R. Bras. Zootec., 53:e20230042, 2024
https://doi.org/10.37496/rbz5320230042

Forage crops
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

Screening of key volatile compounds characterizing the deterioration of maize silage during aerobic exposure

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ABSTRACT - This study was conducted to identify the key volatile compounds that characterize whether silage has deteriorated and to investigate the intrinsic link between the key compounds and silage odor. First, silages with different aerobic exposure durations were sampled, and sensory evaluation integrating aerobic stability monitoring was used to distinguish whether the silage had deteriorated. Subsequently, headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) was utilized to determine the relative content of the compounds. Using the relative content of the compounds in each silage as input, relative odor activity value (ROAV) calculations and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed to determine the odor contribution of the compounds and the compounds with significant differences in relative content (based on variable importance for the projection, VIP) between the deteriorated and non-deteriorated silages. Next, the key compounds were identified by combining the conditions of average ROAV (aROAV) ≥ 1 and VIP > 1. Finally, the OPLS algorithm was used to analyze the intrinsic link of key compounds with the silage odor. The results showed that three out of 63 compounds—4-ethyl phenol, eugenol, and ethyl linoleate—were key compounds to characterize whether the silages deteriorated or not. In addition, ortho-guaiacol, 4-ethyl guaiacol, and 2-methoxy-4-vinyl phenol were the specific key compounds for deteriorated silage. Eugenol and ethyl linoleate were correlated with fruity, sour, and spicy odors. In addition, guaiacol, 4-ethylphenol, 4-ethyl guaiacol and 2-methoxy-4-vinyl phenol contributed to roasted, musty, and putrid odors.

Keywords: corn silage, forage quality, multivariate analysis, sensory analysis, volatile fatty acid

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Received: April 20, 2023
Accepted: September 27, 2023

How to cite: Zhao, K.; Tian, H.; Ren, X.; Yu, Y.; Guo, L.; Li, Y.; Tao, Y. and Liu, F. 2024. Screening of key volatile compounds characterizing the deterioration of maize silage during aerobic exposure. Revista Brasileira de Zootecnia 53:e20230042.
https://doi.org/10.37496/rbz5320230042

Editors:
Gustavo José Braga
Luiz Felipe Ferraretto

1. Introduction

Corn silage is the most widely used ingredient for ruminants (Penagos-Tabares et al., 2023). Silage quality significantly impacts the energy intake and subsequent milk production of cows fed corn silage diets (Tharangani et al., 2021). During ensiling and feeding, poor-quality silage is unavoidable due to aerobic deterioration (Abeni et al., 2021). Odor is the most important sensory quality indicator of silage (Teixeira and Fontaneli, 2017), and it changes most significantly during aerobic exposure (Liu et al., 2022). Among the volatile compounds of the measured substance, there may be only a few key compounds that contribute prominently to odor (Hu et al., 2020). Therefore, deterioration detection based on key compounds is of great importance in production practice.

Volatile compounds in high-quality silage include acids, esters, terpenoids, phenols, alcohols, and alkanes (Figueiredo et al., 2007; Zhang et al., 2021). In addition, 3-methylbutanoic acid was screened as an off-odor compound in high-quality silage (Worku et al., 2021). In poor-quality silage, a review showed the putrefactive butter and putrid odor are associated with butyric acid (Kung et al., 2018). Liu et al. (2022) found that isospathulenol, benzaldehyde, and 2-furanmethanol may contribute to the pungent odor of deteriorated silage. Guan et al. (2020) found that acetic acid was not present in deteriorated silage. However, there are few studies on the key compounds in corn silage for deterioration discrimination during aerobic exposure.

For the key volatile compounds in silage, compounds causing differences in silage odor have been analyzed based on the concentration of the compounds and multivariate analysis methods (Worku et al., 2021). However, the odor of the measured substance is not only related to the concentration of the compounds, but also to the odor contribution (Olivares et al., 2009). In this scenario, the relative odor activity value (ROAV) is used to quantify the odor contribution of compounds, and multivariate analysis methods are integrated to screen for key odor compounds in the measured substance (Tian et al., 2021).

In this study, silage volatiles were first determined during aerobic exposure, and key compounds characterizing whether the silages deteriorated were screened utilizing the integration of ROAV, orthogonal partial least squares-discriminant analysis (OPLS-DA), and OPLS.

2. Material and Methods

2.1. Silage sample preparation

Whole-crop corn (*Zea mays* L., ZhongBei 410, Wo Da Feng Ltd., Shan Xi, Tai Yuan, China) silage was collected in a standard silage pile on a dairy farm in Hohhot City, Inner Mongolia Autonomous Region, China (111.697° E, 40.485° N). The main parameters of the corn silage were as follows: the harvest date was August 2021; the growth stage of the feedstock was the half-milk line; the cut length was 10-20 mm; the compression density was 210±4 kg dry DM/m^3; and the storage period was 90 days. To obtain high-quality silage, each 4000±50 g of silage were manually collected from nine locations along the vertical profile of the pile (3 × 2 m), according to the nine-point sampling method (GB/T 14699.1-2005, 2005). Subsequently, all the silage was thoroughly mixed on a plastic film. Then, 18 high-quality silage samples were equally divided from the above-mixed pile. Of these, each sample weighed 2000±50 g for the subsequent aerobic exposure treatment.

To perform the aerobic exposure treatment, each sample was placed in a polystyrene box (30 × 40 × 30 cm) covered with a sheet of aluminum foil with small holes to avoid moisture loss and ensure sufficient exposure to air (Da Silva et al., 2018). Specifically, each 2-kg sample was placed in the box without compaction (8 cm thick) to ensure that the thickness was sufficient to simulate aerobic exposure. For the aerobic exposure of the silage samples, a total of 18 high-quality silages were divided into nine treatments (two replicates per treatment) and aerobically exposed for different durations. The duration of aerobic exposure for each treatment was 0 days (D0 feed), 0.25 days (D0.25 feed), 0.5 days (D0.5 feed), one day (D1 feed), two days (D2 feed), three days (D3 feed), four days (D4 feed), eight days (D8 feed), and 12 days (D12 feed). After aerobic exposure, each sample was reduced to 60 g based on a quadratic method, of which 50 g was used for sensory quality evaluation and 10 g for measurement of volatile compounds measurement. The quadratic method was carried out as follows: each sample was placed on a clean plastic film and thoroughly mixed into a flat square; the square sample was divided into four sub-samples in diagonal directions, and any pair of orthogonal sub-samples were retained to reduce the weight of the samples; the first and second execution steps were repeated in sequence until 60 g of sub-sample remained. Finally, all samples were immediately frozen at −18 °C.

