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Study on VOCs of Fishmeal during Storage Based on HS-SPME-GC-MS

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ABSTRACT: Fishmeal is widely used in the feed industry as the main protein material. The freshness grade directly affects the quality of the fishmeal. During the storage of fishmeal, the odor would change accordingly as the freshness grades decreased. To study the characteristic volatile organic compounds (VOCs) of fishmeal, stored at 25 °C and 80%RH with different freshness grades, headspace solid-phase microextraction combined with gas chromatography−mass spectrometry (HS-SPME-GC-MS) was used to analyze. The single-factor test was chosen for 50/30 μm divinylbenzene/carboxe/polydimethylsiloxane (DVB/CAR/PDMS) fiber. The equilibrium time of 24 min, the extraction time of 60 min, the extraction temperature of 87 °C, and the addition of a saturated saline volume of 4 mL were determined by Box-Behnken design. There were 15 common VOCs detected during storage, the relative contents of acids increased significantly, ketones, aldehydes, esters, and nitrogen-containing compounds increased, and aromatic compounds and alcohols decreased. Combined with freshness indexes, volatile base nitrogen (VBN) and acid value (AV), hexadecanoic acid, tetradecanoic acid, methyl (Z)-N-hydroxybenzenecarboximidate, (Z)-hexadec-9-enoic acid, 6-ethoxy-2,2,4-trimethyl-3,4-dihydro-1H-quinoline, octadecanal, and [(Z)-octadec-9-enyl] acetate were determined as the characteristic VOCs based on the PLS-DA model. This study may provide data support for the development of fishmeal freshness-detecting instruments.

1. INTRODUCTION

Fishmeal is a high-quality animal protein with high protein and amino acid content.[1] Fishmeal is susceptible to the influence of raw material quality, environmental factors, and storage conditions during processing, preparation, transportation, and storage. Proteolysis and oxidative rancidity of fatty acids gradually intensify undesirable odors such as sourness and burnt notes.[2] Consequently, the freshness grade of fishmeal decreased, resulting in a decrease in the nutrition value. It would not only affect the feeding effect, and endanger animal health, but also threaten human health.[3,4] Odor is one of the most important features to evaluate the freshness grade of fishmeal. In the study of Li et al., it was found that the odor of fishmeal usually consisted of volatile organic compounds (VOCs) such as acids, esters, alcohols, ketones, aldehydes, nitrogen-containing compounds, and sulfur-containing compounds.[5] Therefore, it is necessary to detect the VOCs of fishmeal during storage.

Currently, instrumental analytical techniques are being increasingly used to characterize and quantify VOCs. Gas chromatography−mass spectrometry (GC-MS) has emerged as a well-established technique for odor analysis, owing to its advanced development, extensive availability of chromatographic columns, rapid separation capabilities, and the ability to perform qualitative and quantitative studies.[6,7] Sun et al. found the characteristic aroma profiles of the four most famous Chinese traditional red-cooked chickens by GC-MS.[8] The detection of VOCs involves an essential process of extraction, wherein the accuracy of the extraction plays a key role in the

overall detection process. Solid phase microextraction (SPME) has emerged as a rapid, highly sensitive, solvent-free, and cost-effective methodology for sample preparation in conjunction with GC-MS analysis.[9,10] Extraction conditions also have an impact on extraction efficiency.[11,12] In recent years, more and more researchers have determined the characteristic VOCs in their studies based on headspace solid-phase microextraction combined with gas chromatography−mass spectrometry (HS-SPME-GC-MS). Rong et al. combined HS-SPME-GC-MS to determine the characterization of volatile metabolites in Pu-erh teas with different years, such as linalool and (E)-2-hexenal.[13] However, the characteristic VOCs of fishmeal during storage have not been investigated.

The freshness detection method of fishmeal has been well established, and sensory evaluation and physical and chemical testing methods are usually used. The sensory evaluation method mostly relies on the experience of inspectors, and the results are highly subjective. The physical and chemical testing methods can visualize the freshness grade of fishmeal, but the operation is cumbersome, and even has certain risks.[14] However, there are few studies on the combined freshness of VOCs in fishmeal. Therefore, in this study, we aim to investigate the optimization conditions for VOCs in fishmeal, analyze the composition and relative content of VOCs during storage, and explore the characteristic VOCs that have a significant effect on freshness grades.

