83), 2,8-dimethylundecane (C69), α-methyl-1*H*-indene-1-methanol acetate (C88), 2,6-dimethylheptadecane (C108), 1,2-dimethoxyethylbenzene (C38), 2,6-dimethyl-6-trifluoroacetoxyoctane (C34), modephene (C97), 4'-methylpropiophenone (C53), 2,6-di-tert-butyl-4-methyl-p-quinol (C110), o-xylene (C5), 3-methyl-undecane (C56), tridecane (C84), 2,5,6-trimethyldecane (C19), sabinene (C13), dodecane (C61), β-sesquiphellandrene (C113), 2,3-dimethyldecane (C52), 3,4-dimethylcumene (C63), and 4,7-dimethylundecane (C64). Among them, the first eight volatiles including thieno [2,3-c] pyridine (C71), 2,6,11-trimethyldodecane (C78), 2,3,4-trimethyldodecane (C79), 4,7-dimethylindan (C75), 2,6-dimethylundecane (C65), farnesane (C94), 2,3-dimethyldodecane (C92), and [but-2-en-2-yl] benzene (C51) showed higher discriminatory potential with VIP values greater than 1.5.

3. Materials and Methods

3.1. Chemicals

All solvents used were chromatographic grade. Phenylethyl acetate (internal standard) with purity greater than 99.0% was purchased from Sigma-Aldrich-Fluka (Buchs, Switzerland). The internal standard with a concentration of 22.9 µg/mL was prepared in acetonitrile. A standard series of n-alkanes (C8–C25) were provided by Dr. Ehrensorfer (Augsburg, Germany). Methanol (chromatographic grade purity) and acetonitrile (chromatographic grade purity) were purchased from Merck (LiChrosolv, Germany). All chemicals were stored at 4 °C until use. The SPME holder for manual sampling and fibers of 65 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 1 cm of length) were purchased from Supelco (Aldrich, Bellefonte, PA, USA).

3.2. Samples

The samples for GC-MS analysis are listed in Table 1. Natural Chinese cordyceps were collected from Kangding, Sichuan Province, China. The KD1223 strain of *O. sinensis* fungus isolated from the fruiting bodies of natural Chinese cordyceps was cultured in a 100 rpm shaker with potato dextrose liquid medium supplemented with 10% peptone (PPD) at 13 °C. The fungus was identified by molecular method using the internal transcribed spacer (ITS; ITS1-5.8S-ITS2) of nuclear ribosomal DNA amplification as described before. The identified *O. sinensis* strain was preserved at −80 °C at the Guangdong Institute of Applied Biological Resources, Guangzhou, China.

Artificial cultivation of fruiting bodies on rice media [6] or whole Chinese cordyceps by challenging *T. xiaojinensis* larvae with *O. sinensis* fungus [7,8,11] were established in low altitude Guangzhou, with mimicking environmental conditions. The insect species was identified using a molecular method by the amplification of the Cytochrome b sequences with the primers CB1 (TATGTACTACCATGAGGACAAATATC) and CB2 (ATTACACCTCCTAATTTATTAGGAAT) [46], as described previously [47,48]. The mummified cadavers with mycelia but without fruiting body (before stroma development), and the insect larvae and pupae with or without the injected blastospores (9

months for larvae or 9 months for pupae) were also used for the analysis. The existence of blastospores in the live larvae and pupae was confirmed by hemolymph microscopic examination.

A total of 50 individuals of each sample (for natural and artificial Chinese cordyceps, mummified cadavers, live larvae and pupae with or without blastospores), 30 g of fresh artificial fruiting bodies, and three flasks of fermentation cultures (150 mL/flask) were sampled. Samples were frozen at −80 °C overnight and lyophilized for 48–72 h by vacuum-freeze dryer (Alpha 1-2 LD plus, Marin Christ Gefriertrocknungsanlagen, Osterode, Germany) to consistent weight. The dried samples were grinded at 1000 rpm for 3 min by a multifunctional high-throughput tissue ball mill (GT100, Beijing Grinder Instrument Co., Ltd., Beijing, China) and stored at −80 °C. A quality control (QC) sample was prepared by mixing each collected sample in equal quantities and used for analytical method establishment and methodology examination.

