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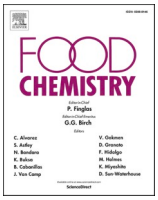

Changes in volatile flavor of yak meat during oxidation based on multi-omics

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ABSTRACT

Hydroxyl radical system combined with GC-IMS and metabolomics were used to assess the effect of oxidation on the formation of volatile flavor emitted from yak meat. The formation of volatile compounds, including heptanal, octanal, nonanal, 2,3-glutaraldehyde, 3-hydroxy-2-butanone, etc. were promoted by oxidation. Among them, 2,3-pentanedione and 3-hydroxy-2-butanone, etc. maybe contributed most to the overall aroma of yak meat, while octanal, nonanal and benzaldehyde maybe related to the formation of off-odor or acidification. Meanwhile, the content of metabolites such as oleic acid, linoleic acid, etc. fatty acids and 3-dehydromangiferic acid, tyrosine were increased or decreased with the time of oxidation. More importantly, the formation of most flavor components in yak meat during the course of oxidation were related to stearidonic acid, acetylleucine, dehydroshikimate, 6-phosphate-glucose etc. differential metabolic components. Moreover, starch and sucrose metabolism (prediction), and amino acid metabolism (enrichment) etc. pathways maybe related with the process of oxidation.

1. Introduction

Yak is an endemic species living in the Himalayas of China (G. Wang et al., 2017). In recent years, yak meat is more and more popular among consumers due to its natural pollution-free, tasty meat and unique volatile flavor. The protein content of yak hindquarter meat is as high as 20% to 23%, and the fat content is about 5% lower than ordinary yellow beef hindquarter meat. Yak meat is rich in amino acids and the content of essential amino acids is also higher than ordinary beef (Bai, Hao, Chai, Niu, Wang, & Liu, 2014). The monounsaturated fatty acid in yak meat is as high as 45.15%, the instability of fat structure leads to its easy oxidation, and the aldehydes generated by oxidation are easy to react with protein, thus promoting protein oxidation. Meanwhile, the reactive oxygen species produced by protein oxidation can also promote lipid oxidation. The Himalayan region is remote and lack of access to transportation. Thus, protein of yak meat is easily oxidized during maturation, transportation and storage, which leads to the destruction of amino acids and other nutrients in meat, and further impacts the flavor of yak meat.

Flavor plays an essential role in the sensory properties of food. The desire of purchase and the acceptability of consumers are directly affected by the quality of food volatile flavor. Volatile flavor is very important in evaluating the nutritional value and freshness of food. At present, researchers are paying more attention to how to use different flavor analysis methods to monitor and predict food quality changes (Q. Zhang, Ding, Gu, Zhu, Zhou, & Ding, 2020).

GC-IMS is a new advanced technology for rapid detection of volatile flavor compounds. It has been widely used in the field of food flavor analysis due to its advantages of high sensitivity, fast detection speed, low analytical temperature and easy operation (S. Wang, Chen, & Sun, 2020). Currently, GC-IMS has been applied to the evaluation of food freshness and deterioration degree, the detection of food odor, and the

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changes of food flavor compounds during processing and storage. In addition, GC-IMS can visualize volatile flavor compounds. Therefore, it has been widely used in food identification and adulteration, flavor changes during food fermentation and analysis and monitoring of flavor compounds during fruit and vegetable storage (Yang et al., 2019).

Metabonomics is an emerging technology that can detect and analyze a variety of metabolites in cells, tissues and organisms. In studies of meat, metabonomics analysis has been used to further understand the post-mortem aging process and improve meat quality, especially meat tenderness and meat color stability (Alessandro & Zolla, 2013). However, the metabonomics has not been reported to decipher the changes of volatile flavor compounds in yak meat during oxidation. Thus, the development patterns and potential mechanism of yak volatile flavor compounds can be explored by determining the relationship between different metabolic pathways and yak volatile flavor precursors.

However, there is a complex relationship between the change of yak meat volatile flavor and meat composition, which involves abundant chemical substances and metabolic pathways. As the precursor of volatile flavor compounds, metabolic compounds also have a great influence on the flavor compounds of yak meat (Setyabrata, Cooper, Sobreira, Legako, Martini, & Kim, 2021). Therefore, the identification of volatile flavor precursors and metabolic pathways in yak meat oxidation process has become a research hotspot.

In this study, GC-IMS and metabolomics were applied to explore the change rule of volatile flavor compounds in the oxidation process of yak meat. Simulation of in vitro oxidation of yak meat by hydroxyl radical oxidation system to establish volatile flavor fingerprinting of yak meat during oxidation. And the metabonomics method was used to further explore the precursor substances and metabolic pathways of yak volatile flavor. That provided a new technical means to explore the change rule of volatile flavor compounds of yak meat, and theoretical support for quality control and prediction of yak meat in the process of ripening, transportation and storage.

2. Materials and methods

2.1. Materials and chemicals

Yak meat (Maiwa yak) bought from Chuanbeige flagship store of Renda Technology Co., Ltd., Mianyang City, Sichuan Province, China.

Ferric chloride, ascorbic acid, hydrogen peroxide, ethylene diamine tetraacetic acid (EDTA), urea, guanidine hydrochloride, glycine, trichloroacetic acid (all analytical pure) were purchased from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China).

2.2. Sample preparation

2.2.1. Preparation of yak meat

Yak hindquarter meat was the main raw material. The yak meat was divided into meat pieces of uniform quality (5 ± 0.1 g) after thawing, cleaning and removing the excess fat and connective tissue. Then, the meat was cleaned with running water, and the water on the surface was dried with paper towels, and then put into a refrigerator at 4 ℃ for later use.