2. MATERIALS AND METHODS

2.1. Regents and Chemicals. The anhydrous ether, ethanol, potassium hydroxide (KOH), hydrochloric acid (HCl), sodium hydroxide (NaOH), boric acid (H_3BO_3), sodium chloride (NaCl), phenolphthalein, methyl-red dye, bromocresol green, and magnesium oxide (MgO) were acquired from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). *3-Methyl-3-buten-1-ol* (used as an internal standard) was acquired from Macklin Biochemical Technology Company (Shanghai, China). Paraffin hydrocarbons (C_6-C_{23}) were acquired from Sigma-Aldrich (USA). All of the chemicals utilized in this study were analytical or chromatographic grade.

2.2. Sample Preparation. The freshly imported Peru fishmeal was used in this study. It was stored under 25 °C and 80% relative humidity (RH) for 1 month in an artificial climate box (RGX-250B, Sailidesi Experimental Analysis Instrument Factory, Tianjin, China). The samples were taken every other day to measure their freshness indexes and VOCs, the total of which was 16.

2.3. Detection Methods of Freshness Indexes. The freshness evaluation indexes of fishmeal, including volatile base nitrogen (VBN) and acid value (AV) referred to the Chinese national standards GB/T 19164.[15] The automatic Kjeldahl nitrogen analyzer (K1160, Hai Neng Science Instrument Co., Ltd., Shandong, China) was used to detect VBN. Three groups of parallel tests were carried out for each index, and the freshness of fishmeal was rated according to national standards.

2.4. Fiber Selection. Four different fused silica fibers (Supelco Inc., Pennsylvanian, USA) were tested, including 85 μm polyacrylate (PA), 65 μm polydimethylsiloxane/divinyl-benzene (PDMS/DVB), 85 μm carboxe/polydimethylsiloxane (CAR/PDMS), and 50/30 μm divinylbenzene/carboxe/polydimethylsiloxane (DVB/CAR/PDMS). The fibers were thermally conditioned according to the supplier's specifications.

The fishmeal on the 30th day was used to test the selectivity and sensitivity of the fiber coating. The different fibers were exposed for 60 min to the headspace of 6 mL of saturated saline to magnetic stirring (LC-MSB-HD, Lichen Company, Shanghai, China) at 60 °C before equilibration for 30 min. The volatiles were immediately desorbed in the GC injection port for 5 min at 250 °C. The absence of artifacts due to compounds remaining in the fiber after desorption was also checked. The selection was performed by comparing the sensitivity and selectivity of the fiber to each VOC detected.

2.5. Selection of Extraction Conditions: Box-Behnken Design (BBD) for DVB/CAR/PDMS Fiber. Design-Expert 8.0.6 was used to investigate the effects of equilibration time (5, 15, and 30 min), extraction time (40, 50, and 60 min), extraction temperature (60, 80, and 100 °C), and the volume of saturated saline added (4, 6, and 8 mL) on the extraction of VOCs of fishmeal. The extraction scheme is shown in Table 1.

Table 1. Coding of Extraction Parameters

	Factors			
Levels	A: Equilibration time (min)	B: Extraction time/min	C: Extraction temperature (°C)	D: Volume of saturated saline added (mL)
−1	5	40	60	4
0	15	50	80	6
1	30	60	100	8

2.6. HS-SPME-GC-MS Analysis. The VOCs were assessed via a gas chromatograph equipped with a trace quadrupole mass detector (GC8890/7000D, Agilent Technologies, Inc., California, USA). Compounds were separated in a capillary column (30m × 0.25 mm × 0.25 μm film thickness, fused silica HP-5MS UI, Supelco Inc., Pennsylvanian, USA). The temperature program was as follows: initial temperature 40 °C held for 2 min, from 40 to 140 °C at 4 °C/min maintained for 2 min, from 140 to 260 °C at 10 °C/min, and final temperature holding for 5 min. Helium was employed as the carrier gas with a constant flow of 1 mL/min. The injector was operated in the splitless mode and its temperature was set at 260 °C. The transfer line temperature was maintained at 280 °C. The quadrupole mass spectrometer was operated in the electron impact (EI) mode and the source temperature was set at 230 °C. Initially, full scan mode data were acquired to determine appropriate masses for the subsequent acquisition in selected ion monitoring mode (SIM), under the mass range of 30−500 amu. All analyses were performed by setting the ionization energy at 70 eV.

