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Analysis and Identification of Differences in Volatile Components of Various Alfalfa Seeds Based on GC-IMS

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Abstract: Volatile components are one key factor in sample identification, differential analysis, quality control and origin traceability. In order to identify and analyze the differences in volatile substances in different alfalfa seeds, this study used gas chromatography–ion mobility spectrometry (GC-IMS), combined with the Gallery Plot plug-in, and PCA, PLS-DA and other analysis methods. In this way, a comprehensive analysis of volatile components in 10 alfalfa seeds, including aerospace varieties, was conducted. A comparative analysis of the characteristics of different sample compounds using topographic maps and fingerprints led to isolation of 48 kinds of 54 volatile compounds. Among them, esters (9 types), olefins (8 types), ketones (8 types), alcohols (6 types) and aldehydes (6 types) were found to be the most abundant volatile compounds in alfalfa seeds. At the same time, PCA and PLS-DA analysis models showed that esters, ketones and alcohols were the main volatiles causing the differences among alfalfa seeds. Among them, the content of various substances in the ZT2 and ZT3 aerospace varieties were higher than that of other varieties, while the types and contents of volatiles in ZT1, ZM2 and GN3 were relatively low. Therefore, in combination with the differences in maturity of each sample, the 10 varieties of alfalfa were finally divided into three categories, and the varieties of the same series were basically classified into one category. This provides a basis and convenience for future seed screening, identification, traceability and forage breeding.

Keywords: VOCs; GC-IMS analysis; *alfalfa* seeds; PCA analysis; PLS-DA analysis; map

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1. Introduction

Alfalfa (*Medicago sativa* L.) is a high-quality leguminous plant, known as the "King of herbage", and plays a very important role in agriculture and animal husbandry [1–4]. Because of its wide adaptability, high yield, good quality and resistance to frequent plugging and unplugging, it is an important source of pasture for animal husbandry all over the world [5,6]. *Alfalfa* has high feed value, economic value and soil improvement value, and is highly valued and sought after by various countries [7,8]. Among them, the United States and Spain are major industrial countries of *alfalfa* pasture [9].

In recent years, China's *alfalfa* industry has made great progress. Breeding technology has been increasingly perfected, and many new varieties have been developed that are cold resistant, drought resistant and salt-alkali resistant [10]. *Alfalfa* is currently being studied intensively by various researchers [11]. This includes research on biotic and abiotic stresses in *alfalfa* and research on the microbial composition and variety improvement of *alfalfa* [12–14]. At the same time, with the continuous improvement of aerospace breeding technology, new varieties of *alfalfa* for aerospace breeding have also appeared. Comparison and exploration of this variety bred in a space environment with varieties grown under ordinary breeding will become one of the focuses of *alfalfa* research in the future.

Alfalfa seeds are not only the key to *alfalfa* breeding, but research has shown that the components of *alfalfa* seeds are complex, and the butanol component has a therapeutic effect on rheumatoid arthritis [15]. In addition, *alfalfa* seeds contain a variety of nutrients and medicinal ingredients. Its polysaccharides, flavonoids and other compounds have anti-inflammatory and anti-aging effects [16]. As a major forage resource, *alfalfa* plays a role in hybrid breeding, pharmaceutical preparations and cosmetics [17]. Therefore, it is necessary to study its volatile organic compounds (VOCs) for preliminary classification and identification, and to conduct subsequent research on its metabolomics and other aspects. Some researchers have shown that many VOCs emitted by seeds are reactive and may be toxic to seeds, and continued reactions will accelerate the deterioration of seeds [18]. Analysis of VOCs has become a feasible method to evaluate molecular markers that influence seed mass loss [19].

Gas chromatography–ion mobility spectrometry (GC-IMS) is a popular technology currently used in food health inspection, drug supervision and biological environment monitoring [20,21]. It enables more efficient and accurate determination of volatile organic compounds in *alfalfa* seeds. At the same time, we combined GC-IMS with principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) models to study the characteristic substances in different *alfalfa* seeds and establish the fingerprints of volatile organic compounds in *alfalfa* seeds [22]. On the one hand, this lays a foundation for exploring the differences between *alfalfa* under aerospace breeding and ordinary breeding. On the other hand, it provides a reference basis for rapid identification and quality identification of *alfalfa* seeds, and provides new ideas for seed traceability and screening breeding.

2. Materials and Methods

2.1. Sample Collection and Preparation

The *alfalfa* seeds selected for this study came from the Lanzhou Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy of Agricultural Sciences. Among them, ZT1 (*Medicago sativa* L. cv. Zhongtian No. 1), ZT2 (*Medicago sativa* L. cv. Zhongtian No. 2) and ZT3 (*Medicago sativa* L. cv. Zhongtian No. 3) are high-yield *alfalfa* varieties bred from the *alfalfa* carried on the Shenzhou 11 spacecraft. ZL1 (*Medicago sativa* L. cv. Zhonglan No. 1) and ZL2 (*Medicago sativa* L. cv. Zhonglan No. 2) are disease-resistant and stress-resistant *alfalfa* varieties newly developed by the institute. ZM1 (*Medicago sativa* L. cv. Zangmu No. 1) and ZM2 (*Medicago sativa* L. cv. Zangmu No. 2) are *alfalfa* varieties newly developed by the institute that are suitable for growing under plateau conditions. LD (*Medicago sativa* L. cv. Longdong), GN (*Medicago sativa* L. cv. Gannong No. 3) and XM2 (*Medicago sativa* L. cv. Xinmu No. 2) are *alfalfa* varieties grown locally for a long time. The selected varieties of *alfalfa* have different advantages and characteristics, and all seeds came from the same experimental base. They were planted on 20 March 2022 and harvested for seed on 15 July 2022 after maturity. The planting area was divided into small areas of 3×5 m^3, with a row spacing of 33.3 cm, 15 rows and a seeding rate of 30 g/15 m^3. Each variety was planted in 3 small areas, planted at the same time and harvested at the same time, so that the external growing conditions were kept as uniform as possible. After collection, samples were stored in a -80 °C freezer until analysis. During sample preparation, plump particles with no loss and a similar size were selected. After removing surface impurities, 0.500 g (accurate to 0.001 g) of the sample was weighed and placed in a 20 mL headspace bottle. A total of 3 samples were taken from each group and used to perform 3 parallel determinations.

2.2. GC-IMS Analysis Methods and Conditions

This analysis refers to the method of Li et al. [23] using an Agilent 490 gas chromatograph equipped with an automatic sampling device (Agilent Technologies, Palo Alto, CA, USA) and an IMS instrument (FlavourSpec®, Gesellschaft für Analytische Sensorsysteme mbH, Dortmund, Germany) for the detection of volatile compounds in samples of differ-

ent varieties of *alfalfa*. The incubation temperature was 80 °C, the incubation time was 20 min and the incubation speed was 500 rpm. The needle temperature was 85 °C, the injection volume was 200 μL, the cleaning time was 0.5 min and the sample was injected in splitless mode.

GC conditions: FS-SE-54-CB-1 quartz capillary column: (15 m × 0.53 mm, 0.5 m, inner diameter: 0.50 μm); chromatographic column temperature: 60 °C; carrier gas: N_2 (purity ≥ 99.999%). The temperature of the chromatographic column was 40 °C. The carrier gas was high-purity nitrogen (purity ≥ 99.999%). The carrier gas flow program was: initial 2.0 mL/min, keep for 2 min, increase linearly to 10.0 mL/min at 2–10 min, linearly increase to 100.0 mL/min at 10–20 min, keep at 100.0 mL/min at 20–30 min and then stop the flow for a total run time of 30 min.