2.2.2. Oxidation treatment of yak meat

The pretreated yak meat samples were divided into 5 parts, and the water on the surface of the meat was absorbed by paper towel. Then, the meat samples were placed in a Fenton oxidation system (1.0 mmol/L $FeCl_3$, 0.1 mmol/L Vitamin C and 10 mmol/L H_2O_2), and the ratio of meat sample to oxidizing liquid was 2:1 (m/v). And the meat samples needed to be sealed and protected from light. The oxidized meat samples with different oxidation time were obtained after 0, 2, 4, 6 and 8 h of oxidation, respectively. After a period of reaction, EDTA (1.0 mmol/L) was added to chelate Fe^{2+} in the oxidation system to terminate the reaction. The samples were stored in refrigerator at 4 ℃ for 2 h for later use.

2.2.3. Extraction of myofibrillar protein

The myofibrillar protein (MP) was isolated from yak meat according to the method of Zhang et al. (2020). The MP were stored in refrigerator at 4 ℃ for 2 h for later use. The MP content was determined by Biuret method using bovine serum albumin as a standard protein, which was used within 24 h. The MP concentration was normalised to 10 mg/mL with NaCl phosphate buffer (0.4 mol/L, pH 7.0).

2.3. The quality characteristics

2.3.1. pH

The pH value was determined according to the method of Huang et al. (2021) with slight modifications. An appropriate amount of oxidized yak meat sample was grinded after bloting surface moisture with absorbent paper. Oxidized samples (10 g) were added into distilled water (100 mL), homogenized for 1 min (10,000 r/min) (T25, IKA company, Staufen, Germany), and then filtered. The filtrate was used to scaled the pH value (Mettler-Toledo Instrument Co., Ltd., Shanghai, China). Five replicates were done for each sample.

2.3.2. Cooking loss

The cooking loss was measured by the method described by Luo et al. (2021). The meat samples were heated in a constant temperature water bath at 80 ℃ for 5 min. The samples were removed and rinsed by running water. The cleaned sample was cooled to room temperature and kitchen paper was used to wipe off the residual liquid left on the surface of the meat after cooking. And then the cooking loss was calculated.

2.3.3. TVB-N

The volatile basic nitrogen (TVB-N) was measured as previously reported with some modifications (Dong et al., 2021). The meat samples were cut up, dissolved with water and filtered. The Nessler was added into the filtrate. The absorbance of the solution was measured at 420 nm using water as control.

2.4. Oxidation index

2.4.1. TBARS

Thiobarbituric acid reactive substances (TBARS) was according to the method of Dong et al. (2020). Meat sample (0.3 g) was mixed with 3 mL TBA and 17 mL TCA-HCl in boiling water bath for 30 min, then 5 mL chloroform was added. And the absorbance value was measured at 532 nm after centrifugation (3,000 g, 10 min) (H1650R, Xiangyi Co., Ltd, Hunan, China) .

2.4.2. Determination of carbonyl content

The carbonyl content was measured according to the method of Geng et al. (2018) and Zhang et al. (2020). The myofibrillar protein was diluted to 0.1 mg/mL with 0.4 mol/L NaCl phosphate buffer solution (pH 7.0), and the carbonyl value was measured by 2, 4-dinitrophenylhydrazine reaction.

2.4.3. Determination of total sulfhydryl content

The determination of total sulfhydryl content was carried out in the present study according to the previous literature with some modifications (Y. Wang, Zhou, Li, Wang, Cai, & Chen, 2018; Geng, Xie, Wang, & Wang, 2021). The myofibrillar protein was dissolved in phosphate buffer solution (0.05 mol/L, pH 7.0). The absorbance was determined by 5,5 ’-dithiobis (2-nitrobenzoic acid). And the total sulfhydryl content was calculated by the molar extinction coefficient of 13,600 L/(mol•cm).

2.5. Determination of volatile flavor compounds

2.5.1. Sample collection and processing

One sample of each with different oxidation times was taken and grinded with a meat grinder. 5 ± 0.1 g of the mince sample was weighed into a 20 mL headspace injection vial and set aside. Three replicates of each sample were made and used for GC-IMS (Flavorspec, G.A.S. Instrument, Germany) analysis.

2.5.2. GC-IMS analysis

Chromatographic column type: FS-SE-54-CB-1 (15 m × 0.53 mm, 1 μm), analysis time: 30 min, column temperature: 60 ℃, carrier gas/drift gas: N_2. The headspace injection was performed automatically with an injection volume of 500 μL, incubation time of 15 min, incubation temperature of 55 ℃, injection needle temperature of 85 ℃, and incubation speed of 500 r/min.

2.5.3. Statistical analysis

The Analytical software LAV (Laboratory Analytical Viewer) and the built-in NIST database and IMS database of GC-IMS Library Search software were used for qualitative analysis of characteristic volatile flavor compounds. The Reporter plug-in in LAV was used to directly compare the spectral differences between samples. Fingerprint was compared with gallery plot plugin to visually and quantitatively compare the volatile matter differences between yak meat samples treated with different oxidation time. The Dynamic principal component analysis was performed by the dynamic PCA plug-in.

2.6. Metabonomics analysis

2.6.1. Extraction of metabolites

The extraction methods of metabolites were carried out according to the previous literature and modified appropriately (Want et al., 2013). Oxidized yak meat samples were accurately weighed into a 2 mL LEP tube of 100 ± 2 mg. 1 mL of tissue extract was added to oxidized yak meat samples (75% 9:1 methanol: chloroform, 25% H_2O) (-20 ℃). After the steel ball was added, the samples were put into a high-throughput tissue grinding machine (55 Hz, 1 min) (TISSUELYSER-II, Jingxin Pharmaceutical Machinery Co., Ltd., Shanghai, China) and repeat twice.