2.7. Data Processing and Detection of VOCs. The identification of VOCs was based on computer matching with the reference mass spectra of NIST 17. L library and by comparison of mass spectra and retention times with commercial standards. Under the same GC-MS settings, paraffin hydrocarbons (C_6-C_{23}) were used as the standard for GC-MS analysis and the retention indexes (RI) of the detected compounds were calculated and compared qualitatively with the NIST17.L mass spectra library,[16] and the results were reported only if the match was greater than 70% (with a maximum value of 100%). The RI is calculated by the following formula.

$$\text{RI} = 100[z + (t_{R_i}^T - t_{R_z}^T / t_{R_{z+1}}^T - t_{R_z}^T)] \tag{1}$$

Figure 1. Chromatograms of VOCs of fishmeal by four fibers. (a) Chromatograms of VOCs of fishmeal by 85 μm PA repeated three times, (b) by 65 μm PDMS/DVB, (c) by 50/30 μm DVB/CAR/PDMS, (d) by 85 μm CAR/PDMS, and (e) a comparison of the chromatograms of the four fibers to detect the VOCs of fishmeal.

where t_R^T, $t_{R_z}^T$, and $t_{R_{z+1}}^T$ are the elution times of the unknown constituent, pre-eluted normal alkane, and posteluted normal alkane, respectively; and z is the number of carbon atoms in the pre-eluted hydrocarbon RI.

Quantitative detections of VOCs were performed by the internal standard method that involved calibration plots with the concentration levels of the analyte and the ratio between

the peak area of the analyte and that of the internal standard, *3-methyl-3-buten-1-ol*, calculated using the following formula.

$$C_1 = S_1/S_0 C_0 \tag{2}$$

where S_1 is the peak area of the analyte (mau min), S_0 is the peak area of the internal standard (mau min), and C_0 is the content of the internal standard (μL/mL).

Table 2. Design Schemes and Results

No.	A: equilibration time	B: extraction time	C: extraction temperature	D: volume of saturated saline added	Y_1: the total peak area (amu min)	Y_2: types of VOCs	Y_3: the relative content of internal standard (%)
1	5	40	80	6	9.18×10^9	42	1.5
2	30	40	80	6	7.60×10^9	48	2.5
3	5	60	80	6	2.01×10^{10}	55	15.4
4	30	60	80	6	2.53×10^{10}	62	8.15
5	15	50	60	4	1.66×10^{10}	58	17.5
6	15	50	100	4	3.56×10^{10}	47	4.7
7	15	50	60	8	5.49×10^9	32	37.8
8	15	50	100	8	1.81×10^{10}	29	9.1
9	5	50	80	4	1.94×10^{10}	65	14.2
10	30	50	80	4	2.36×10^{10}	72	11.1
11	5	50	80	8	1.24×10^{10}	38	26.9
12	30	50	80	8	1.87×10^{10}	62	15.2
13	15	40	60	6	2.95×10^9	35	11.9
14	15	60	60	6	2.66×10^{10}	46	18.0
15	15	40	100	6	2.23×10^{10}	56	2.9
16	15	60	100	6	3.21×10^{10}	49	5.1
17	5	50	60	6	6.10×10^9	33	37.9
18	30	50	60	6	8.29×10^9	39	35.4
19	5	50	100	6	2.59×10^{10}	43	16.7
20	30	50	100	6	2.95×10^{10}	40	4.6
21	15	40	80	4	4.79×10^9	33	4.1
22	15	60	80	4	2.29×10^{10}	51	8.2
23	15	40	80	8	4.42×10^9	40	5.1
24	15	60	80	8	1.28×10^{10}	53	19.9
25[a]	15	50	80	6	1.04×10^{10}	54	11.4
26[a]	15	50	80	6	1.27×10^{10}	50	4.8
27[a]	15	50	80	6	1.07×10^{10}	48	13.8
28[a]	15	50	80	6	1.84×10^{10}	62	14.5
29[a]	15	50	80	6	1.55×10^{10}	41	15.9

[a]Note:represents zero points.