2.2. Discrimination of undeteriorated and deteriorated silages

2.2.1. Sensory quality evaluation

Silage quality was evaluated using the DLG scheme (Table 1), which included four sensory properties (odor, texture, color, and visible mold) and five quality grades (very good, good, need for improvement, bad, and very bad) (DLG, 2004). The sensory analysis was performed by five panelists (three males and two females, aged 20–30 years) and at room temperature (23±2 °C). Silages (50±0.1 g) were placed in a 120-mm diameter glass Petri dish and presented with a two-digit random number in ascending order for presentation. The mean value of the five evaluators' results was used as the quality grade of silage.

Table 1 - Sensory evaluation criteria

Index	Grading				Score
Odor	Aromatic fruity or pronounced bakery odors				0
	Slightly alcohol or slightly vinegary odors				1
	Strong alcohol or roasted odors				3
	Musty or slight butyric acid odors				5
	Putrid or foul odors				7
Texture	Similar to raw materials				0
	Brittle structure of some stems and leaves				1
	Stem and leaf structure, greasy and slimy				2
	Decay				4
Color	Similar to raw materials				0
	Slightly discolored				1
	Badly discolored				2
Mold	Visible mold				7
Total grade	Very good, grade 1	Good, grade 2	Need for improvement, grade 3	Bad, grade 4	Very bad, grade 5
Total score	0-1	1-4	4-5	5-8	8-20

2.2.2. Aerobic stability monitoring

To determine the aerobic stability of silage, the temperature of each sample was recorded by a datalogger (CR10X, Campbell Scientific, Inc., Logan, UT) inserted into its geometric center. The ambient temperature was measured with a thermometer (TES-1310, Taishi Electronics, Inc., Taibei, China). In addition, silage and ambient temperatures were measured using two devices to ensure that both temperature data were obtained simultaneously and accurately. Specifically, two sets of temperature data were collected when the silage reached the aforementioned aerobic exposure time. Aerobic stability was defined as the number of hours the silage remained stable before rising more than 2 °C above ambient temperature (Ranjit and Kung, 2000).

2.2.3. Criteria for determining silage deterioration

Based on the sensory evaluation, silages of grades 1-5 were rated as very good, good, need for improvement, bad, and very bad, respectively, indicating that silages of grades 1-3 and 4-5 may be able to be categorized into two main groups (overall good and overall bad). Previous studies had shown that the signs of silage deterioration due to aerobic exposure were the loss of aerobic stability, presence of mold stains on the surface, and presence of a moldy odor (Adesogan et al., 2004; Kung, 2014). In this study, we identified whether silage was deteriorated based on quality grade and quality characteristics

(odor, mold): silage was considered not deteriorated if it was aerobically stable, free of moldy odor and any moldy stains, and within grades 1-3; silage was considered deteriorated if it was aerobically unstable, with moldy odor and moldy stains, and within grades 4-5.

2.3. Measurement of volatile compounds

Prior to testing, the fiber (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane [DVB/CAR/PDMS], Supelco Inc., Bellefonte, PA, USA) was pre-aged at 300 °C for 30 min. Samples were ground into powder in liquid nitrogen. The silage powder (10±0.001 g) was transferred into a 20-mL headspace sample vial, which was sealed with a Teflon/silicone septum. Then, the vials were stabilized at 65 °C for 10 min. After that, the aged fiber was exposed to the headspace of the vial at 65 °C for 10 min. Finally, the fiber was withdrawn from the vial and desorbed at 280 °C for 5 min in splitless mode.

An HP 7890B-7000C GC-MS instrument analyzed the volatile compounds in the fiber with an HP-5MS capillary column (60 m × 0.25 mm, 0.25 μm; Agilent Technologies, California, USA). Helium (He, purity > 99.999%) was used as the carrier gas flowing at 1.2 mL/min. The oven temperature programming was as follows: 40 °C initial temperature for 5 min, increased to 250 °C at 10 °C/min, and then to 280 °C at 5 °C/min for 5 min. The MS conditions were as follows: 230 °C for ion source temperature, EI ionization source, 70 eV for ionization energy, 250 °C for transmission line temperature, 250 °C for interface temperature, and 150 °C for quadrupole temperature. The mass scan range was m/z 35-550.

The compounds were identified by comparing the mass spectral information of samples with the NIST 05 data and matching of the retention indices with the corresponding standards (positive and negative matches > 800; RI). The RI (Eq. 1) of compounds were calculated by n-alkanes (C7-C40; Sigma-Aldrich Co., St. Louis, MO, USA).

$$RI = 100 \times \left[n + \frac{\lg tRy - \lg tRn}{\lg tR\,(n+1) - \lg tRn}\right] \quad (1)$$

wherein n is the number of carbon atoms and tRn < tRy < tR (n + 1); tRy is the adjusted retention time of the test compound y in minutes; tRn is the n-alkane composed of n carbon number, and the retention time is in minutes; and tR (n + 1) is the retention time of a normal structure with a carbon number of n + 1 in minutes.

Moreover, the relative content of volatile compounds was calculated by the percentage of each substance peak area to the total peaks area in GC-MS total ion chromatograms (TIC), which was the peak area normalization method.

2.4. Determination of key volatile compounds

2.4.1. Relative odor activity value calculation

The ROAV method is used to measure the odor contribution of volatile compounds to the measured object (Liu et al., 2008). The formula of ROAV is shown in Eq. 2.

$$ROAV \approx 100 \times \frac{CR_i}{CR_{max}} \times \frac{T_{max}}{T_i} \quad (2)$$

wherein CR_i and T_i are the relative concentration and odor detection threshold of the compound i, respectively, and CR_{max} and T_{max} are the relative concentration and odor detection threshold of the compounds that contributed most to the overall odor in the sample, respectively.