IMS conditions: Drift tube length: 98 mm; drift tube temperature: 45 °C; drift gas: nitrogen (purity ≥ 99.999%); drift gas flow rate: 150 mL/min; linear voltage in the tube: 500 V/cm. The radioactive source is: beta ray (tritium); the mode is: positive ion mode; the average number of spectral scans is: 12 times.

2.3. Statistical Analysis

In this study, the laboratory analysis software VOCal 0.1.0 was used to compare the differences in the spectra at different angles, and to select useful characteristic peak areas. Qualitative analysis of substances was aided by the National Institute of Standards and Technology (NIST) and the IMS databases in the GC-IMS library search software. The spectral differences between samples were compared by visualizing the two-dimensional top view and three-dimensional spectra of the Reporter plug-in of the GC-IMS [24] and the specific content differences of volatile organic compounds between samples were compared using the fingerprint results in the Gallery Plot plug-in. The Dynamic PCA plug-in was used for data plotting, and PCA and K-means cluster analysis were performed on the data. The PLS-DA model was established using SIMCA 14.1. All data were analyzed using SPSS 18.0. At the same time, MetaboAnalyst 5.0 and Excel 2021 were used for auxiliary data processing.

3. Results

3.1. Analysis of HS-GC-IMS Topographic Map Differences of Volatile Compounds in Different Varieties of Alfalfa Seeds

In this study, 10 *alfalfa* seed samples were rapidly analyzed by gas chromatography–ion mobility spectrometry (GC-IMS). With the help of the Reporter plug-in that comes with the LAV 2.2.1 software, the GC-IMS three-dimensional comparative spectra of the volatile components of different *alfalfa* seed samples were obtained (Figure 1). Among them, the darker the color, the higher the component content. It can be intuitively observed from Figure 1 that the types of volatile organic compounds in different *alfalfa* seeds are relatively similar, but there are certain differences in the size and color of different ion peaks. It shows that the content of the same substance in different samples has certain differences.

The three-dimensional topography of the sample is represented as a two-dimensional top view. As shown in Figure 2, the red vertical line at 1.0 ms on the abscissa is the reactive ion peak (RIP peak), and each point on both sides of the RIP peak represents a volatile substance. The results show that the drift time is between 1.0–3.85 ms, and most of the volatile components complete the time migration within 100–250 s. The drift time of volatiles with higher content in seeds is mainly concentrated around 100 s and 240 s. According to the color distribution and depth difference, it is shown that the peak signal intensity of ZT2 and ZT3 seeds during the migration time is higher, and the volatiles show diversity. The peak signal intensity of seeds ZT1 and GN3 is low. The peak signal intensities of other samples are similar. This shows that there are certain differences in the content of volatile substances among seeds.

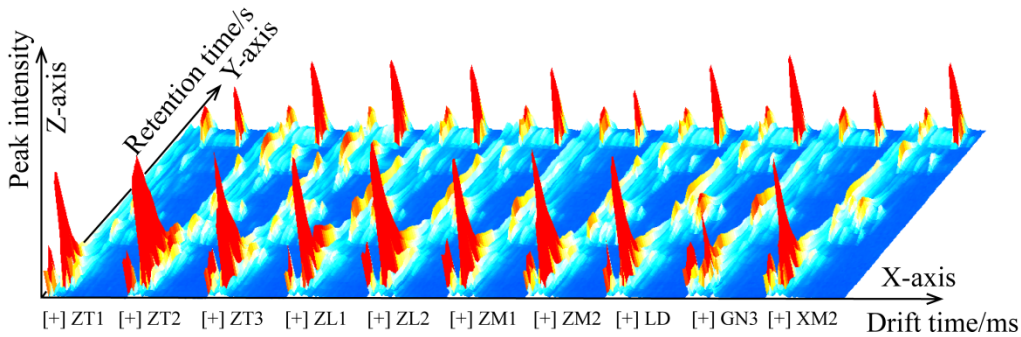

Figure 1. Three-dimensional topography of volatile components in *alfalfa* seeds. Note: In the three-dimensional topography, the X-axis represents the ion migration time, the Y-axis represents the retention time of the sample components and the Z-axis represents the ion peak intensity. The redder the colour, the higher the volatile compound content of the sample.

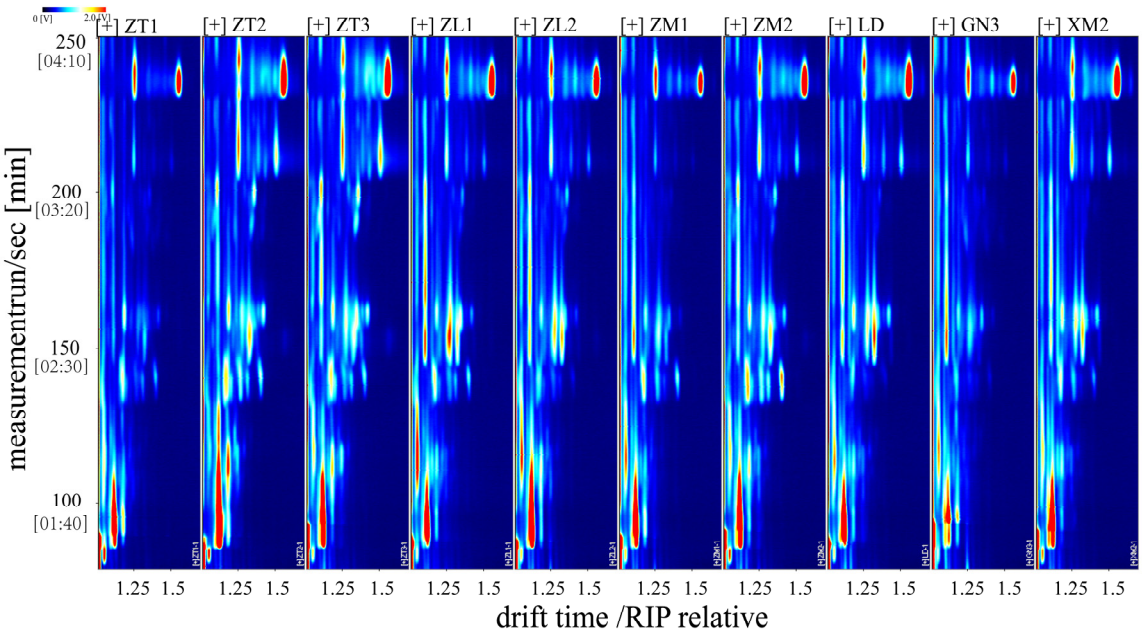

Figure 2. Two-dimensional topographic map of volatile components of *alfalfa* seeds. Note: In the figure, the horizontal axis represents the ion migration time, the vertical axis represents the retention time of the volatile components of the sample and the color represents the signal intensity of the substance. White indicates low intensity, red indicates high intensity and darker colors indicate higher intensity.

Based on the two-dimensional and three-dimensional topographic map results, this study uses the ZT1 seed as a reference, and the relative difference results of the components among various seeds are shown in the figure (Figure 3). The ZT2 and ZT3 seeds have the highest relative content of volatile substances and the most types. The main differences are concentrated in the two migration times of 150 s and 200–250 s. The volatile content of seeds ZL1 and ZL2 is relatively similar. There is a big difference in the volatile content between ZM1 and ZM2. The spectra of LD and XM2 are relatively close, and the relative contents of ZM1 and GN3 are the lowest. The above results show that the volatiles of aerospace-bred seeds are significantly higher than those of common varieties. There are significant differences in the volatile content of seeds under different breeding conditions, which can be further analyzed to classify and identify seeds.