2.6.2. LC-MS analysis

LC conditions: AcCity UPLC® HSS T3 1.8 m (2.1 × 150 mm) chromatographic column was used for gradient elution. The automatic sampler temperature was set at 8 ℃, the flow rate was 0.25 mL/min, the column temperature was 40 ℃, the injection volume was 2 μL. The mobile phase was positive ion 0.1% formic acid water (A_2) − 0.1% formic acid acetonitrile (B_2); Anion 5 mM ammonium formate water (A_3) - acetonitrile (B_3). MS conditions: electrospray ion source (ESI), positive and negative ion ionization mode, positive ion spray voltage of 3.50 kV, negative ion spray voltage of 2.50 kV, sheath gas of 30 Arb, auxiliary gas of 10 Arb. The capillary temperature was 325 ℃, and the scanning range was 81 ~ 1000 with a resolution of 70,000. HCD was used for secondary cracking with a collision voltage of 30 eV, and the unnecessary MS/MS information was removed by dynamic elimination (Jinqiu Wang et al., 2019; J. Wang et al., 2021).

2.6.3. Multivariate statistical analysis

To further discover the effect of oxidation time on the volatile flavor compounds of yak meat, multivariate statistical analysis was performed on yak meat samples. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal-partial least squares discriminant analysis (OPLS-DA) were used to downscale and categorize the metabolic data to obtain more reliable and intuitive results. All metabolites were obtained from the following databases: Metlin (http://metlin.scripps.edu/), MoNA (http://mona.fiehnlab.ucdavis.edu//) and Panomic's own standard database (Suzhou BioNovoGene Biomedical Tech Co., LTD., Suzhou, China). All metabolic pathways were obtained from the KEGG database b(https://www.genome.jp)

2.7. Data processing

The measurements data were presented as the mean values of at least triplicate experiments, and the data were analyzed and plotted by GraphPad Prism 7.05 software (San Diego, CA, USA). One-way ANOVA was used to estimate the difference between the means ($P < 0.05$).

3. Results and discussion

3.1. pH, cooking loss, and TVB-N

With the prolonging of oxidation time, the pH value of yak meat first decreased and then increased (Fig. 1a). This phenomenon was caused by the disruption of oxygen in yaks after slaughter, anaerobic respiration was carried out in the body of yaks, glycogenolysis produced lactic acid, and adenosine triphosphate decomposed into phosphoric acid, resulting in a significant decline in the pH value of yaks ($P < 0.05$); After that, with the prolongation of oxidation time, lactic acid was gradually degraded and proteins in yak meat were decomposed into alkaline substances such as ammonia and amino acids, which made yak meat tend to be alkaline and pH value increased significantly. Meanwhile, the metabolomics results showed that lysine was significantly up-regulated and aspartic acid was significantly down-regulated with increasing oxidation time, lysine being a basic amino acid and aspartic acid being an acidic amino acid, which was also similar to the pH results.

Cooking loss is one of the important indicators to measure the quality of meat, which can reflect the water retention of meat to a certain extent. And the water retention of meat directly affects the color, tenderness and nutritional composition of meat products (Jinqiu Wang et al., 2021). The meat lost components mainly including water, certain sarcoplasmic proteins and intramuscular fat soluble collagen in the cooking process. If the cooking loss rate was higher, the poorer the eating quality. The cooking loss of yak meat increased significantly (Fig. 1a) ($P < 0.05$). And with the extension of oxidation time, the proteins in yak meat denatured and contracted, and the structure of myofibrillar protein was destroyed, so the water-holding capacity of the meat decreased and the cooking loss increased. The water retention of meat mainly depends on the ability of protein to bind water molecules. The key lies in the hydration of protein surface and the capillary action of the lattice of muscle fibers. In general, muscle proteins are amphiphilic polymer, and protein conformation changes induced by protein oxidation expose the hydrophobic sites originally located in the interior of the molecule. Thus, the hydrophobicity of the protein was improved and the surface hydration of the protein was reduced, which further led to the decrease of the water retention of the meat.

TVB-N value is an important index to evaluate meat freshness. During meat storage, biological macromolecules such as protein and fat are decomposed under the action of microorganisms and enzymes to produce basic nitrogen-containing substances such as ammonia and amines, thus leading to the increase of TVB-N value (Dong et al., 2021). With the increase of oxidation time, the content of TVB-N in yak meat increased significantly (Fig. 1b). At 2 h of oxidation, the TVB-N value reached 15.84 mg/100 g, which already exceeded the national standard for first-grade fresh meat (15 mg/100 g). And the TVB-N value of yak meat reached 20.06 mg/100 g after 6 h of oxidation, which exceeded the national standard for fresh meat (20 mg/ 100 g) and belonged to spoiled meat. During the processing of yak meat, with the prolongation of oxidation time, microorganisms multiply and produce a large number of metabolites with spoilage and odor such as trimethylamine, amino acids and sulfides, resulting in a rapid increase of TVB-N value.