The ROAV values varied from 0 to 100; the higher the ROAV value, the greater the contribution of compounds to overall odor. The ROAV ≥1 was the main odor compound of the sample, and 0.1 ≤ ROAV < 1 was an important odor modifier.

2.4.2. Analysis of differential compounds by OPLS-DA

The OPLS-DA is a supervised algorithm for pairwise comparative analysis to find key variables (Ye et al., 2022). In this study, this algorithm was utilized to screen for volatile compounds with significant changes in relative content. First, the relative contents of 63 volatile compounds were used as input to construct the OPLS-DA model. Subsequently, a 200-permutation test was performed to demonstrate the stability of the model. Finally, the variable importance for the projection (VIP) output from OPLS-DA was used to measure the differentiation of compounds between undeteriorated and deteriorated silages.

2.5. Relationship between key volatile compounds and silage odor

The OPLS algorithm was used to confirm the relationship between key compounds and silage odor. First, the relative contents of 63 compounds and the odors of 18 silages were used separately as inputs to construct the OPLS model. Subsequently, a biplot was plotted based on the odor information of the compounds and silage. In the biplot, compounds located near silage odor points illustrated a strong correlation with the corresponding odor.

2.6. Statistical analysis

Origin 2022 (Northampton, MA, USA) was used to plot the temperature of silage and the relative content of volatile compound groups. GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used to plot the changes in the relative content of volatile compounds in silage. Median normalization, log transformation, and autoscaling were applied to preprocess of the generated data before the OPLS-DA and OPLS analysis, which was established using SIMCA 14.1 (Umetrics, Sweden).

3. Results

3.1. Discrimination of undeteriorated and deteriorated silages

3.1.1. Sensory quality evaluation

According to the DLG evaluation method (Table 2), D0-D1 feeds were undeteriorated (grades 1-3), and D2-D12 feeds were deteriorated (grades 4-5). The sensory properties of silages varied with increasing aerobic exposure time. In terms of odor, the fruity odor of the silages gradually disappeared. At the same time, the roasted, moldy, and rotten odors increased steadily.

Table 2 - Results of sensory quality evaluation

ID	Odor		Texture score	Color score	Mold score	Grade
	Score	Properties				
D0	0	Fruity, faint sour	0	0	0	1
D0.25	0.8	Fruity, faint alcoholic, faint sour, spicy	0	0	0	1
D0.5	2.8	Faint fruity, faint roasted, faint alcoholic, strong sour, spicy	0	0.3	0	2
D1	3	Faint fruity, faint roasted, strong sour, spicy	0.8	0.7	0	3
D2	4.4	Roasted, faint musty, faint ammonia	1.6	1.3	0	4
D3	5	Roasted, musty, ammonia	1.9	1.7	0	5
D4	6.2	Strong roasted, faint putrid, musty	3.2	2	4.2	5
D8	7	Strong roasted, putrid, musty	4	2	7	5
D12	7	Strong roasted, putrid, musty	4	2	7	5

Data expressed as the means (n = 5).

3.1.2. Aerobic stability monitoring

For the temperature change in silages (Figure 1), the average temperatures of the D2, D3, D4, D8, and D12 feeds were 27.4, 35.8, 29.2, 31.7, and 27.3 °C, respectively, all of which were more than 2 °C above the ambient temperature. This phenomenon indicated that these feeds were non-aerobically stable. Moreover, a moldy odor started to appear from the D2 feed and small moldy spots (Table 2), probably due to the multiplication of mold. Also, D0-D1 feeds were classified as grades 1-3 and D2-D12 feeds were classified as grades 4-5. Therefore, it was determined that deterioration occurred in the D2-D12 feeds according to the criteria in section 2.2.3. In this study, D0-D1 feeds were defined as undeteriorated grade (fresh-grade) silages, and D2-D12 feeds were defined as deteriorated-grade silages. The following studies were conducted between fresh- and deteriorated-grade silages.

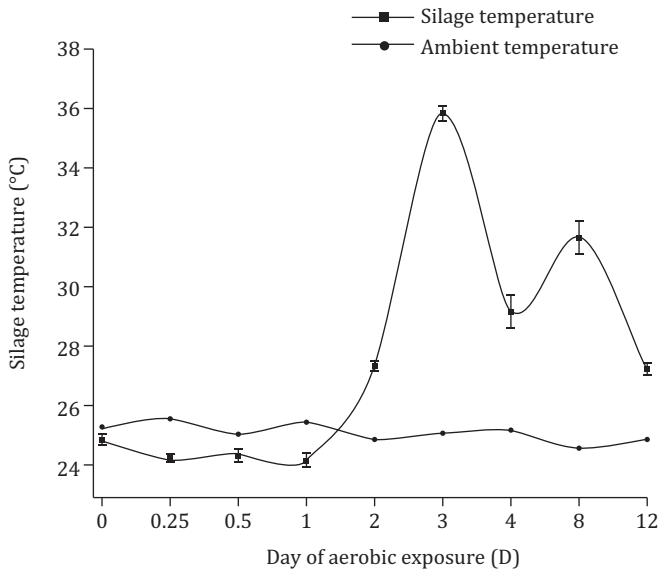


Figure 1 - Temperature changes during aerobic exposure.

3.2. Volatile component analysis

There was a significant difference between the relative contents of various functional groups of compounds in silages (Figure 2), with the main families of compounds being acids (54.67-70.87%), esters (6.17-29.58%), and phenols (3.19-9.26%), while terpenes (0.99-6.35%), alkanes (1.21-4.85%), and heterocyclics (0.34-0.88%) had lower contents. In addition, a wide variety was found in the relative contents of chemical families between the fresh- and deteriorated-grade silages. The relative content of esters ranged from 20.99 to 28.86% in fresh-grade silages, while their content ranged from 6.08 to 20.99% in deteriorated-grade silages. Deteriorated-grade silages had higher relative contents of acids (64.42 to 70.17%), phenols (3.41 to 9.60), terpenoids (1.54 to 6.35%), and alkanes (1.80 to 4.85%) than fresh-grade silages (54.35 to 60.73% for acids, 2.57 to 3.47% for phenols, 0.99 to 1.43% for terpenoids, and 1.21 to 1.65% for alkanes). In particular, the contents of n-hexylferulic acid, palmitic acid, linoleic acid, (Z)-oleic acid, linolenic acid, ethyl palmitate, ethyl linoleate, (Z)-ethyl oleate, and ethyl linolenate were higher (above 3% in individual silages).