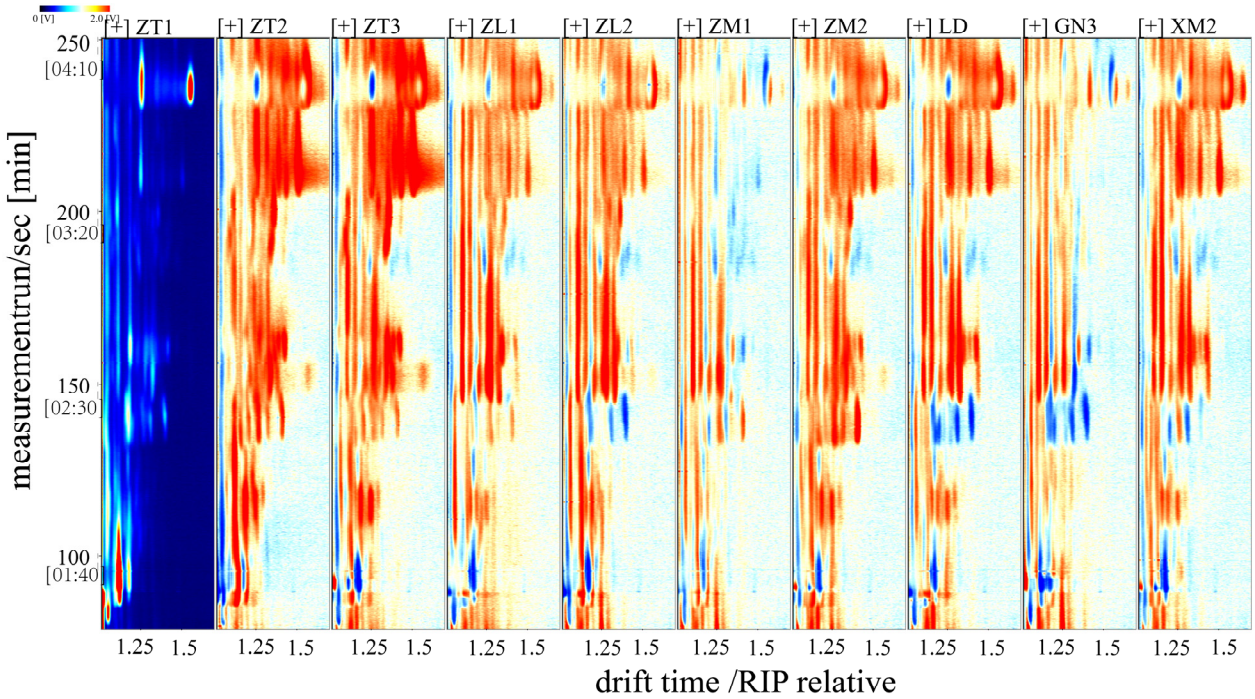

Figure 3. Two-dimensional difference map of volatile components of *alfalfa* seeds. Note: In the figure, the control group is sample ZT1, red means that the content of the same substance is higher than that of the control group and blue means that the content of the same substance is lower than that of the control group.

3.2. Qualitative Analysis of Volatile Compounds in Different Varieties of Alfalfa Seeds

Based on the above differential spectra, in order to further explore the specific volatile components of different samples, the NIST database and IMS database in the GC-IMS Library Search were used to qualitatively identify the volatile substances [25]. Figure 4 represents the spectrum of the qualitative determination results of the volatile substances in the ZT3 sample seed. In the figure, each calibrated value represents a volatile substance, and missing values indicate that the content of such substances in this sample is too low or not detected. According to the results, it can be seen that there are obvious differences in the color depth of different volatiles, which indicates that the contents of different volatiles are different. The distribution of each point in the spectrum shows that the separation results of the overall volatiles are better, and each substance can be clearly distinguished, which is beneficial to the classification and identification of samples.

Based on the above spectra, this study detected a total of 48 types of 54 qualitative volatile substances in different *alfalfa* seeds using substances already included in the NIST and IMS databases. These included monomers and dimers with six compounds (propyl hexanoate, 1.8-Cineole, Heptanal, Hexanal, 1-propene-3-methylthio, Octamethylcyclotetrasiloxane). This phenomenon is due to the fact that compounds with high proton affinity can cause ions to form dimers as they move in the drift cell, with similar retention times but different migration times [26,27]. The specific results are shown in Table 1. The above-mentioned 48 kinds of volatile substances were classified and analyzed statistically, and the substances divided into 13 different types. Among them, there are nine kinds of esters, eight kinds of hydrocarbons, eight kinds of ketones, six kinds of alcohols, six kinds of aldehydes, two kinds of heterocyclic compounds, acids and furans, and ethers and terpenes. There is one each of alkenes, silicides, chlorides and organic bases. Among them, there are nine kinds of ester compounds, eight kinds of hydrocarbon compounds, eight kinds of ketone compounds, six kinds of alcohol compounds, six kinds of aldehyde compounds and two kinds of heterocyclic, acid and furan, compounds, while ethers, terpenes, silicides, chlorides and organic bases each have one. This indicates that the volatile components in

alfalfa seeds are relatively diverse, which provides a basis for reference and comparison for subsequent classification and identification.

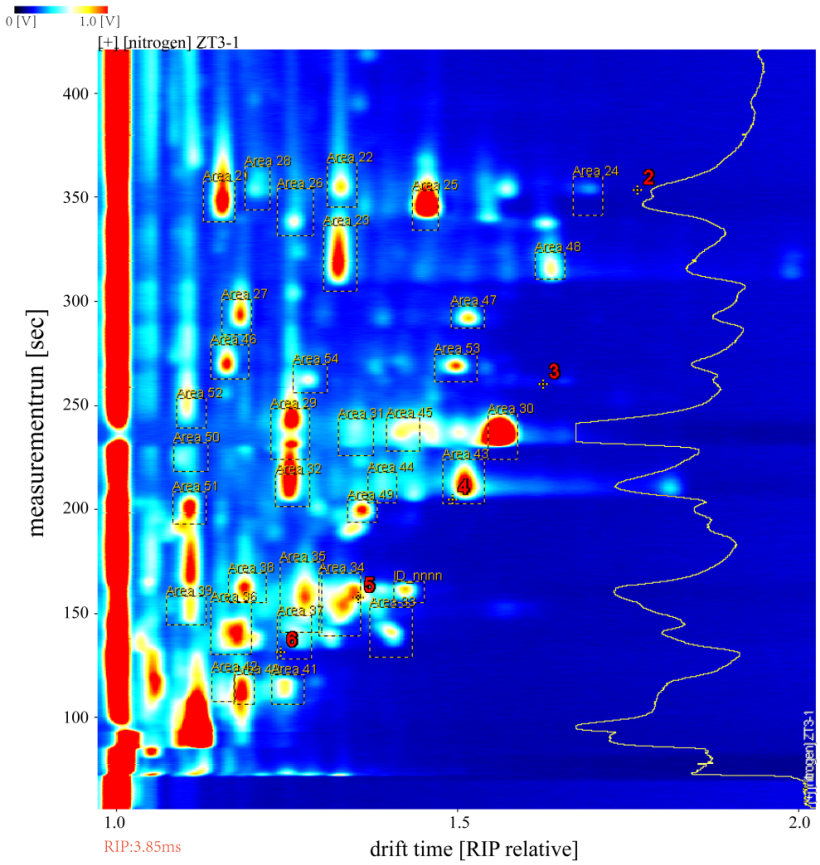

Figure 4. Qualitative spectrum results of volatile substances using GC-IMS. Note: The figure shows the identification results of ZT3 *alfalfa* seeds, and each framed area represents a defined substance.