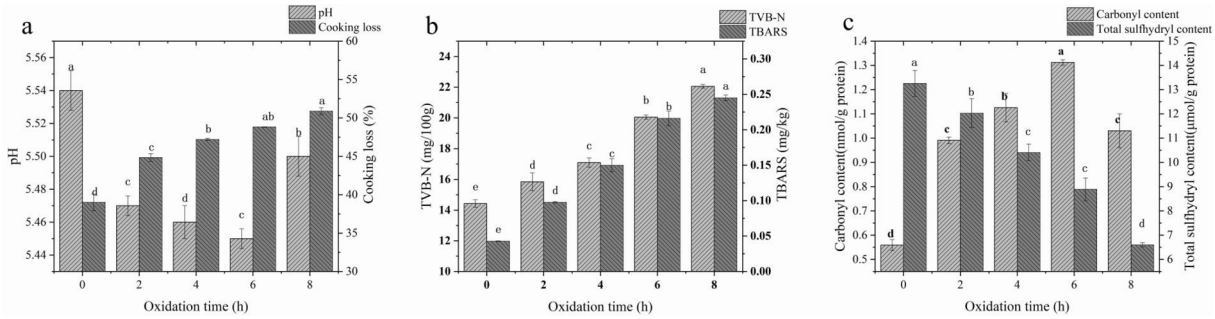

Fig. 1. Effect of oxidation times on the pH, cooking loss (a), TVB-N, TBARS (b), carbonyl and total sulfhydryl (c) of yak meat. Different letters in the same subplot indicate significant differences ($P < 0.05$).

3.2. TBARS, carbonyl and total sulfhydryl

The main product of lipid oxidation is peroxide, which further decomposes under the action of enzymes to produce small molecules such as aldehydes, ketones and fatty acids. And the malondialdehyde formed can react with thiobarbituric acid. Therefore, the TBARS value can more accurately evaluate the degree of lipid oxidation (X. Wang et al., 2021). Compared with the control group (Fig. 1b), the TBARS value increased significantly ($P < 0.01$) with increasing oxidation time before 6 h, and the trend of increasing TBARS value slowed down after 6 h. In general, the increase in TBARS values of yak meat samples before 6 h ($P < 0.05$) represented the continuous production of aldehydes during oxidation. And the slowing down of the increasing trend after 6 h was due to the reaction of malondialdehyde generated by oxidation with other macromolecules, which caused a slow increase in TBARS values.

Many oxidation products were produced by proteins oxidation. The carbonyl group was the main product of proteins that have been oxidized. Hence, the carbonyl value was generally used to evaluate the degree of oxidation of proteins. The carbonyl content of yak meat myofibrillar protein showed a general trend of increasing and then decreasing during the oxidation process (Fig. 1c), and reached the maximum value at 6 h of oxidation. The increased of the content of carbonyl group may be triggered by the conversion of some amino acids in the side chains of the protein skeleton to carbonyl groups due to the attack of –OH. α-aminoadipate hemialdehyde and γ-glutamate hemialdehyde were formed carbonyl compounds that were biomarkers of protein oxidation in meat (H. Zhang, M. Ai, et al., 2020). The carbonyl content showed a significant trend of decrease after the oxidation treatment reached to 6 h. Which may be caused by the newly formed carbonyl group that attacked the nucleophile in the protein, resulting in carbonyl ammonia condensation reaction, which led to the decrease of carbonyl group content.

Sulfhydryl content is also an important index to characterize the degree of protein oxidation. Myofibrillar protein is rich in sulfhydryl groups, which can be easily converted to disulfide bonds under oxidation conditions, leading to a decrease in sulfhydryl group content. The total sulfhydryl content of yak meat myogenic fibrin decreased significantly ($P < 0.05$) with the increasing oxidation time (Fig. 1c), and reached 6.56 μmol/g at 8 h. This indicated that the degree of protein oxidation gradually increased with time, which was also consistent with the results of carbonyl determination. Sulfhydryl groups were presented in proteins in two main forms, one was presented on the surface of the protein and the other was encapsulated inside the protein (H. Zhang, X. Huang, et al., 2020). The total sulfhydryl group of myofibrillar protein was converted into intermolecular disulfide bond by hydroxyl radical attack during processing, which directly affected the structure of protein and then affected its functional properties.

3.3. Changes in volatile flavor compounds during oxidation of yak meat

3.3.1. Qualitative results of volatile flavor compounds of yak meat in different oxidation times by GC-IMS

The qualitative analysis of volatile components was carried out by GC-IMS Library Search software, and the matching was carried out by GC-IMS Library (Table 1). The results indicated that the volatile substances that can be identified during the oxidation process of yak meat were 54 monomers and dimer of some of their substances. The monomers mainly include 11 aldehydes, 10 alcohols, 7 ketones, 2 esters, 1 ether, 1 acid, 1 phenolic, 1 furan and 1 sulfide. With the extension of oxidation time, aldehydes such as pentanal, (E)-2-pentanal, hexanal, E-2-hexanal, heptanal, E-2-heptenal, octanal, E-2-octenal, nonanal, benzaldehyde, alcohols such as propanol, butanol, pentanol, hexanol, 2-hexanol, (E)-acetic acid-2-hexen-1-ol ester, 1-octen-3-ol, octen-3-ol, 1,8-eucalyptol, ketones such as 2-acetone, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, acetylacetone, esters such as butyl butyrate, ethyl propionate, dimethyl sulfide, methyl butyrate, pentanol furan and other volatile substances were produced and gradually increased. Only ethanol decreased gradually with the prolonging of oxidation time. In the results, we also founded interesting results. Substances such as 3-hydroxy-2-butanone, maltol, pentanone and 2,3-pentanedione gradually increased with the oxidation time until 6 h, but the content decreased after 6 h. The contents of sulfides such as dimethyl sulfide and dimethyl sulfide gradually increased before 4 h of oxidation and decreased significantly after 4 h.

As shown in Table 1, aldehydes, especially unsaturated aldehydes, were the main volatile flavor compounds formed during the oxidation of yak meat. Aldehydes were important intermediates in the melad reaction or lipid oxidation reaction and can be involved in the interaction between amino acids and carbonyl groups. And aldehydes, such as hexanal, nonanal and heptanal, were key volatile flavor compounds in yak meat oxidation process because of their low perception threshold and relatively large content. (S. Song et al., 2017). Hexanal and heptanal were oxidation products of linoleic acid and arachidonic acid, respectively, while octanal and nonanal were produced by oxidation of oleic acid, which gave yak meat its beef fat and green grass volatile flavors. Meanwhile, some aldehydes were derived from the carbonyl groups that produced by protein oxidation. Benzaldehyde was derived from the degradation product of phenylalanine through Strecker degradation reaction. However, benzaldehyde produced an unpleasant taste that can give an impact on the aroma of yak meat.