A total of 63 volatile compounds were detected in all silages (Figure 3), including 19 acids, 17 esters, 11 phenols, four terpenes, three alkanes, three heterocycles, three aldehydes, two alcohols, and one amide.

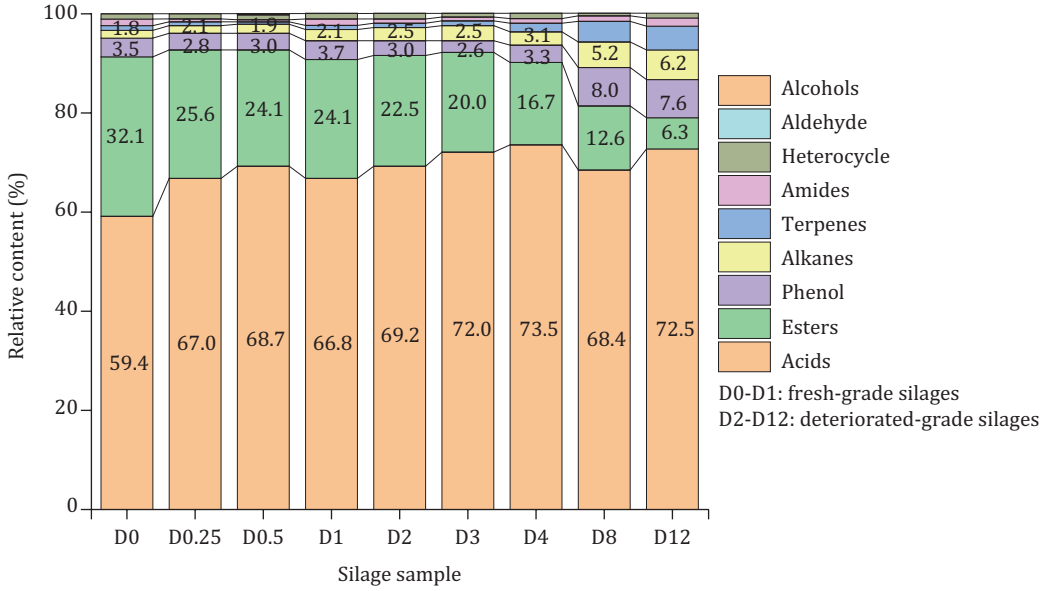
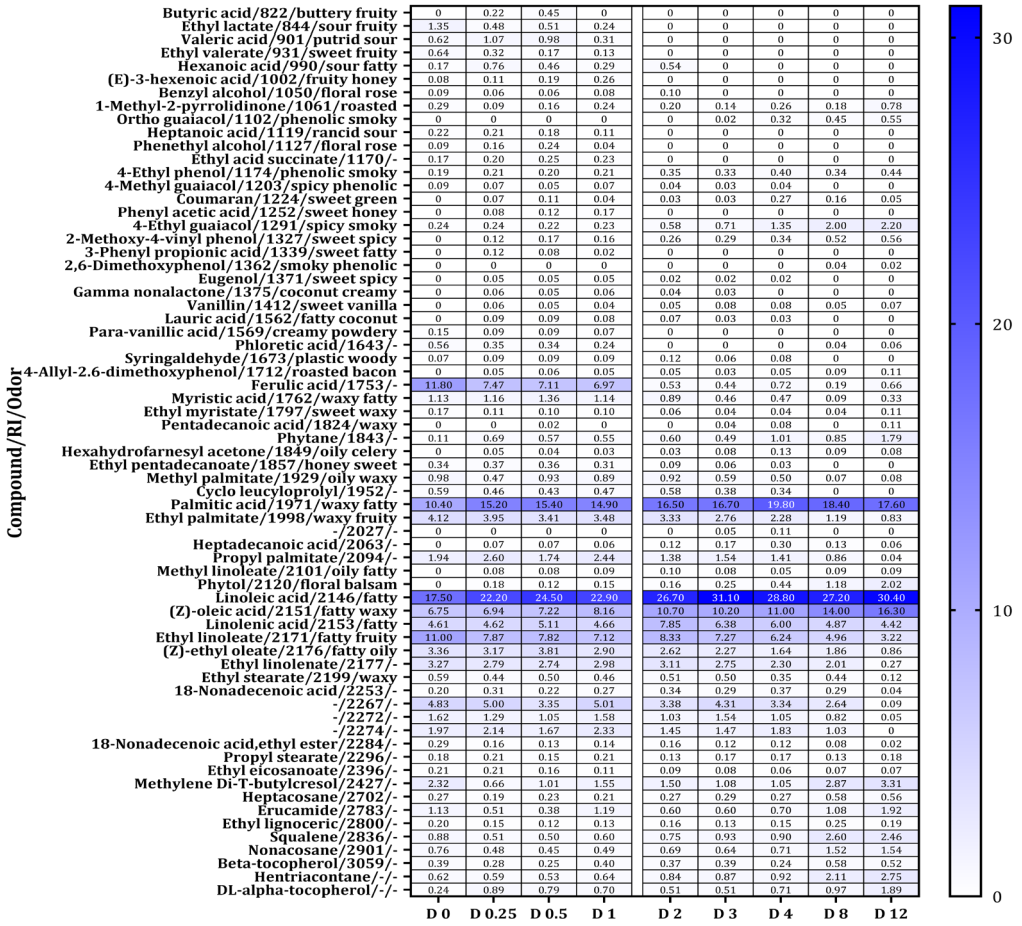


Figure 2 - Changes in the relative content of the volatile compound groups during aerobic exposure.