Table 1. Qualitative results of volatile substances in different *alfalfa* seeds.

Count	Compound	CAS#	Formula	MW	RI	Rt [s]	Dt [RIP Rel]
		Esters					
1	propyl hexanoate (M)	C626777	$C_9H_{18}O_2$	158.2	1095.9	744.70	1.47585
2	propyl hexanoate (D)	C626777	$C_9H_{18}O_2$	158.2	1096	745.00	1.94982
3	Methyl cyanoacetate	C105340	$C_4H_5NO_2$	99.1	886.5	343.95	1.46022
4	Pentafluoropropionic acid, hexyl ester	C134639013	$C_9H_{13}F_5O_2$	248.2	893	352.15	1.69438
5	Acetic acid, 2-methylpropyl ester	C110190	$C_6H_{12}O_2$	116.2	781.3	235.11	1.42551
6	Trifluoroacetic acid, isobutyl ester	C17355838	$C_6H_9F_3O_2$	170.1	655.8	139.93	1.18171
7	S-methyl ethanethioate	C1534083	C_3H_6OS	90.1	682.2	150.89	1.10985
8	methyl acrylate	C96333	$C_4H_6O_2$	86.1	581.3	113.09	1.15077
9	3-methylpentane	C96140	C_6H_{14}	86.2	575	111.07	1.18570
10	Propyl acetate	C109604	$C_5H_{10}O_2$	102.1	652.4	138.59	1.26556
		Hydrocarbons					
11	Butylbenzene	C104518	$C_{10}H_{14}$	134.2	1051.2	635.43	1.44650
12	1,3,5-trimethylbenzene	C108678	C_9H_{12}	120.2	987.2	504.93	1.17659
13	Propylbenzene	C103651	C_9H_{12}	120.2	953.4	443.63	1.25661
14	m-Methylstyrene	C100801	C_9H_{10}	118.2	987.9	506.20	1.07917
15	Styrene	C100425	C_8H_8	104.2	894.3	353.87	1.32933
16	c-1,2-Dimethylcyclopentane	C1192183	C_7H_{14}	98.2	731	186.69	1.48007
17	t-1,2-Dimethylcyclopropane	C20520643	C_5H_{10}	70.1	519.3	94.74	1.18570
18	2,2,4,4-Tetramethylpentane	C1070877	C_9H_{20}	128.3	773.4	226.74	1.10278

Table 1. *Cont.*

Count	Compound	CAS#	Formula	MW	RI	Rt [s]	Dt [RIP Rel]
		Ketones					
19	Acetophenone	C98862	C_8H_8O	120.2	1063.3	663.18	1.33408
20	5-nonanone	C502567	$C_9H_{18}O$	142.2	1058.5	652.03	1.19062
21	Furaneol	C3658773	$C_6H_8O_3$	128.1	1071.6	683.07	1.22718
22	3-Octanone	C106683	$C_8H_{16}O$	128.2	991.6	513.35	1.25225
23	4,5-dihydro-3(2H)-thiophenone	C1003049	C_4H_6OS	102.2	979.5	490.23	1.15626
24	2,6 dimethyl-4-heptanone	C108838	$C_9H_{18}O$	142.2	957.7	450.97	1.15335
25	2-Hexanone	C591786	$C_6H_{12}O$	100.2	770.3	223.50	1.25262
26	2,3-pentanedione	C600146	$C_5H_8O_2$	100.1	692.6	156.49	1.26955
		Alcohols					
27	1.8-Cineole (M)	C470826	$C_{10}H_{18}O$	154.3	1049.6	631.82	1.25368
28	1.8-Cineole (D)	C470826	$C_{10}H_{18}O$	154.3	1040	610.53	1.40812
29	alpha-Terpineol	C98555	$C_{10}H_{18}O$	154.3	1164.9	951.50	1.26643
30	n-Hexanol	C111273	$C_6H_{14}O$	102.2	863.3	316.45	1.32465
31	4-Methyl-2-pentanol	C108112	$C_6H_{14}O$	102.2	745.9	199.84	1.36425
32	3-methylbutan-1-ol	C123513	$C_5H_{12}O$	88.1	731.3	186.89	1.23836
33	(Z)-2-Penten1ol	C1576950	$C_5H_{10}O$	86.1	756.4	209.73	1.25374
		Aldehydes					
34	Benzaldehyde	C100527	C_7H_6O	106.1	968.9	470.82	1.19116
35	Heptanal (M)	C111717	$C_7H_{14}O$	114.2	887.9	345.67	1.15189
36	Heptanal (D)	C111717	$C_7H_{14}O$	114.2	894	353.44	1.57075
37	Hexanal (M)	C66251	$C_6H_{12}O$	100.2	781.7	235.53	1.25564
38	Hexanal (D)	C66251	$C_6H_{12}O$	100.2	782.7	236.66	1.56163
39	2,4,6-Trimethyl-1,3,5-trioxane	C123637	$C_6H_{12}O_3$	132.2	756.8	210.15	1.51448
40	(E)-2-pentenal	C1576870	C_5H_8O	84.1	758.3	211.57	1.38775
41	1,3,5-Trioxane	C110883	$C_3H_6O_3$	90.1	651.9	138.37	1.31147
		Heterocyclic compound					
42	Oxirane, 2,3-dimethyl-, trans-	C21490631	C_4H_8O	72.1	526.6	96.75	1.11983
43	Oxetane, 2-methyl-	C2167397	C_4H_8O	72.1	581.9	113.31	1.24859
		Acid					
44	2-methylbutanoic acid	C116530	$C_5H_{10}O_2$	102.1	844.4	295.69	1.18264
45	butanoic acid	C107926	$C_4H_8O_2$	88.1	782.1	235.96	1.35384
		Furan					
46	Furan, 2-ethenyl-	C1487189	C_6H_6O	94.1	746.9	200.78	1.10685
47	2-ethyl furan	C3208160	C_6H_8O	96.1	708.8	168.57	1.11184
		Ethers					
48	1-propene-3-methylthio(M)	C10152768	C_4H_8S	88.2	697.2	159.84	1.34740
49	1-propene-3-methylthio(D)	C10152768	C_4H_8S	88.2	698.1	160.51	1.42825
		Terpenes					
50	Limonene	C138863	$C_{10}H_{16}$	136.2	1044.1	619.55	1.16778
		Silicide					
51	Octamethylcyclotetrasiloxane (M)	C556672	$C_8H_{24}O_4Si_4$	296.6	1004.3	537.80	1.40826
52	Octamethylcyclotetrasiloxane (D)	C556672	$C_8H_{24}O_4Si_4$	296.6	1005.4	539.86	1.82381
		Chloride					
53	1,2-Dichloroethane	C107062	$C_2H_4C_{l2}$	99.0	653.6	139.04	1.40530
		Organic base					
54	N,N-diethylethanamine	C121448	$C_6H_{15}N$	101.2	700.2	162.08	1.19269

Note: M in the table represents the monomer of the compound, and D represents the dimer of the compound. MW represents the molecular weight of the compound, RI represents the retention index in the capillary gas chromatography column, Rt [s] represents the retention time calculated with orthoketone C4–C9 as the external reference and Dt [RIP rel] represents the migration time in the drift tube. CAS# indicates the CAS number.