Ketones, as products of lipid oxidation, most of them had fruity and creamy aromas. And ketones had a higher threshold value, much higher than other aldehydes, so they had a positive effect on the volatile flavor of yak meat. Ketones generally played a coordinating role in the overall volatile flavor of meat products. It has been reported that 2-heptanone played an important role in changing the volatile flavor of meat and meat products and can be used as a marker of product deterioration (C. Li, Wu, Li. & Dai, 2013).

Table 1
Changes in volatiles of yak meat during the course of oxidation by hydroxyl radical.

Compounds	Molecular formula	Molecular weight	Oxidation time (h)				
			0	2	4	6	8
14	*	623.31	386.39 ± 13.40^{a}	276.98 ± 17.79^{b}	223.59 ± 14.67^{c}	187.55 ± 13.74^{d}	164.76 ± 13.80^{e}
ethanol	C_2H_6O	98.68	1346.79 ± 10.84^{a}	1038.04 ± 14.44^{b}	761.82 ± 121.03^{c}	637.41 ± 114.61^{d}	130.99 ± 17.13^{e}
2	*	111.75	8695.67 ± 184.89^{a}	6919.73 ± 534.45^{b}	5316.71 ± 147.37^{c}	3293.14 ± 30.58^{d}	726.86 ± 121.29^{e}
3-hydroxy-2-butanone-M	$C_4H_8O_2$	198.41	3917.2 ± 1231.22^{bc}	4107.92 ± 128.60^{bc}	4314.82 ± 146.01^{b}	5193.36 ± 153.13^{a}	3742.97 ± 158.39^{c}
maltol	$C_6H_6O_3$	756.19	65.93 ± 13.49^{c}	76.23 ± 13.37^{ab}	79.43 ± 10.54^{ab}	82.72 ± 12.90^{a}	73.59 ± 10.91^{b}
dimethyl trisulfide	$C_2H_6S_3$	557.68		115.82 ± 17.58^{b}	164.94 ± 16.17^{a}	162.53 ± 13.63^{a}	122.40 ± 18.33^{b}
2-Butanone-M	C_4H_8O	134.76		350.06 ± 15.18^{d}	405.72 ± 118.21^{c}	446.04 ± 113.48^{b}	536.62 ± 14.15^{a}
Methyl sulfide	C_2H_6S	119.67	216.48 ± 17.46^{d}	226.42 ± 110.73 ^{cd}	354.14 ± 114.18^{a}	291.70 ± 120.70^{b}	250.81 ± 120.96^{c}
1	*	111.88	785.36 ± 123.32^{d}	936.71 ± 122.26^{c}	1584.10 ± 132.53^{a}	1550.79 ± 21.24^{a}	1407.48 ± 13.80^{b}
1-butanol	$C_4H_{10}O$	166.71	345.31 ± 112.16^{d}	335.47 ± 18.48^{d}	406.28 ± 18.45^{c}	500.97 ± 112.36^{b}	667.18 ± 120.77^{a}
2-pentanone-M	$C_5H_{10}O$	177.80	269.88 ± 112.64^{c}	716.91 ± 130.67^{b}	1021.67 ± 104.83^{a}	1068.48 ± 115.84^{a}	1024.92 ± 25.97^{a}
2,3-pentanedione	$C_5H_8O_2$	182.06		163.96 ± 111.38^{b}	218.48 ± 111.35^{a}	219.55 ± 125.22^{a}	183.63 ± 15.30^{b}
4	*	174.08		706.48 ± 116.82^{c}	827.12 ± 153.45^{b}	854.72 ± 125.87^{b}	1067.67 ± 97.61^{a}
Hexanal-M	$C_6H_{12}O$	269.03	217.83 ± 111.85^{d}	2468.97 ± 138.54^{a}	2995.68 ± 81.33^{b}	3186.88 ± 86.39^{a}	3224.88 ± 88.78^{a}
2-Butanone-D	C_4H_8O	134.13	132.83 ± 17.88^{d}	211.30 ± 14.67^{c}	233.44 ± 10.49^{c}	295.47 ± 129.56^{b}	339.59 ± 123.47^{a}
1-pentanol-M	$C_5H_{12}O$	242.34	110.23 ± 16.07^{e}	2099.55 ± 67.17^{d}	2682.12 ± 54.61^{c}	2974.64 ± 107.12^{b}	3206.26 ± 42.30^{a}
2-heptanone-M	$C_7H_{14}O$	378.46	163.93 ± 16.43^{d}	1072.83 ± 71.32^{c}	1294.10 ± 138.76^{b}	1679.86 ± 64.17^{a}	1720.98 ± 103.63^{a}
Octan-3-ol	$C_8H_{18}O$	552.89		1924.80 ± 1111.48^{c}	2701.02 ± 41.11^{b}	2834.49 ± 90.78^{ab}	2986.92 ± 174.85^{a}
1-octen-3-ol-M	$C_8H_{16}O$	545.12		1990.97 ± 76.63^{c}	3058.57 ± 21.05^{b}	3247.03 ± 133.89^{b}	4189.53 ± 140.10^{a}
Butyl butyrate	$C_8H_{16}O_2$	571.37	139.66 ± 16.56^{d}	537.24 ± 117.04^{c}	546.76± 44.65^{c}	706.67 ± 123.28^{b}	816.64 ± 130.62^{a}
2-Hexanone-M	$C_6H_{12}O$	255.18	112.42 ± 17.80^{d}	286.89 ± 120.44^{c}	401.25 ± 112.60^{b}	425.03 ± 124.86^{b}	517.01 ± 121.05^{a}
Hexanal-D	$C_6H_{12}O$	265.15		1592.34 ± 32.47^{d}	2792.70 ± 179.69^{c}	4347.97 ± 377.15^{b}	7782.39 ± 234.23^{a}
1-pentanol-D	$C_5H_{12}O$	239.68		659.15 ± 18.92^{d}	1178.85 ± 90.88^{c}	1576.17 ± 205.95^{b}	2356.46 ± 181.72^{a}
(E)-2-Hexen-1-ol	$C_6H_{12}O$	377.00		72.83 ± 12.23^{d}	87.07 ± 12.82^{c}	96.88 ± 15.00^{b}	122.18 ± 15.61^{a}