3. RESULTS AND DISCUSSION

3.1. Fiber Selection. The influence of the type of SPME fiber on the extraction efficiency of fishmeal VOCs was first evaluated. Figure 1 shows significant differences in the extraction of VOCs of fishmeal samples on the 30th day by different fibers due to the diversity of polarity and size of the SPME coatings. The peaks of the chromatogram are shaped and narrow, suggesting that the fibers provide enhanced extraction efficacy for VOCs under the specified conditions. 85 μm PA was more effective in the detection of the long-chain VOCs, but the number of VOCs detected was relatively small, and alcohols, paraffin hydrocarbons, ethers, esters, and aldehydes were not completely detected (Figure 1a). The total of peak area of VOCs detected by 65 μm PDMS/DVB was the largest, and it could detect heterocyclic aromatic compounds, ketones, acids, alcohols, esters, aldehydes, ethers, long-chain paraffin hydrocarbons, long-chain olefins, and nitrogen-containing compounds, but the types of VOCs were relatively few. In the later stage of storage, the freshness decreased, and VBN increased significantly. Trimethylamine, a freshness marker VOC was not detected by 65 μm PDMS/DVB (Figure 1b). 50/30 μm DVB/CAR/PDMS and 85 μm CAR/PDMS detected a relatively large number of VOCs in a wide range of categories, which could detect trimethylamine (Figure 1c and Figure 1d). Compared with 85 μm CAR/PDMS, 50/30 μm DVB/CAR/PDMS had better repeatability and was more conducive to the detection of VOCs of fishmeal,

which may be due to its combination of 85 μm CAR/PDMS and 65 μm DVB/PDMS fiber characteristics with good repeatability. Meanwhile, 50/30 μm DVB/CAR/PDMS had the highest extraction efficiency for the internal standard. Therefore, it was determined that 50/30 μm DVB/CAR/PDMS fiber was used for the detection of VOCs during fishmeal storage.

3.2. Optimization of Extraction Conditions Based on the BBD. To optimize the extraction conditions, 50/30 μm DVB/CAR/PDMS fiber was determined to detect VOCs of fishmeal stored on the 30th day. The total peak area (Y_1), types of VOCs (Y_2), and relative content of internal standard (Y_3) were used as response values to evaluate the extraction efficiency. The experimental scheme consisted of 24 factorial points and 5 zero points, in which the factorial points were the three-dimensional vertices formed by the independent variable values in each factor, and the zero point tests were repeated 5 times to estimate the experimental errors. The results are listed in Table 2.

To further determine the significance of the effects of each factor (A: equilibration time, B: extraction time, C: extraction temperature, D: volume of saturated saline added) on the response values (Y_1: the total peak area, Y_2: types of VOCs, Y_3: relative content of internal standard), the results were processed by Design-Expert 8.0.6 software, and the analyses of variance (ANOVA) for each index are shown in Table 3. Table 3 shows that factors B, C, and D had highly significant effects on the total peak area ($P < 0.01$), and the factors

Table 3. Analysis of Variance of the Total Peak Area (Y_1), Types of VOCs (Y_2), and the Relative Content of Internal Standard (Y_3)

	Source	Sum of squares	Degrees of freedom of variance source	Mean of square	F-value	P-value
The total peak area (Y_1)	Model	1.73×10^{21}	4	4.33×10^{20}	27.71	<0.0001**
	A	4.08×10^{19}	1	4.08×10^{19}	2.61	0.1194
	B	5.13×10^{20}	1	5.13×10^{20}	32.82	<0.0001**
	C	9.63×10^{20}	1	9.63×10^{20}	61.57	<0.0001**
	D	2.17×10^{20}	1	2.17×10^{20}	13.86	0.0011**
	Residual	3.75×10^{20}	24	1.56×10^{19}		
	Lack of fit	3.29×10^{20}	20	1.65×10^{19}	1.42	0.4011
	Pure error	4.63×10^{19}	4	1.16×10^{19}		
	Total	2.11×10^{21}	28			
Types of VOCs (Y_2)	Model	2018.52	14	144.18	1.32	0.3033
	A	184.08	1	184.08	1.69	0.2145
	B	313.25	1	313.25	2.88	0.1120
	C	25.11	1	25.11	0.23	0.6385
	D	349.16	1	349.16	3.21	0.0950
	AB	1.25	1	1.25	0.011	0.9162
	AC	16.37	1	16.37	0.15	0.7040
	AD	50.49	1	50.49	0.46	0.5070
	BC	81.00	1	81.00	0.74	0.4030
	BD	6.25	1	6.25	0.057	0.8141
	CD	16.00	1	16.00	0.15	0.7072
	A^2	1.64	1	1.64	0.015	0.9041
	B^2	72.79	1	72.79	0.67	0.4273
	C^2	817.30	1	817.30	7.51	0.0160
	D^2	28.61	1	28.61	0.26	0.6163
	Residual	1524.52	14	108.89		
	Lack of fit	1351.72	10	135.17	3.13	0.1415
	Pure error	172.80	4	43.20		
	Total	3543.03	28			
The relative content of internal standard (Y_3)	Model	2391.46	8	298.93	12.39	<0.0001**
	A	105.91	1	105.91	4.39	0.0491*
	B	182.13	1	182.13	7.55	0.0124*
	C	1109.76	1	1109.76	45.99	<0.0001**
	D	244.80	1	244.80	10.15	0.0047**
	A^2	119.45	1	119.45	4.95	0.0377*
	B^2	327.60	1	327.60	13.58	0.0015**
	C^2	178.76	1	178.76	7.41	0.0131*
	D^2	20.43	1	20.43	0.85	0.3685
	Residual	482.57	20	24.13		
	Lack of fit	405.70	16	25.36	1.32	0.4324
	Pure error	76.87	4	19.22		
	Total	2874.03	28			