RI - calibration data from n-alkane; odor: search mainly in flavor database (www.thegoodscentscompany.com).
Data were expressed as the mean value (n = 2).

Figure 3 - Relative content changes of volatile components in silages with different aerobic exposure.

A total of 61 and 52 compounds were present in fresh-grade silages and deteriorated-grade silages, respectively. There were 31 mutual compounds in all samples, which might define the odor tone of the silages. The two different grade silages contained unique components. For instance, fresh-grade silages had 11 unique components, including butyric acid, valeric acid, (E)-3-hexenoic acid, heptanoic acid, phenyl acetic acid, 3-phenyl propionic acid, para-vanillic acid, ethyl lactate, ethyl valerate, ethyl acid succinate, and phenethyl alcohol. Ortho-guaiacol and 2,6-dimethoxyphenol were detected only in deteriorated-grade silages. These unique compounds might have increased the odor variance between the two different grade silages.

3.3. Determination of key volatile compounds

3.3.1. Calculation of relative odor activity values

In this study, 37 compounds had odor thresholds reported by previous studies, and thus, the present study analyzed the odor contribution of these compounds. Prior to the analysis, it was necessary to designate a compound with the highest contribution. Given the high relative content of linolenic acid in all silages (3.76 to 8.20%) and the low level of odor threshold (0.005 mg/kg), the ROAV of linolenic acid was defined as 100. For the other compounds, 15 volatiles—including six phenols, five esters, two aldehydes, one terpene, and one acid—had a considerable contribution to the overall odor (Table 3). Among them, five and seven compounds had an average ROAV (aROAV) ≥ 1 in fresh- and deteriorated-grade silages, respectively (Table 4). The details were as follows: ethyl valerate, 4-ethyl phenol, eugenol, linolenic acid, and ethyl linoleate with aROAV ≥ 1 in fresh-grade silages and ortho-guaiacol, 4-ethyl phenol, 4-ethyl guaiacol, 2-methoxy-4-vinyl phenol, eugenol, linolenic acid, and ethyl linoleate with aROAV ≥ 1 in deteriorated-grade silages. Notably, 4-ethyl phenol, eugenol, linolenic acid, and ethyl linoleate had aROAV ≥ 1 in both types of silage. This phenomenon illustrated that these compounds contribute significantly to the odor of deteriorated or undeteriorated silage and are potentially key compounds.

Table 3 - Odor active compounds (ROAV ≥ 0.1) in silages during aerobic exposure

Compound	Odor threshold	Fresh-grade silage					Deteriorated-grade silage					
		D0	D0.25	D0.5	D1	aROAV	D2	D3	D4	D8	D12	aROAV
Ethyl valerate	0.0058	11.9	5.9	2.9	2.4	5.8	0.0	0.0	0.0	0.0	0.0	0.0
Ortho guaiacol	0.00084	0.0	0.0	0.0	0.0	0.0	0.0	1.8	32.2	55.2	75.2	32.9
4-Ethyl phenol	0.013	1.5	1.7	1.5	1.7	1.6	1.7	1.9	2.5	2.6	3.8	2.5
4-Methyl guaiacol	0.021	0.4	0.3	0.2	0.3	0.3	0.1	0.1	0.1	0.0	0.0	0.0
4-Ethyl guaiacol	0.08925	0.2	0.2	0.2	0.2	0.2	0.4	0.6	1.2	2.3	2.8	1.4
2-Methoxy-4-vinyl phenol	0.01202	0.0	1.1	1.3	1.4	0.9	1.3	1.9	2.3	4.4	5.3	3.0
Eugenol	0.00071	0.0	8.0	6.4	7.3	5.4	1.7	2.1	2.7	0.0	0.0	1.3
Gamma-nonalactone	0.0097	0.0	0.6	0.4	0.6	0.4	0.2	0.2	0.0	0.0	0.0	0.1
Vanillin	0.053	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1
Ethyl palmitate	2	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.0	0.0	0.0
Phytol	0.64	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3	0.1
Linolenic acid	0.005	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Ethyl linoleate	0.45	2.6	1.9	1.7	1.7	2.0	1.1	1.2	1.1	1.1	0.8	1.1
(Z)-ethyl oleate	0.87	0.4	0.4	0.4	0.3	0.4	0.1	0.2	0.1	0.2	0.1	0.1
Ethyl stearate	0.5	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0

ROAV - relative odor activity value; aROAV - average relative odor activity value.
Odor thresholds (mg/kg) of compounds in water were obtained from van Gemert (2011).
Data were expressed as the means ± standard deviations (n = 2).

Table 4 - Compounds with aROAV ≥ 1 in 2-grade silage

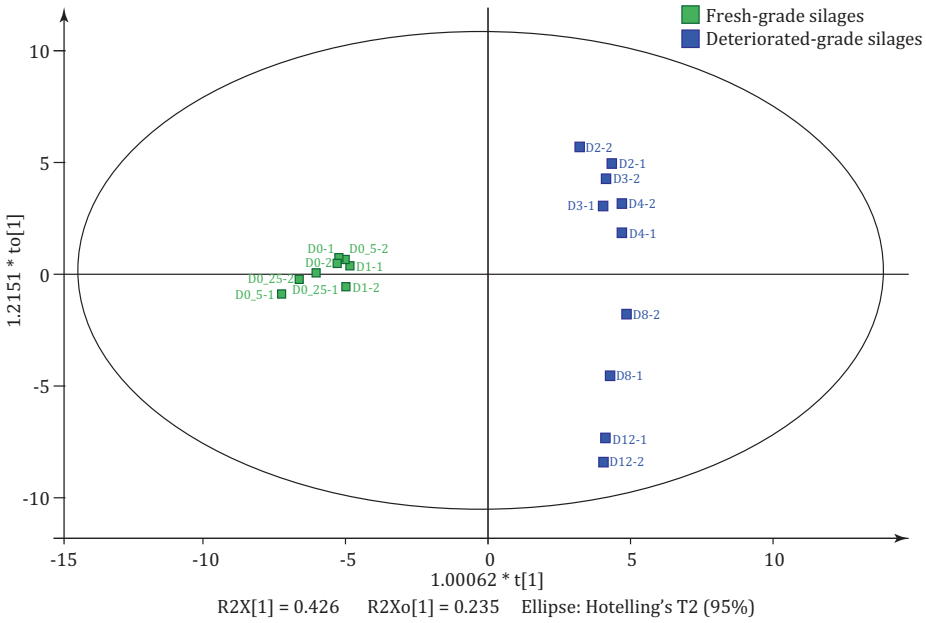
Silage	Compound	aROAV
Fresh-grade silages	Ethyl valerate	5.8
	4-Ethyl phenol	1.6
	Eugenol	5.4
	Linolenic acid	100.0
	Ethyl linoleate	2.0
Deteriorated-grade silages	Ortho guaiacol	32.9
	4-Ethyl phenol	2.5
	4-Ethyl guaiacol	1.4
	2-Methoxy-4-vinyl phenol	3
	Eugenol	1.3
	Linolenic acid	100.0
	Ethyl linoleate	1.1