19	*	762.35		88.32 ± 12.37^{c}	98.54 ± 15.86^{c}	118.40 ± 13.85^{b}	143.13 ± 113.53^{a}
Heptanal-M	$C_7H_{14}O$	394.75	157.06 ± 18.65^{e}	484.36 ± 111.68^{d}	582.26 ± 119.05^{c}	1143.98 ± 12.40^{b}	1642.30 ± 175.85^{a}
Pentanal-M	$C_5H_{10}O$	184.06	151.36 ± 13.88^{e}	284.15 ± 13.97^{d}	355.93 ± 12.74^{c}	637.06 ± 118.37^{b}	755.99 ± 15.97^{a}
2-heptanone-D	$C_7H_{14}O$	377.68		357.73 ± 15.71^{d}	533.50 ± 123.46^{c}	785.90 ± 125.21^{b}	1150.28 ± 117.30^{a}
2-pentyl furan	$C_9H_{14}O$	564.47		482.78 ± 123.45^{c}	855.02 ± 155.96^{b}	866.17 ± 182.13^{b}	1421.05 ± 166.72^{a}
Octanal-M	$C_8H_{16}O$	592.56	242.36 ± 17.26^{d}	292.85 ± 1 4.23^{d}	408.38 ± 118.26^{c}	861.53 ± 161.53^{b}	1409.09 ± 63.19^{a}
Nonanal-M	$C_9H_{18}O$	781.36	382.14 ± 119.80^{d}	453.66 ± 120.41^{d}	550.28 ± 128.29^{c}	1158.47 ± 81.84^{b}	1890.13 ± 60.40^{a}
Benzaldehyde	C_7H_6O	497.92	55.52 ± 12.43^{d}	70.46 ± 15.22^{c}	76.33 ± 11.14^{c}	90.89 ± 13.68^{b}	136.55 ± 15.72^{a}
1-hexanol-M	$C_6H_{14}O$	362.15	181.33 ± 14.76^{e}	801.14 ± 18.38^{d}	1164.56 ± 26.35^{c}	1328.10 ± 137.63^{b}	2003.73 ± 147.69^{a}
7	*	194.14		60.67 ± 17.47^{d}	127.05 ± 19.88^{c}	234.60 ± 118.22^{b}	285.42 ± 117.60^{a}
2-Methylbutyric acid	$C_5H_{10}O_2$	399.72		42.84 ± 12.24^{d}	67.10 ± 14.33^{c}	93.49 ± 15.14^{b}	143.50 ± 19.31^{a}
2-Hexanone-D	$C_6H_{12}O$	254.10		161.05 ± 16.15^{c}	384.88 ± 120.31^{b}	418.11 ± 125.96^{b}	887.65 ± 129.21^{a}
3	*	142.83		125.54 ± 16.54^{d}	155.59 ± 15.09^{c}	225.26 ± 19.64^{b}	273.62 ± 113.83^{a}
10	*	487.08		130.70 ± 16.98^{d}	205.57 ± 111.53^{c}	275.96 ± 120.42^{b}	412.77 ± 125.24^{a}
2-pentanone-D	$C_5H_{10}O$	179.13		452.01 ± 121.88^{d}	950.23 ± 134.30^{c}	1610.52 ± 14.72^{b}	3524.83 ± 52.82^{a}
11	*	551.48		100.69 ± 14.74^{c}	194.84 ± 111.44^{b}	212.80 ± 124.36^{b}	271.15 ± 113.46^{a}
E-2-octenal	$C_8H_{14}O$	699.10		133.22 ± 17.79^{c}	167.05 ± 13.72^{b}	189.28 ± 114.19^{b}	512.35 ± 125.33^{a}
Octanal-D	$C_8H_{16}O$	593.18				107.89 ± 114.83	265.01 ± 115.20
E-2-heptenal-M	$C_7H_{12}O$	492.68		74.40 ± 19.62^{d}	123.54 ± 18.43^{c}	226.23 ± 128.58^{b}	745.53 ± 16.90^{a}
Heptanal-D	$C_7H_{14}O$	391.64		41.78 ± 110.68^{c}	76.54 ± 15.94^{c}	236.16 ± 140.23^{b}	720.18 ± 143.94^{a}
(E)-2-hexenal	$C_6H_{10}O$	325.67	91.50 ± 14.18^{d}	99.89 ± 16.03^{d}	119.56 ± 16.84^{c}	137.5 ± 14.06^{b}	229.94 ± 18.60^{a}
2-hexanol	$C_6H_{14}O$	292.49		24.21 ± 12.96^{c}	40.41 ± 12.27^{bc}	59.64 ± 15.18^{b}	115.44 ± 121.06^{a}
E-2-Pentenal	C_5H_8O	225.92		31.66 ± 12.17^{c}	39.92 ± 16.71^{bc}	51.28 ± 12.59^{d}	145.3 ± 118.19^{a}
ethyl propanoate	$C_5H_{10}O_2$	193.71		46.69 ± 16.85^{c}	68.11 ± 16.89^{b}	75.11 ± 15.60^{b}	170.45 ± 112.62^{a}
1-propanol	C_3H_8O	125.20		72.36 ± 13.41^{c}	136.43 ± 110.12^{b}	138.99 ± 14.16^{b}	302.93 ± 135.56^{a}
2-Propanone	C_3H_6O	111.37	55.17 ± 15.17^{d}	74.03 ± 16.20^{d}	150.69 ± 13.64^{c}	253.48 ± 114.99^{b}	827.6 ± 137.64^{a}
5	*	184.13		67.68 ± 14.43^{d}	221.04 ± 18.33^{c}	303.26 ± 128.58^{b}	760.49 ± 150.36^{a}
9	*	491.66			54.28 ± 14.84^{b}	64.22 ± 16.89^{b}	101.02 ± 12.63^{a}
12	*	564.06	135.64 ± 16.21^{c}	140.50 ± 11.40^{c}	272.38 ± 132.20^{b}	282.17 ± 110.39^{b}	551.31 ± 112.84^{a}
15	*	649.68	52.26 ± 12.52^{d}	69.46 ± 17.26 ^{cd}	94.7 ± 13.20^{c}	163.9 ± 112.38^{b}	326.84 ± 144.26^{a}
Pentanal-D	$C_5H_{10}O$	184.42			62.20 ± 111.39^{c}	241.11 ± 130.43^{b}	739.67 ± 133.36^{a}
6	*	193.42					136.57 ± 110.86
8	*	388.36					49.75 ± 17.42
1-Hexanol-D	$C_9H_{18}O$	780.21		93.31 ± 16.37^{d}	144.77 ± 16.30^{c}	196.62 ± 19.14^{b}	478.89 ± 142.97^{a}
13	*	586.79		72.55 ± 15.51^{c}	77.85 ± 13.62^{c}	91.62 ± 14.36^{b}	120.53 ± 18.51^{a}