affected in the order were extraction temperature, extraction time, and volume of saturated saline added. The regression model of the total peak area was highly significant ($P < 0.01$), and the lack of fit was not significant ($P = 0.4011$), indicating that the obtained regression model was well fitted to the actual results. The factors C, and D had highly significant effects on the relative content of internal standard ($P < 0.01$), and the factors A, and B were significant ($P < 0.05$). The factors affected in the order were extraction temperature, extraction time, volume of saturated saline added, and equilibration time. The regression model of the relative content of internal standard was highly significant ($P < 0.01$), and the lack of fit was not significant ($P = 0.4324$), indicating that the obtained regression model was well fitted to the actual results. The regression model and the lack of fit of the types of VOCs were not significant. By eliminating the nonsignificant factors in the model, the response functions of the total peak area (Y_1) and

the relative content of internal standard (Y_3) are presented by formulas 3 and 4, respectively.

$$Y_1 = 1.637 \times 10^{10} + 6.540 \times 10^9 \cdot B + 8.957 \times 10^9 \cdot C - 4.279 \times 10^9 \cdot D \tag{3}$$

$$Y_3 = 11.31 - 2.97A + 3.90B - 9.62C + 4.52A^2 - 7.11B^2 + 5.25C^2 \tag{4}$$

Based on the Design-Expert 8.0.6 software, the response surface diagram was constructed to visualize the influence of factors (A: equilibration time, B: extraction time, C: extraction temperature, and D: volume of saturated saline added) on the total peak area as shown in Figure 2 and on the relative content of the internal standard as shown in Figure 3. Combined with the regression model solved by the optimization module in

(a) *A*: equilibration time and *B*: extraction time

(b) *A*: equilibration time and *C*: extraction temperature

(c) *A*: equilibration time and *D*: volume of saturated saline added

(d) *B*: extraction time and *C*: extraction temperature

(e) *B*: extraction time and *D*: volume of saturated saline added

(f) *C*: extraction temperature and *D*: volume of saturated saline added

Figure 2. Response surface diagram of the influence of various factors on the total peak area.

(a) *A*: equilibration time and *B*: extraction time

(b) *A*: equilibration time and *C*: extraction temperature

(c) *A*: equilibration time and *D*: volume of saturated saline added

(d) *B*: extraction time and *C*: extraction temperature

(e) *B*: extraction time and *D*: volume of saturated saline added

(f) *C*: extraction temperature and *D*: volume of saturated saline added

Figure 3. Response surface diagram of the influence of various factors on the relative content of the internal standard.

Design-Expert 8.0.6 software, the maximum total peak area and the minimum relative content of the internal standard were taken as the targets. The optimized extraction conditions combinations were obtained with the equilibration time of 24 min, extraction time of 60 min, extraction temperature of 87 °C, and 4 mL of saturated saline added.

3.3. VOCs of Fishmeal during Storage. The optimized method was then applied to detect the VOCs of fishmeal during storage. A total of 145 VOCs were detected during storage. The number of VOCs of fishmeal at different storage periods is shown in Figure 4a. The central part of Figure 4a shows 15 representing 15 VOCs that could be detected at all times during storage, which were: naphthalene, 2-methylnaphthalene, 6-ethoxy-2,2,4-trimethyl-3,4-dihydro-1H-quinoline, ethoxyquinoline, 1-(5-acetyl-4-methyl-1-phenyl-4H-pyridin-3-yl)ethenone, hexadecanoic acid, tetradecanoic acid, benzaldehyde, hexadecanal, 3,5,5,9-tetramethyl-6,7,8,9-tetrahydrobenzo[7]annulen-2-ol, 6,7,8,9-tetrahydro-3H-benzo[e]indole-1,2-