aROAV - average relative odor activity value.

3.3.2. Analysis of differential compounds by OPLS-DA

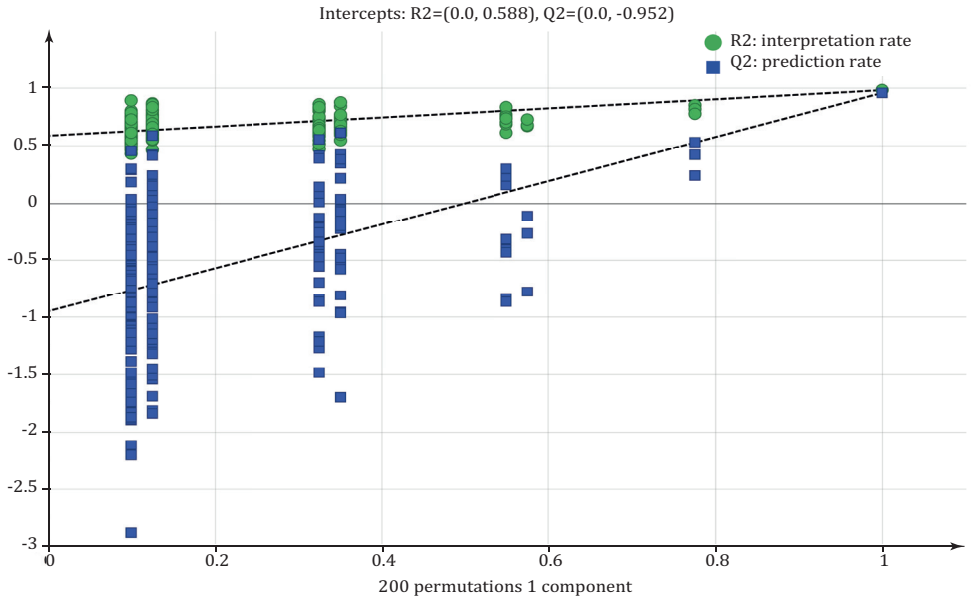
For the OPLS-DA of the compounds, there were significant differences between the two types of silages (Figure 4), indicating that volatile compounds could effectively discriminate silage deterioration. Moreover, the model showed satisfactory performance with R2x, R2y, and Q2 values of 0.774, 0.983, and 0.959, respectively. The validity of the model was confirmed by the 200-permutation test, in which the Q2 regression line intersected the vertical axis at a point less than zero and all blue Q2 values on the left were lower than the original points on the right (Figure 5).

The VIP quantifies the contribution degree of each variable to classification (Su et al., 2022). There were 30 compounds with VIP > 1, which were the differential compounds between the two silage grades (Figure 6). There were six compounds regarded as key compounds (VIP > 1, aROAV ≥ 1) (Figure 5



OPLS-DA - orthogonal partial least squares discriminant analysis.

Figure 4 - Score plot for the OPLS-DA model.



OPLS-DA - orthogonal partial least squares discriminant analysis.

Figure 5 - Permutation plot for the OPLS-DA model.