(*continued on next page*)

Table 1 (*continued*)

Compounds	Molecular formula	Molecular weight	Oxidation time (h)				
			0	2	4	6	8
16	*	666.65	178.77 ± 19.26^{b}	191.55 ± 19.67^{b}	184.89 ± 110.54^{b}	184.09 ± 111.35^{b}	265.4 ± 117.49^{a}
E-2-heptenal-D	$C_7H_{12}O$	492.06					95.06 ± 18.34
1,8-cineole	$C_{10}H_{18}O$	666.65					73.18 ± 18.02
17	*	727.03		153.51 ± 110.45^{c}	185.46 ± 11.05^{b}	201.77 ± 119.12^{b}	266.32 ± 116.19^{a}
18	*	748.38					77.39 ± 15.62

Note: Data are expressed as the mean ± SD from triplicate determinations. Different letters in the same column indicate significant differences ($P < 0.05$), n = 3. M: monomer, D: dimer, T: polymer.

Alcohols were produced mainly due to the degradation of linoleic acid in muscle by lipoxygenases and peroxidases. most of which had pleasant smell such as sweet, fresh, fruit and vegetable aroma and flower aroma, which can increase the volatile flavor of meat products. The main alcohols detected during the oxidation of yak meat were pentanol, hexanol, and 1-octen-3-ol and so on. The production of amyl alcohol may be caused by the automatic oxidation of polyunsaturated fatty acids. Meanwhile, the content of 1-Octen-3-ol monomer and dimer had significant changes during the oxidation process of yak meat. The change of 1-Octen-3-ol content was generally used to reflect the racification degree of meat products (Q. Zhang et al., 2020), which was generally produced by the glycolytic pathway of carbohydrates and the Enrlich pathway of amino acids. Because of the high threshold of alcohols, alcohols generally did not have a great effect on the volatile flavor of yak meat, and only a few higher levels of alcohols have some effect on the volatile flavor of yak meat. Therefore, alcohols did not contribute much to the volatile flavor of yak meat, but they played a synergistic role in the overall volatile flavor of yak meat.

Esters were generally synthesized by esterification reactions between acids resulting from the degradation of fats or proteins and alcohols, or by ester exchange reactions (alcoholysis) of fatty acids in triglycerides and ethanol. Furanoid had a gravy aroma. Since furanoid can be formed at a relatively low temperature and a relatively short time, furanoid already appeared when yak meat was oxidized for 2 h, and the content of furanoid gradually increased with the extension of oxidation time. Giri et al. reported that 2-pentylfuran and other furanoids were derived from the Maillard reaction and Strecker degradation of proteins (Giri et al., 2010).

3.3.2. PCA of yak volatile flavor at different oxidation times

The Dynamic PCA plug-in program was used to create PCA plots to intuitively analyze the differences in volatile flavor compounds of yak meat samples after different oxidation times (Fig. 2a). PCA can simplify the data and reveal the interrelationship between different samples, and it had been widely used as a multivariate statistical analysis technique for sample variation analysis (Huang et al., 2012).