Figure 4. Changes in VOCs during storage of fishmeal. (a) Flower plot of numbers of VOCs. (b) Relative content stacking histogram.

dione, 4-methoxy-6-[3-(4-methoxy-6-oxopyran-2-yl)-2,4-bis(3-methylphenyl) cyclobutyl]pyran-2-one, 3,3,6,8-tetramethyl-2,4-dihydronaphthalen-1-one, pentadecane, heptadecane. Among them, ethoxyquinoline is an antioxidant with excellent performance and good economy, commonly used in premixes, fishmeal, and fat-added products. It can be used to prevent vitamin A, vitamin D, vitamin E, and fat oxidation and has a certain antimildew and preservation effect. 6-ethoxy-2,2,4-trimethyl-3,4-dihydro-1H-quinoline is a related compound produced by antioxidants during the processing of fishmeal. Naphthalene exists in the environment, released from natural events as a product of incomplete combustion, which has been detected in Pu-erh teas, sunflower seed oils, and grilled lamb shashliks.[17−19] Hexadecanoic and tetradecanoic acids are both long-chain saturated fatty acids found naturally in most animal fats. Hexadecanal is a long-chain saturated aldehyde with a weak aroma of flowers and waxes, which may be produced by fatty acid oxidation during the processing of fishmeal.[20]

The relative contents of VOCs of fishmeal during storage are shown in Figure 4b, and the details are shown in Table 1. The total relative content of VOCs shows an increasing-decreasing-significantly increasing trend, and the overall trend is increasing. The relative content of heterocyclic aromatic compounds decreased in the whole storage, and nitrogen-containing heterocyclic aromatic compounds (pyrazines and pyrroles), oxygen-containing heterocyclic aromatic compounds (furans), sulfur-containing heterocyclic aromatic compounds (thiophenes) appeared in the later storage due to Maillard reaction. The relative content of acids showed an increasing trend, probably deriving from the degradation of glycerol and phospholipids, which increased significantly in the later storage. The relative content of esters increased significantly on the sixth day of storage, and then decreased steadily, with an overall increasing trend, probably due to the combined action of enzymes and microbial metabolism.[5] The relative contents of ketones, aldehydes, and alcohols fluctuated during the storage. Due to the reproduction and growth of microorganisms and the activity of enzymes, the decom-

position of nitrogen-containing compounds was promoted. So, the relative content of nitrogen-containing compounds such as oximes, amines, and nitriles increased during storage.

3.3.1. Heterocyclic Aromatic Compounds. The relative content of pyridines (3,5-Diacetyl-4-methyl-1-phenyl-1,4-dihydropyridine and 3-phenylpyridine), pyrroles (2-[(3,4-Dimethyl-2H-pyrrole-2-ylidene)methyl]-3,4-dimethyl-1H-pyrrole), and furans (2-(2z)-2-pentenyl-furan, 2-ethylfuran, and dibenzofuran) increased in later storage. It may be that the Maillard reaction would generate lower molecular weight heterocyclic compounds, which would combine with nitrogen and sulfur to form new heterocyclic aromatic compounds.

3.3.2. Acids and Esters. Among the acids, the relative contents of hexadecanoic acid and tetradecanoic acid increased significantly. Yang et al. found that hexadecanoic acid and tetradecanoic acid could be detected in Pseudosciaena crocea before defat and deodorization, and speculated that saturated fatty acids were the main cause of fishy odor.[21] During the storage of fishmeal, the fishy odor became strong. Therefore, it is speculated that saturated fatty acids are the main cause of the fishy odor of fishmeal.

Esters are mainly formed by the esterification of alcohols with carboxylic acids, decomposed liquids by microorganisms and enzymes. Most esters have the characteristic aroma of sweet and caramel.[22] High humidity storage conditions increased the activity of microorganisms and enzymes, which promoted the esterification reaction. So, the relative content of esters increased. The relative content of long-chain esters increased in the later stage of storage, in special methyl (Z)-N-hydroxybenzenecarboximidate, which could be detected in baked materials and aquatic products.

3.3.3. Ketones, Aldehydes, and Alcohols. Ketones originate from the oxidation of unsaturated fatty acids or the oxidative decomposition of amino acids and the Maillard reaction, showing green aromas, which have an enhanced effect on bloody odor.[23] Due to the Maillard reaction, thioketones (5-hexyloxolane-2-thione) were detected in the later stage of storage, and the relative content increased. Ketenes can interact with aldehydes to enhance the fishy odor. Non-adeca-10,13-dien-2-one was detected in the later stage of storage, and the relative content showed an increasing trend.