3.3. GC×GC-QTOFMS Analysis for Volatile Components

The analysis of volatile composition and analytical method validation were referenced by the method of previous reports [19,21,24]. The volatile constituents of *O. sinensis* and host insects were analyzed by comprehensive two-dimensional gas chromatography (7890B-SSM1800, Agilent Technologies, Santa Clara, CA, USA and J&X Technologies, Shanghai, China) coupled with a high-resolution quadrupole time-of-flight mass spectrometry (QTOFMS) (7250, Agilent Technologies). First, 100 mg of samples were accurately weighed into a 20 mL vial, and then the SPME fiber that was equilibrated at 270 °C for 30 min in an autosampler (PAL RSI 120, CTC Technologies, Alexandria, VA, USA) was exposed to the headspace of the bottle for 20 min at 60 °C. Then, the SPME fiber was introduced into the GC splitless injector and kept there for 3.0 min to allow thermal desorption of the analytes. All samples were conducted in triplicate to check the repeatability and reliability of the method development. Reproducibility is expressed as the relative standard deviation (RSD). To compare the techniques of GC-MS and GC×GC-MS, a quality control sample (mix of each collected sample) was analyzed by both techniques under the same chromatographic conditions. The analytical system was equipped with simultaneous 1DGC and GC×GC in one instrument, which can conduct both techniques at the same time without any change of columns. The samples were introduced by a splitless injector (SSL) system equipped with an autosampler. Peak separation was performed on a weak-polar column HP-5 MS (5% phenyl-95% dimethylpolysiloxane, 30 m × 250 µm, 0.25 µm) in the first dimension and a more polar column DB-17 MS (50% phenyl-50% dimethylpolysiloxane, 1.2 m × 180 µm, 0.18 µm) in the second dimension (both from Agilent Technologies, USA).

The 1DGC and GC×GC conditions were the same. The GC injector was kept at 250 °C in splitless mode. The carrier gas was helium with a flow rate of 1.0 mL/min for the first dimensional column. The initial oven temperature was 50 °C; it was held for 3 min and then ramped at a rate of 4 °C/min to 230 °C and held for 1 min. For the GC×GC system, the carrier gas was helium with a flow rate of 3.14 mL/min for second dimensional column, and the cold zone temperature of modulator was set at −50 °C. The temperatures of the entry hot zone and exit hot zone were +30 and +120 °C relative to oven temperatures, respectively, with a cap temperature of 320 °C for both hot zones. The modulation period was 4 s.

The MS transfer line temperature was kept at 280 °C, and the ion source temperature was kept at 200 °C. Electron impact ionization was 70 eV. Data were collected as a mass range of 50–500 *m/z* at a sampling rate of 50 scan/s, and a solvent delay of 8 min was used.

3.4. Data Analysis

Qualitative and semi-quantitative methods primarily were referenced with similar reports [26,27]. Compound identification was based on mass spectra comparison with NIST 17 library (NIST/EPA/NIH 2017) with the minimum requirements of match factor above 800. Further confirmation was carried out using one-dimensional retention index (RI) and accurate mass, as described in many previous studies [49]. In order to compare the reference RI values with experimental RI values obtained in this

work, a standard mixture of n-alkanes (C8–C25) was injected (0.5 μL) in the GC×GC-QTOFMS system under the same conditions used for the samples. The semi-quantitative method was performed based on peak area normalization. The 1-DGC data were processed using Agilent Mass Hunter Qualitative Analysis Navigator B.08.00. The GC×GC data were analyzed by a dedicated GC×GC data processing software Canvas (V1.4.0, J & X Technologies).