3.3. Gallery Plot Fingerprint Analysis of Volatile Compounds in Different Varieties of Alfalfa Seeds

In order to more intuitively and quantitatively compare the differences of volatile organic compounds between different samples, the qualitative results of the above substances were compared with the actual samples one by one, so as to find out the representative classification characteristics and specific substances. In this study, fingerprints were drawn using the Gallery Plot plug-in that comes with the LAV software. As shown in Figure 5,

each point represents a volatile compound. In the figure, it can be found that different samples have their own characteristic peak areas. Among them, the fingerprints of samples ZT2 and ZT3 have a high degree of similarity, and their overall content is significantly higher than that of other varieties, especially considering the total content of 5-nonanone to n-Hexanol in Area A, where 15 substances are significantly higher than other varieties. The content of t-1,2-Dimethylcyclopropane in ZT1 is higher than other varieties. The contents of methyl acrylate and 2,3-pentanedione in ZL1 are significantly higher than those in other varieties. ZL2 and ZM1 have no particularly significant substances. The contents of 1,2-Dichloroethane, 1.8-Cineole(M), Propyl acetate, Trifluoroacetic acid and isobutyl ester in ZM2 are significantly higher than other samples. The content of propyl hexanoate (M) in the LD sample is higher than that of other varieties, the content of Octamethylcyclotetrasiloxane (D) in XM2 is the highest among all samples and the spectral characteristics of the two are similar. There is no relatively high content of volatiles in sample GN3, which is one of the samples with the lowest overall substance content.

In addition, the fingerprint results show that the five volatiles of propyl hexanoate (M), Octamethylcyclotetrasiloxane (M), 2,6 dimethyl-4-heptanone, Hexanal (M) and Styrene in Area B are significantly higher in the 10 samples. The content is high, and the difference is small. In contrast, c-1,2-Dimethylcyclopentane and Methyl cyanoacetate are the two substances with the lowest overall content in the profile, and the variability between samples is also small. Other volatiles have significant differences among different samples ($p < 0.05$). Based on Figures 5b and S1, ZT2 and ZT3 had the most types and the highest content of volatile substances, while ZM1 and GN3 had lower types and contents of volatile substances. The above differential results show that there are great differences in the volatiles of *alfalfa* seeds under different breeding conditions. Especially ZT2 and ZT3, the aerospace breeding varieties, have more obvious differences in seed volatility than other varieties under breeding.

3.4. Principal Component Analysis of Volatile Compounds

Based on the above spectral results, this study further conducted principal component analysis (PCA) on the detected substances [28]. After preprocessing the original data through normalization and centering, the PCA analysis of volatile components was obtained as shown in Figure 6a. The explained variance of PC1 is 46.58%, the explained variance of PC2 is 17.36% and the cumulative contribution rate is higher than 60%, indicating that this model can be used as a separation model. Through the calculation of K-means clustering, the p value is $0.032 < 0.05$, indicating that there is a significant difference between the groups that can be used for analysis. The R value is 0.1427, indicating that there is an overall positive correlation between the groups. As shown in Figure 6b, the K-means unsupervised method was used to divide the PCA results of the 10 samples based on the first two principal component analyses into three groups through Euclidean distance. ZL1, ZL2, XM2 and LD are in group 1, ZT2 and ZT3 are in group 2 and ZT1, ZM1, ZM2 and GN3 are in group 3. The volatile compound compositions of samples within each group are similar, while the compounds between different groups differ significantly. The distribution of specific compounds in different groups is shown in Figure 6c. The origin of the Y coordinate represents the average proportion of each substance in the whole. Positive values mean it is higher than the average, and negative values mean it is lower than the average. At the same time, this study made a double graph (Figure S2) of the distribution of the PCA samples and volatiles, and analyzed it in conjunction with Figures 6c and S2. Among the samples in group 2, the proportion of overall compounds is higher; especially the content of ketones and alcohols is much higher than that of other groups. While the content of ester compounds in the samples of group 1 is relatively high, the overall content of samples in group 3 is low. This is consistent with the previous fingerprint results.

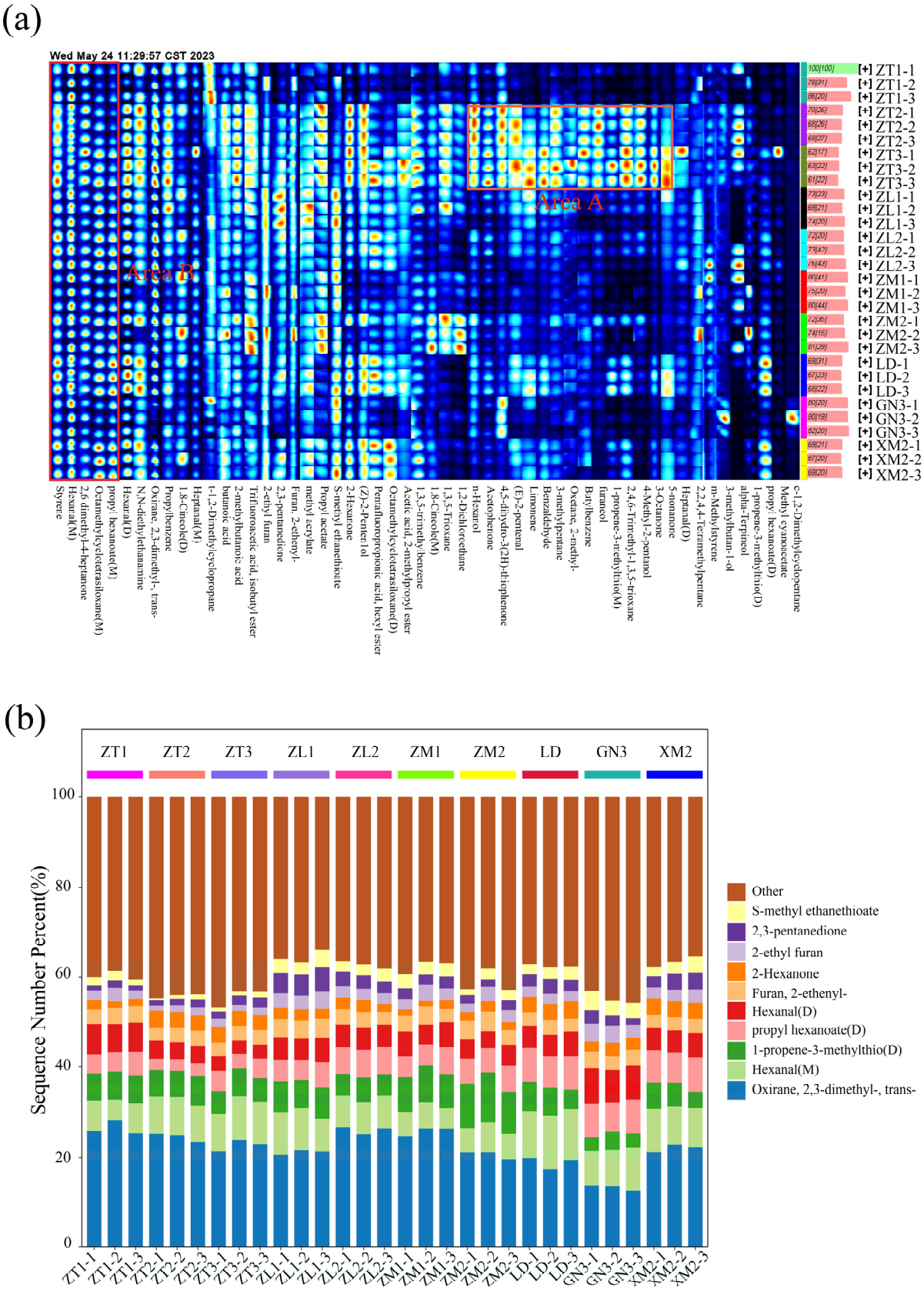

Figure 5. GC-IMS fingerprints and abundance maps of the top ten volatiles in different *alfalfa* seed samples. Note: Each column in (**a**) represents the same substance in all samples, and each row represents all substances in a sample. The darker the color, the higher the substance content. (**b**) shows the abundance maps of the top ten volatiles.