Aldehydes originate from the decomposition of peroxides formed by the oxidation of unsaturated fatty acids, which would bring obvious odor changes due to their low odor threshold.[24] Benzaldehyde has the odor of ester and cucumber, and the relative content gradually decreased.[25,26]

Alcohols mainly present light, sweet, mellow, and fruit odors. The relative content of alcohols increased first and then decreased, which was the same as the study of Li et al.[5] The relative content of alcohols decreased, which may be due to the increase in microbial and enzyme activities under high humidity storage conditions, which promoted the conversion of alcohols to esterification.

3.3.4. Others. Hydrocarbons originate from the decomposition of fatty acid alkoxy radicals. The relative content of paraffin hydrocarbons increased, the content of olefins and alkynes decreased, and no olefins and alkynes were detected in the later periods of storage. Paraffin hydrocarbons ($C_6−C_{19}$) have been detected in VOCs of crustacean aquatic products, but the thresholds are relatively high and the contribution to odors is not significant. The relative content of nitrogen-containing compounds increased. In the later storage, trimethylamine with a fishy odor was detected, which could

Figure 5. Changes in freshness indexes, VBN and AV, during storage.

be used as an odor characteristic of freshness. (1Z)-1-(3,5-Di*tert*-butyl-4-hydroxyphenyl)ethanone oxime could be detected in the middle of storage, whose relative content increased in the later storage. Under acidic conditions, amides and ketoximes are converted and present in different forms of nitrogen-containing compounds in different stages of fishmeal storage.

3.4. Detection of Freshness Indexes. VBN and AV were used as the freshness indexes of fishmeal, and the changes during storage are shown in Figure 5. In the early storage, the AV increased slowly. On the eighth day of storage, the AV increased significantly and continued to maintain a high value. It was because of the storage under high humidity, which promoted the growth of microorganisms and exacerbated the rancidity and oxidation of fat. Finally, the AV was more than 5 mg/g on the 30th day. Oppositely, the VBN decreased steadily in early storage. On the eighth day of storage, the VBN decreased significantly, with a value of less than 100 mg/100g, and the fishmeal was in an early stage of corruption, which proved that the VBN was not suitable for early spoilage.[27] After 16 days of storage, VBN increased slowly. After 24 days, the decomposition rate of nitrogen-containing compounds was exacerbated, the content of ammonia, dimethylamine, and trimethylamine increased, and the VBN increased significantly, exceeding 160 mg/100 g. Combined with the Chinese national standards GB/T 19164 and the changes of each freshness index during the storage, the freshness grades of fishmeal were divided shown in Figure 5. Among them, *A* represents the superior freshness when 100 mg/100 g ≤ VBN ≤ 130 mg/100 g, and 3 mg/g ≤ AV ≤ 4 mg/g;[5,15] *B* represents the early stage of corrupting, where the rancidity and oxidation of fat were exacerbated, and VBN decreased; *C* represents the middle stage of corruption, where fatty acid rancidity and oxidation reached a certain level, and VBN increased slowly; *D* represents the complete corruption of fishmeal, where the AV and VBN increased significantly.

3.5. Multivariate Statistical Analysis. In this experiment, a model of the correlation between the relative content of VOCs measured by GC-MS and fishmeal at four freshness grades during storage was established to identify the differences in VOCs in different storage based on partial least-squares discriminant analysis (PLS-DA). The variable

importance in the projection (VIP) method was used to explore the characteristic VOCs in the PLS-DA model.[28] The overall sample size in the model was 48 (16 samples with different storage time × 3 times). The Y variable in the model refers to the four freshness grades, as detailed in Figure 5. The X variable referred to the relative content of VOCs detected by GC-MS.

According to the PLS-DA score plot shown in Figure 6a, the first three components in the models accounted for 93.6% of the total variables. The accuracy of the PLS-DA model was 0.87, the goodness of fit (R^2) was 0.90, and the goodness of prediction (Q^2) was 0.79, which showed that the PLS-DA model had a good fitting ability. The PLS-DA score plot shows that superior freshness fishmeal and complete corruption could be completely distinguished during storage (freshness grades *A* and *D*, respectively). Samples with similar freshness grades slightly overlapped in the confidence intervals and the differences were not obvious. This may be because the odor of fishmeal changed slowly and was not easily distinguishable during storage. The permutation test method was used to investigate the modeling effect of PLS-DA, and the results are shown in Figure 6b, where after 100 cross-validations, only less than one randomly grouped model was superior to the established PLS-DA model $(P < 0.01)$. The result shows that the PLS-DA model was reliable.