To visualize the clustering among categories and identify the differentially changed components responsible for the separation, supervised partial least squares discriminant analysis (PLS-DA) and variable importance in projection (VIP) score were carried out using SIMCA 14.1 software (Umetrics, Umea, Sweden). A data set consisting of a 16×120 matrix was conducted by PLS-DA. The rows represent the samples analyzed and the columns represent the relative contents of the volatile metabolites determined by GC×GC-QTOFMS. All variables were scaled with unit variance (UV) prior to PLS-DA. To gain the chemical markers for discrimination of the three groups in the PLS-DA model, VIP values were calculated and inspected for identified volatile compounds. Generally, VIP values > 1 and $p < 0.05$ are considered as significant contributors to the model [40,50,51]. In this study, seven-fold cross-validation and 200 response permutation testing (RPT) methods were used to investigate the quality of the model.

4. Conclusions

This study presents the volatile metabolite profiles by HS-SPME-GC×GC-QTOFMS from *O. sinensis* fungus and insect host-based products. A total of 120 volatile compounds including 36 alkanes, 25 terpenes, 17 aromatic hydrocarbons, 10 ketones, 5 olefines, 5 alcohols, 3 phenols, and 19 other compounds were identified. There are great differences in the volatile compounds among the three categories of *O. sinensis* fungus, *Thitarodes* hosts of *O. sinensis*, and the Chinese cordyceps. In general, natural and mature artificial Chinese cordyceps (fungus–insect complexes), fruiting bodies, and fermented products had more volatile compounds than insect larvae, insect pupae, and immature artificial Chinese cordyceps. Twenty-four volatile compounds were identified in all the 16 samples, including 4-carene (C10), 3-carene (C18), 2,2,4,4-tetramethyloctane (24), 2-propyltoluene (C29), 2,4,6-trimethyldecane (C31), 2,6-dimethyl-6-trifluoroacetoxyoctane (C34), linalool (C41), 2-nonen-1-ol (C42), 6-ethyl-2-methyloctane (C44), (+)-α-terpineol (C61), 2,6-dimethylundecane (C65), 2,8-dimethylundecane (C68), 4-methyldodecane (C73), 4,7-dimethylindan (C75), 2,3-dimethylundecane (C76), 2,4-dimethyldodecane (C77), 2,6,11-trimethyldodecane(C78), 4,6-dimethyldodecane (C84), 2,3,5,8-tetramethyldecane (C86), farnesane (C94), tetradecane (C97), seychellene (C108), 2,6-di-tert-butyl-4-methyl-p-quinol (C109), and hexadecane (C118). Alkanes are the dominant volatile compounds in all products. 2,5,6-trimethyldecane and 2,6,7-trimethyldecane are the major volatile compounds in all products except the larval ones, while 2,2,4,4-tetramethyloctane dominates in the larval products. From the view of the differences in volatile compounds, it seems that the fermented products are not the same as the natural and artificial Chinese cordyceps. Based on the volatile compounds, three classes (*O. sinensis* fungus, *Thitarodes* insect, and fungus–insect complexe) were confirmed by partial least squares-discriminant analysis (PLS-DA). Thieno [2,3-c] pyridine, 2,6,11-trimethyldodecane, 2,3,4-trimethyldodecane, 4,7-dimethylindan, 2,6-dimethylundecane, farnesane, 2,3-dimethyldodecane, and [but-2-en-2-yl] benzene are potential discriminatory compounds. The present results suggested that HS-SPME-GC×GC-QTOFMS combined with multivariate data analysis is an ideal method for analyzing and distinguishing different *O. sinensis* and insect hosts-based products. The information provided in this study is of importance for the further identification of bioactive components and for proposals of possible mechanisms to obtain those bioactive compounds in a different form than the traditional fungus-insect interaction.

Author Contributions: R.H. and X.Q. conceived and designed the experiments; L.C. and X.Q. prepared experimental samples; X.Q. performed the experiments and analyzed the data; X.Q. and R.H. wrote the manuscript. All the authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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

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Sample Availability: Samples of the different *Ophiocordyceps sinensis* and insect host products in this study are available from the authors.