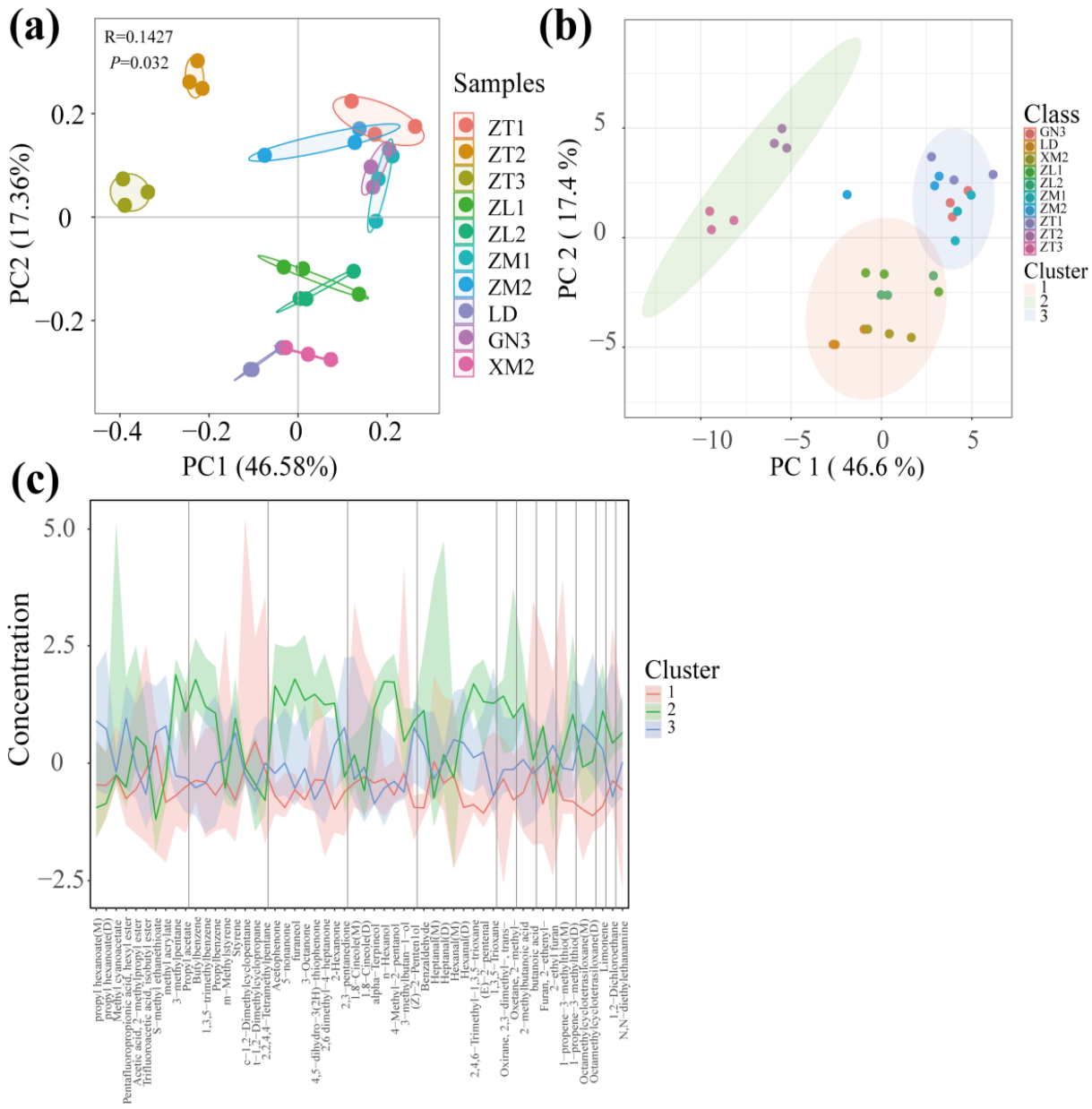

Figure 6. PCA analysis results of volatile compounds in different samples. Note: (**a**) is the PCA plot of different samples. (**b**) is the K-means cluster analysis of 10 samples. (**c**) shows the distribution of compounds under different clusters. In (**a**,**b**), PC1 and PC2 represent the contribution of the first two principal components, and the R value and *p* value represent the correlation coefficient and significance coefficient calculated by K-means clustering. (**b**,**c**) show the 10 samples divided into three groups with significant differences by Euclidean distance, and the distribution of volatiles under different clusters.

3.5. PLS-DA Model Analysis of Sample Volatile Compounds

Based on the results of the PCA analysis, this study constructed a PLS-DA model. PLS-DA also performed normalization and centering preprocessing on the original data. Meanwhile, cross-validation and permutation tests were used in PLS-DA to evaluate the performance metrics of the models [29]. In this study, 54 qualitative compounds were used as x variables, and different varieties of *alfalfa* seeds were used as y variables to establish a PLS-DA correlation model. According to this model, all samples could be divided into three categories, among which the aerospace varieties ZT2 and ZT3 had the best separation effect and were classified into one category. Other varieties were divided

into two categories based on the explained variance of PC2, and the sample classification was basically consistent with PCA. The model is shown in Figure 7a, where R^2 = 0.82485, Q^2 = 0.68559 and both are greater than 0.5, indicating that the model is more stable. The explained variance of PC1 is 40.3%, the explained variance of PC2 is 22% and the total explained variance of the first two principal components is higher than 60%, indicating that the model explanation degree is good. From the permutation test results in Figure 7b, it can be seen that after as many as 200 instances of cross-validation, the regression line of model Q^2 intersects with the abscissa, and the original value on the right is generally higher than the values of Q^2 and R^2 on the left. The slopes of the regression lines of R^2 and Q^2 are both greater than 1, and the intercept of the regression line of Q^2 is negative, indicating that the model has no overfitting phenomenon and has good predictive ability [30].