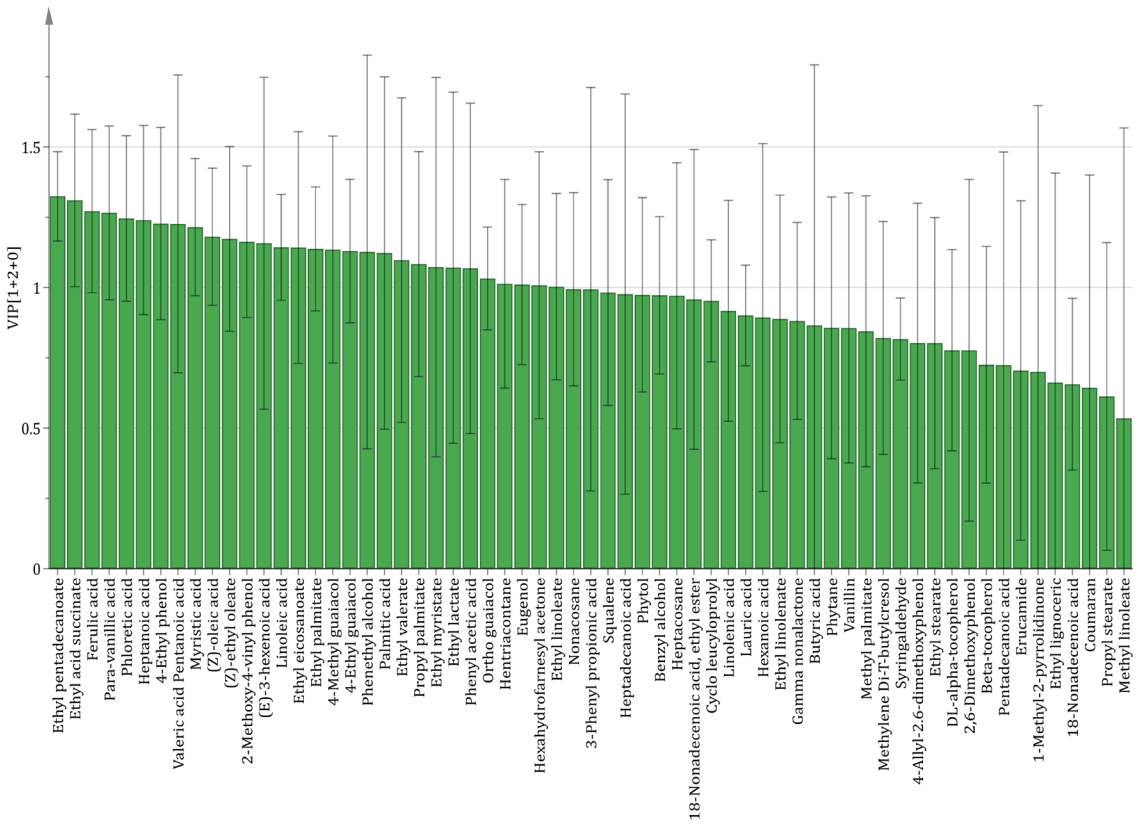
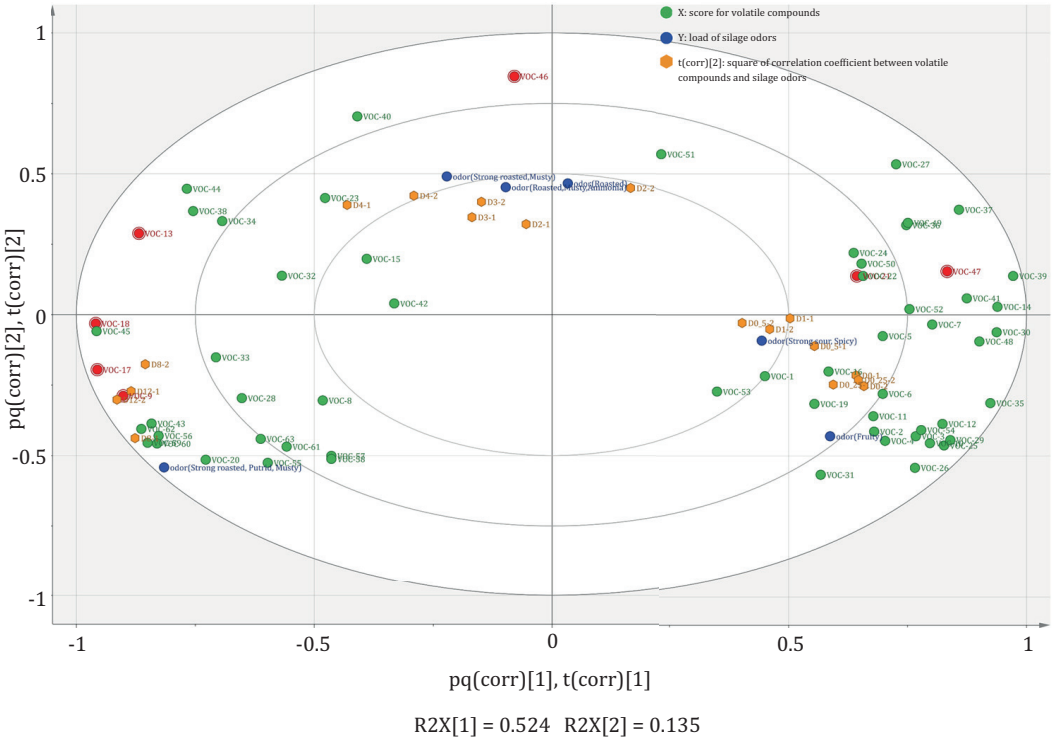


Figure 6 - Variable importance for the projection (VIP) value for volatile compounds.

and Table 3). Among them, 4-ethyl phenol, eugenol, and ethyl linoleate were the key compounds that characterized whether the silages deteriorated or not since these compounds had aROAV ≥ 1 in both types of silages. In addition, ortho-guaiacol, 4-ethyl guaiacol, and 2-methoxy-4-vinyl phenol were the specific key compounds for deteriorated-grade silage, because the aROAV ≥ 1 of the three compounds was found only in this type of silage. In terms of odor, ethyl linoleate had a fruity odor; eugenol had a sweet odor; and ortho-guaiacol, 4-ethyl phenol, 4-ethyl guaiacol, and 2-methoxy-4-vinyl phenol all had a smoky odor, further confirming the variation in fruity, sweet, and smoky odors between the two silage grades.

3.4. Relationship between key volatile compounds and silage odor

The OPLS was used to investigate the relationship between key compounds and silage odor (Figure 7). In terms of the performance of the OPLS model, R2X, R2Y, and Q2 were 0.945, 0.958, and 0.724, respectively, which demonstrated acceptable performance. Combined with the results of sensory evaluation, in the OPLS, we defined fruity odor corresponding to D0 and D0.25 feeds, strong sour and spicy odor corresponding to D0.5 and D1 feeds, roasted odor corresponding to D2 feeds, roasted, musty, and ammonia odor corresponding to D3 feeds, strong roasted and musty odor corresponding to D4 feeds, and strong roasted, malodorous, and musty odor corresponding to D8 and D12 feeds (Figure 7). Moreover, we defined a group of odor points that have a tendency to cluster: strong sour and spicy point and fruity point were a group, which were located in the positive direction of the X-axis and negative direction of the Y-axis; strong roasted and musty point, roasted point and roasted, musty and ammonia point were a group, which were located in the positive direction of the X-axis; strong



OPLS - orthogonal partial least squares.
VOC-9: ortho guaiacol; VOC-13: 4-ethyl phenol; VOC-17: 4-ethyl guaiacol; VOC-18: 2-methoxy-4-vinyl phenol; VOC-21: eugenol; VOC-46: linolenic acid; VOC-47: ethyl linoleate.

Figure 7 - Biplot of OPLS among the silage samples, odors, and volatile compounds.

roasted, malodorous, and musty points are grouped in the negative direction of the X- and Y-axes. It was noted that eugenol and ethyl linoleate were positively correlated with fruity and sour and spicy odors, respectively. In addition, guaiacol, 4-ethyl guaiacol, and 2-methoxy-4-vinyl phenol contributed to the roasted, musty, and putrid odor groups. 4-Ethylphenol contributed to both the upper and left groups of odors on the plot.