The influence on and explanatory power of each variable for the classification of each group of samples were assessed by calculating VIP. A higher VIP value indicated a more significant difference between the content of a variable in the different groups, which was a key indicator of fishmeal in different freshness grades. In addition, the key VOCs were usually screened out in combination with Fisher's test $(P < 0.05)$. So, hexadecanoic acid, tetradecanoic acid, methyl (Z)-*N*-hydroxybenzenecarboximidate, (Z)-hexadec-9-enoic acid, 6-ethoxy-2,2,4-trimethyl-3,4-dihydro-1H-quinoline, octadecanal, and [(Z)-octadec-9-enyl] acetate were selected as the characteristic VOCs to distinguish the freshness grade differences of fishmeal (VIP > 1 and $P < 0.05$) shown in Figure 6d in detail.

Figure 6. PLS-DA model of freshness grades and relative content of VOCs during fishmeal storage. (a) Score plot, (b) permutation test results, (c) VIP scores, and (d) significance test results.

4. CONCLUSIONS

In this research, the VOCs of fishmeal stored at 25 °C and 80% RH were studied based on HS-SPME-GC-MS and the characteristic VOCs during storage. The results obtained were as follows:

(1) The extraction conditions of HS-SPME-GC-MS were optimized. The single-factor test was used to determine that the 50/30 μm DVB/CAR/PDMS fiber had the highest extraction efficiency. The extraction conditions combination was optimized by BBD as follows: equilibration time of 24 min, extraction time of 60 min, extraction temperature of 87 °C, and the addition of saturated saline volume of 4 mL

(2) The changes in VOCs during fishmeal storage were obtained. There were 15 common VOCs detected during storage, including naphthalene, 2-methylnaphthalene, 6-ethoxy-2,2,4-trimethyl-3,4-dihydro-1H-quinoline, ethoxyquinoline, 1-(5-acetyl-4-methyl-1-phenyl-4H-pyridin-3-yl)ethanone, hexadecanoic acid, tetradecanoic acid, benzaldehyde, hexadecanal, 3,5,5,9-tetramethyl-6,7,8,9-tetrahydrobenzo[7] annulen-2-ol, 6,7,8,9-tetrahydro-3H-benzo[e]indole-1,2-dione, 4-methoxy-6-[3-(4-methoxy-6-oxopyran-2-yl)-2,4-bis(3-methylphenyl)cyclobutyl]pyran-2-one, 3,3,6,8-tetramethyl-2,4-dihydronaphthalen-1-one, pentadecane, and heptadecane. The relative content of acids increased significantly; ketones, aldehydes, esters, nitrogen-containing compounds, and paraffin hydrocarbons showed an increasing trend; heterocyclic aromatic compounds, alcohols, olefins, and alkynes decreased during storage.

(3) The correlation between VOCs and freshness grades was established. The AV and VBN were used as the freshness indexes of fishmeal, and the freshness grades were divided according to freshness indexes. Combined with the relative content of each VOC and freshness grades during storage, the PLS-DA model was established. The prediction accuracy of the model was 0.87, the goodness of fit R^2 = 0.90, and the goodness of prediction Q^2 = 0.79, which had a good fitting ability. According to the VIP value and Fisher's test, hexadecanoic acid, tetradecanoic acid, methyl (Z)-N-hydroxybenzenecarboximidate, (Z)-hexadec-9-enoic acid, 6-ethoxy-2,2,4-trimethyl-3,4-dihydro-1H-quinoline, octadecanal, and [(Z)-octadec-9-enyl] acetate were determined as the characteristic VOCs.

The findings of this study demonstrate the viability of utilizing odor for fishmeal freshness detection, establishing a theoretical foundation for the subsequent design and development of an electronic nose instrument for fishmeal freshness detection. Moreover, the study furnishes empirical evidence to inform the judicious selection of gas sensors. Future research endeavors may focus on enhancement of odor analysis methodologies, broaden the scope of fishmeal freshness detection, and engage in collaborative partnerships with the feed industry to facilitate the practical implementation of research outcomes, thus engendering more efficacious mechanisms for fishmeal quality management.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c03323.

The types and relative content of VOCs detected in fishmeal are shown in Table S1 (PDF)

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Notes

The authors declare no competing financial interest.

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