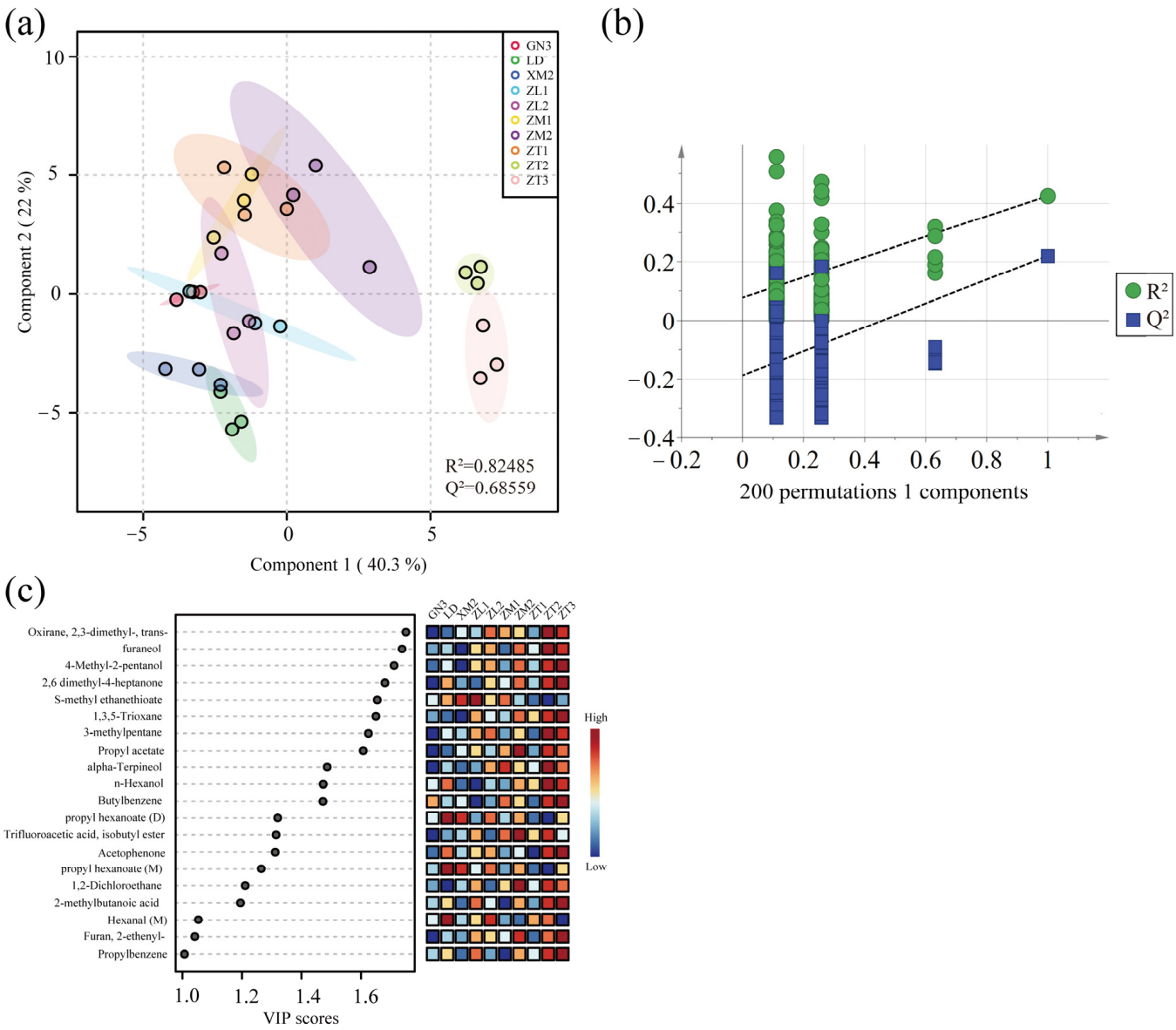

Figure 7. PLS-DA model of volatile compounds in different samples. Note: (**a**) is the score chart of the PLS-DA model for different samples, R^2 is the fitting ability of the model and Q^2 is the estimation of the predictive ability of the model. It is better to be greater than 0.5, and the closer to 1, the better. (**b**) shows the confidence test results of different samples; the abscissa indicates the degree of retention of the sample during the permutation test and the ordinate indicates the value of R^2/Q^2. (**c**) shows the VIP score results of different samples.

In order to further analyze the differential composition of samples, this study calculated and tested the variable importance in projection (VIP) score of the PLS-DA model. As shown in Figure 7c, the larger the VIP value, the more significant the difference of the volatile compound under different samples, and VIP > 1 is usually used as the screening index [31]. A total of 20 (VIP > 1) marker volatile compounds were screened in this study. Among them, there are five types of ester compounds, three types of ketone compounds, three types of alcohol compounds, two types of olefins and aldehydes and one each of acids, furans, heterocyclic compounds and chlorides. Oxirane, 2,3-dimethyl-, trans- has the largest VIP value of 1.751, indicating that this substance is the most important differentiating factor. In addition, esters, ketones and alcohols are the three main types of substances that affect the differences of different samples, and the PCA cluster analysis also shows that the distribution of these three types of substances is quite different.

3.6. GC-IMS Correlation Analysis Based on Seed Maturity

Based on the above PCA and PLS-DA model analyses in this study, a seed maturity analyzer was used to measure the chlorophyll fluorescence intensity in the seed coats of different samples. The lower the fluorescence intensity, the lower the chlorophyll content and the higher the seed maturity. On the contrary, the maturity of the seeds is lower, so the maturity of the seeds is obtained [32]. The difference is shown in Figure 8a. The results show that the difference in maturity was similar to the previous clustering and grouping, indicating that some substances in the volatiles may be related to the maturity of seeds. In this regard, this study analyzed the correlation between the top ten volatiles and seed maturity. As shown in Figure 8b, the part circled in red represents the correlation between seed maturity and volatiles. It shows that Oxirane, 2,3-dimethyl-, trans- had significant positive correlation with maturity ($p < 0.01$). Furan, 2-ethenyl- was significantly negatively correlated with maturity ($p < 0.05$). Other volatiles were negatively correlated with maturity, but not significantly.

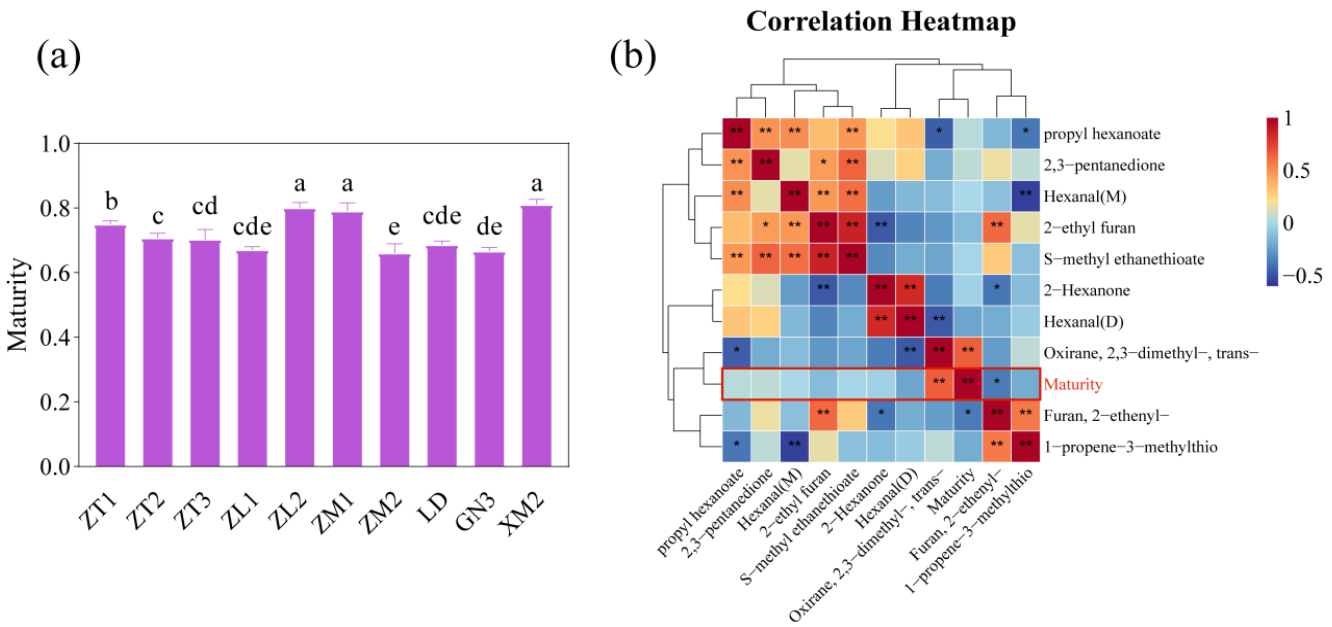

Figure 8. Correlation results of volatiles and seed maturity. Note: (**a**) is the difference in maturity of seeds of different varieties. The same letter indicates no significant difference, and different letters indicate significant difference. (**b**) is a Spearman correlation heat map between the top ten most abundant volatiles and seed maturity. Among them, "Maturity" indicates seed maturity, * means $p < 0.05$, ** means $p < 0.01$.