4. Discussion

4.1. Changing patterns of relative content of volatile compounds during aerobic exposure

In this study, the changing pattern of relative contents of some compounds with aerobic exposure was analyzed by previous studies. The relative content of butyric, valeric, and hexanoic acids gradually decreased with the increasing duration of aerobic exposure, which may be due to the depletion of yeast (Zhang et al., 2018). The relative content of long-chain fatty acids (number of carbon atoms > 6), such as palmitic, linoleic, (Z)-oleic, and linolenic, increased with aerobic exposure. This could be a consequence of their weaker volatility (Tong et al., 2007). Esters were important for silage odor characterization (Worku et al., 2021). Ethyl lactate, ethyl valerate, ethyl palmitate, and ethyl linoleate were the ethyl esters that have been identified in silage (Figueiredo et al., 2007; Zhang et al., 2021). The relative content of DL-alpha-tocopherol was increased by aerobic exposure time. This phenomenon was caused by the proliferation of microorganisms during the aerobic exposure period (Liu et al., 2019). Zong et al. (2022) found higher squalene content in spoiled alfalfa silages than in quality forages. Similarly, the relative squalene content of the D8 and D12 feeds was several times higher than that of the D0 feed.

4.2. Effect of key compounds on the odor of 2-grade silage

In this study, 4-ethyl phenol, eugenol, and ethyl linoleate were the key compounds that characterized whether the silages deteriorated. We considered the effects of these compounds on 2-grade silage odors as follows: in fresh-grade silage, the odor of 4-ethyl phenol might have been masked, eugenol contributed a spicy odor, and ethyl linoleate contributed a fruity odor; in deteriorated-grade silage, 4-ethyl phenol contributed a roasted odor and eugenol and ethyl linoleate odors might have been masked. Specific reasons were given as follows:

The 4-ethyl phenol had a smoky odor (Figure 3), which was shown to be associated with the roasted odor of silage (Figure 7). The aROAV of 4-ethyl phenol in deteriorated-grade silage was 2.5, and the aROAV of guaiacol (smoke flavor) was 32.9 (Table 3), both having similar odors. Therefore, these two compounds might work together to provide a roasted odor to deteriorated-grade silage. In addition, odors of low odor intensity may be masked by odors of higher odor intensity (Ma et al., 2021). The 4-ethyl phenol had an aROAV of 1.6 in fresh-grade silage at a relatively low level, suggesting that its odor may be masked by compounds with higher aROAV.

Eugenol was spicy (Figure 3) and was shown to be associated with the spicy odor of silage (Figure 7). Eugenol had a higher aROAV in fresh grade silage than most compounds, except for ethyl valerate and linoleic acid (Table 3). This indicated that the odor of eugenol may be revealed. In contrast, the aROAV of 1.3 for eugenol in deteriorated-grade silage was lower, suggesting that its odor may be masked by compounds with high aROAV.

Ethyl linoleate had a fruity odor (Figure 3) and was shown to be associated with the fruity odor of silage (Figure 7). The aROAV for ethyl linoleate in fresh-grade silage was 2.0 and the similar odor of ethyl valerate (fruity) had an aROAV of 5.8 (Table 3). This suggested that ethyl linoleate may synergistically contribute to the fruity odor of fresh-grade silage with ethyl valerate. The relatively low aROAV of 1.1 for ethyl linoleate in deteriorated-grade feeds suggests that its odor may be masked by compounds with higher aROAV.

4.3. Limitations and future works

In similar studies, principal component analysis (PCA) and ROAV were used to analyze key compounds in chicken breast (Bi et al., 2021); the ROAV, PCA, and sensory evaluation were integrated to analyze key compounds in safflower seed oil (Wang et al., 2020); the OPLS-DA combined with ROAV mined 11 key compounds characterizing black tea grades (Su et al., 2022). Although the above studies provided satisfactory results, methods to quantify the intrinsic link between key compounds and the odor of the measured object were scarce. Therefore, this study innovatively utilized OPLS to analyze the relationship between key compounds and silage odor. The results showed that OPLS could effectively mine the intrinsic link between silage odor and key compounds.

However, the limitations of this study were as follows: silage volatiles were not quantified during aerobic exposure; only volatiles associated with silage fruity, spicy, and roasted odors were screened, and additional key compounds were not found to fully characterize silage odors. Therefore, future work could aim to quantitatively determine the volatile compounds in silage with reference to similar studies, and introduce more intelligent algorithms (e.g., the partial least square (PLS)) for in-depth screening of key compounds that characterize silage odor (Su et al., 2022).

5. Conclusions

In this study, HS-SPME-GC-MS identifies a total of 63 volatile compounds in silages during aerobic exposure. The following, screened from ROAV integrated with OPLS-DA, are the key compounds used to characterize whether the silages deteriorate: 4-ethyl phenol, eugenol, and ethyl linoleate. The OPLS innovatively mines the relationship between these three key compounds and silage odor: 4-ethyl phenol affects the roasted odor of deteriorated-grade silage, and eugenol and ethyl linoleate affect the spicy and fruity odors of fresh-grade silage, respectively. In practical application scenarios, these key compounds have the potential to be used as markers for monitoring silage spoilage.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Zhao, K. and Ren, X. **Data curation**: Yu, Y. and Guo, L. **Formal analysis**: Li, Y. **Project administration**: Tian, H. and Liu, F. **Visualization**: Tao, Y. **Writing – original draft**: Zhao, K. and Ren, X. **Writing – review & editing**: Tian, H.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (32071893), the Science and Technology Program Project of Inner Mongolia Autonomous Region (2022YFDZ0024), the Natural Science Foundation of Inner Mongolia Autonomous Region (2023MS03044), the Postgraduate Research Innovation Program of Inner Mongolia Autonomous Region (DC2300002028), and the Innovation Training Program for College Students of Inner Mongolia Autonomous Region (202310129026).

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