This study conducted a one-way ANOVA on sample content for all 54 qualitative volatiles. As shown in Table S1, only 8 volatile compounds (Methyl cyanoacetate, 2,6 dimethyl-4-heptanone, 1.8-Cineole (D), Heptanal (M), Heptanal (D), Furan, 2-ethenyl-, 1-propene-3-methylthio (M), N,N-diethylethanamine) had no significant difference between groups ($p > 0.05$), and the remaining 46 volatiles had significant differences ($p < 0.01$), among which 37 substances had extremely significant differences between groups ($p \leq 0.001$). Therefore, it is shown that the characterized volatiles are basically significantly different in different seeds. This shows to a certain extent that the volatiles of seeds are an important way to distinguish between different varieties and are likely to become a scientific basis for identifying varieties.

4. Discussion

Based on the above research, it can be clearly seen from the multi-dimensional topographic map and difference map (Figures 1–3) that the overall migration time of volatile substances in the 10 *alfalfa* seeds is consistent. This shows that the composition of seeds under different breeding conditions is similar. However, there are significant differences in content distribution between different seeds ($p < 0.05$). Under the premise that the environmental conditions such as the place of origin are the same, it shows that the adaptability of different *alfalfa* seeds to the environment is different [33]. This shows that seed planting is subject to different environmental limitations. It will provide some inspiration for further in-depth study of its omics content in the future.

Alcohols and aldehydes determined the basic volatility of *alfalfa* seeds in this study, while volatile flavor was determined by esters, ketones or other components. Most of these ingredients have the function of spices. This is similar to related studies in other substances by Yuan et al. and Lv et al. [34,35]. According to the fingerprint results (Figure 5), with ZT1 as a reference, the *alfalfa* seeds of ZT2 and ZT3 have the highest relative volatile types and contents. Among them, more than ten volatile compounds, mainly alcohols and aldehydes, including benzaldehyde, n-hexanol, etc., are all substances with large differences. Xia et al. also had the same changes in their research on Andrographis paniculata for aerospace breeding [36]. This indicates that aerospace breeding will increase the levels of various VOCs in subsequent generations. This phenomenon may provide a new idea or approach for subsequent breeding. At the same time, studies have shown that benzaldehyde, etc., are key GC-IMS markers for identifying seed viability [37]. Therefore, changes in these components mean differences in seed viability. This also provides a new consideration method for the identification of seed quality.

This study characterized a total of 48 types of 54 volatile substances, classified them according to their molecular structures and divided them into a total of 13 different types of compounds. Among them, the top five dominant categories are esters (9 types), hydrocarbons (8 types), ketones (8 types), alcohols (6 types) and aldehydes (6 types). This is similar to the research results of Jia et al. [38]. However, some signal peaks have not yet been characterized due to the currently limited types of compounds included in the NIST database and IMS database for qualitative analysis. Therefore, the database can be expanded in the future by combining methods such as HPLC and GC-MS [39]. This study shows through PCA analysis (Figure 6) that different *alfalfa* varieties showed better separation effects. Among them, ZT2 and ZT3 from aerospace breeding showed excellent separation effects compared with other varieties. Among them, ZT1 does not have this obvious feature. The reason may be that ZT1 is the result of the first generation of breeding, and ZT3 and ZT2 are the results of the second and third generation of breeding. Combined with the research of Yang et al. [40], this study analyzed that the genetic signal factors of seeds have changed due to the special environmental conditions of space. Therefore, after several generations of breeding, the composition of seeds has changed more and more obviously. And as shown in Figure S2, the number of volatiles related to ZT2 and ZT3 is significantly greater than that of other varieties, which also verifies that there is a significant increase in volatiles in the seeds of aerospace varieties. In addition, there are also certain separation results among

other varieties. Among them, ZL1 and ZL2 are in one group, and ZM1 and ZM2 are in one group, which shows that this classification analysis can help trace the origin of seeds [41]. Subsequently, through PLS-DA model construction, cross-validation and confidence testing were conducted on the volatile compounds that caused the sample differences [42], and it was concluded that 20 substances had a significant contribution to the differences between samples (Figure 7). Esters, ketones and alcohols are the three main types of substances that cause sample differences.

This study combines analysis of the seed maturity of the samples. According to Spearman correlation analysis (Figure 8), there is a significant positive correlation ($p < 0.01$) between seed maturity and Oxirane, 2,3-dimethyl-, trans-, and a significant negative correlation with Furan, 2-ethenyl- Correlated ($p < 0.05$), negatively correlated with most other volatiles, but not significant ($p > 0.05$). This indicates a closer relationship between seed maturity and volatiles. Changes in different volatiles have a certain impact on seed maturity [43,44]. These volatile components affect seed quality, and seed quality reflects its acceptance and adaptability to the environment [45]. This study performed a one-way between-group analysis of variance for all volatiles. The final results show that only 8 of the 54 substances (Methyl cyanoacetate, 2,6 dimethyl-4-heptanone, 1.8-Cineole (D), Heptanal (M), Heptanal (D), Furan, 2-ethyl-, 1-propene-3-methylthio (M), N,N-diethylethanamine) had no significant difference ($p > 0.05$). Among them, 37 substances had extremely significant differences between groups ($p \leq 0.05$). Such analysis can facilitate subsequent tracking and exploration of different substances, and provide new ideas and scientific basis for classification and identification of varieties. Similarly, Fang et al. and Gao et al. also confirmed the role of seed volatile research in classification and identification [46,47]. The differences in volatile substances in seeds and their effects on seed growth and development will be one of the main directions for subsequent research. This also provides new considerations for *alfalfa* research in aerospace breeding.

5. Conclusions

This study used GC-IMS technology to determine the volatile substance characteristics of different *alfalfa* seeds. Among them, the VOCs content of ZT2 and ZT3 varieties bred for spaceflight is generally higher, and the classification characteristics are the most obvious. It shows that aerospace breeding has a greater impact on the intrinsic components of seeds. Through qualitative analysis, PCA analysis and PLS-DA model construction, the differences in VOCs between different varieties were analyzed. The results showed that among the 54 volatile compounds, 48 had significant differences between groups ($p < 0.05$). Esters, ketones and alcohols are the main substances responsible for seed differences. Seed maturity analysis revealed that Oxirane, 2,3-dimethyl-, trans-, Benzaldehyde, etc., are closely related to seed maturity and vigor. It shows that different volatiles affect the characteristics of seeds, thereby changing the adaptability of seeds to the external environment. By clustering and grouping the 10 samples, the grouping results showed that the Zhongtian series, Zhonglan series and Tibetan clover series were each clustered into one group, indicating that the clustering results were consistent with the source of the seeds. Therefore, this study provides new ideas and a scientific theoretical basis for *alfalfa* seed identification and screening, *alfalfa* source tracing and new variety breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030578/s1, Figure S1: Difference histogram of the top ten most abundant substances among seeds; Figure S2: Dual plots of PCA analysis of seeds and compounds. Table S1: ANOVA results of one-way analysis of variance for seed volatile content.

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Data Availability Statement: Data will be made available on request.

Conflicts of Interest: Authors have no conflicts of interest to declare.

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