The contribution rate of the first principal component PC1 was 77%, and the contribution rate of the second principal component PC2 was 13% (Fig. 2a). The total contribution rates of the first and second components were 90%, which indicated that the total contribution rates of PC1 and PC2 contained most of the information of oxidized yak meat samples, and could represent the main characteristics of volatile flavor of yak meat during the oxidation process. As can be seen from the Fig. 2a, the yak meat was well divided into three clusters. The samples oxidized for 0 h were located on the left side of PC1, the samples oxidized for 8 h were located on the right side of PC1, while the samples oxidized for 2 h, 4 h, and 6 h were concentrated in the middle and upper part. This indicated that there was a significant difference in the composition of volatile compounds between unoxidized yak meat and yak meat with different oxidation times. The spatial distribution of the sample points showed that the volatile flavor compounds changed dramatically with the oxidation of yak meat. Therefore, the composition of volatile flavor compounds can be divided into three periods: the first period was 0 h (unoxidized); the second periods were 2 h, 4 h and 6 h (mid-oxidation); and the third period was 8 h (end-oxidation).

3.3.3. Differential volatile flavor substances of yak meat during different oxidation processes

The Gallery Plot plugin of LAV software was used to more comprehensively compare the differences of volatile compounds in yak meat at different oxidation times. Five groups of yak meat samples with different oxidation times were selected, and each experiment was repeated three times. All the peaks to be analyzed in the obtained GC-IMS two-dimensional spectrum were automatically generated by fingerprints (Fig. 2b). The results showed that there were large differences in volatile flavor compounds between the samples. There were few volatile substances in the control group, and the content and type of volatile substances in the yak meat increased with the oxidation time.

As shown in area A (Fig. 2b), in fresh yak meat (0 h), volatile flavor compounds such as 3-hydroxy-2-butanone, maltol, pentanone monomer, and 2,3-pentanedione, whose content gradually increased with oxidation time up to 6 h, decreased slightly after 6 h of oxidation. It was shown that before 6 h, glucose was degraded by enzymatic catalysis to produce pyruvate, which was synthesized by enzymatic action as α-acetyl lactate; α-acetyl lactate produced. 3-hydroxy-2-butanone and 2,3-pentanedione by oxidative decarboxylation. After 6 h, the alkoxy radical in 3-hydroxy-2-butanone was broken and the propyl radical combined with the hydroperoxy radical produced by the oxidation of fatty acids, and the furan-like substances were produced after further reaction to remove the hydroxyl radical. Therefore, the content of 3-

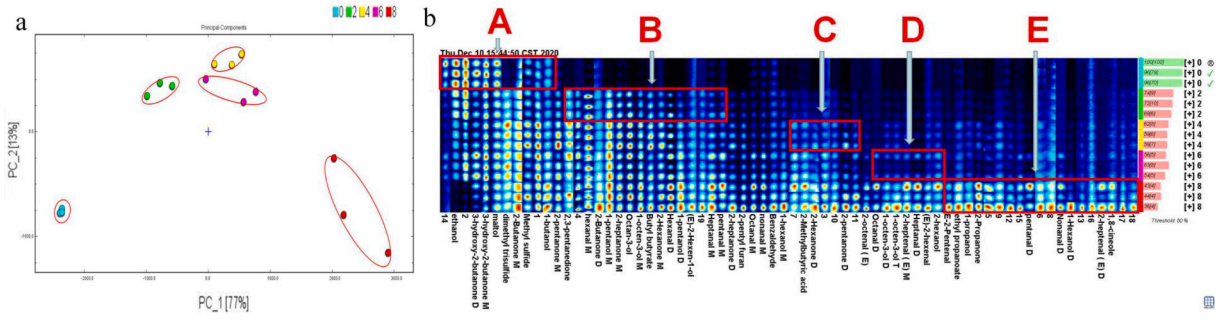

Fig. 2. Variation in volatiles from yak meat during the course of oxidation (0–8 h) (a: principal component analysis, b: volatiles fingerprint).

hydroxy-2-butanone was significantly reduced and the content of furan-like substances was significantly increased (F. Wang et al., 2021). 2.3-Pentanedione can be oxidized to produce the sodium salt of 2-carbonyl-butyrate, which played an important role in glycolysis as a substrate for lactate dehydrogenase. Maltol was the product of the merad reaction between maltose and amino acids, so its content gradually increased before 6 h. However, maltol can be used as an intermediate product to assist amino acids in Strecker degradation to produce aldehydes, the oxidation of aldehydes to produce acids, and the reaction of acids with alcohols to produce butyl butyrate, ethyl propionate and other esters, so the content decreased significantly after 6 h. After 6 h 2-pentanone monomer content decreased probably because the monomer gradually aggregated into a dimer, so the content of 2-pentanone D increased significantly.

Meanwhile, the content of sulfides such as dimethyl trisulfide and dimethyl sulfide gradually increased before 4 h of oxidation and decreased significantly after 4 h. Dimethyl trisulfide was considered to be a characteristic product of the methionine melad reaction, where methionine degradation produced methyl mercaptan, and oxidation of methyl mercaptan produced dimethyl trisulfide (Zhao et al., 2013). The dimethyl sulfide was a product of methyl mercaptan oxidation. Although the concentration of sulfur-containing compounds was low, their very low threshold made them an important component of yak meat volatile flavor. It had been shown that 2-pentanone, 2-butanone, and furanone were derived from the reaction of sulfides with amino acids, so their content gradually decreased after 4 h of oxidation. The ethanol content gradually decreased with increasing oxidation time. Frank et al. (2016) reported that ethanol was mainly produced by the oxidation of the degradation products of linoleic acid. As an essential fatty acid that cannot be synthesized by human body, linoleic acid existed in large quantities in the oils and fats of animals and plants. The ethanol produced will condense with the oxidized aldehydes to form esters, so its content gradually decreased.

After 2 h of oxidation, acetylacetone, *n*-he xanal, butanone, *n*-pentyl alcohol, 1-octen-3-ol monomer, butyl butyrate and *n*-hexanal were detected in region B, and the content continued to rise. After oxidation for 4 h, methylbutyric acid and 2-hexanone and 2-pentanone in region C were detected in gradually increasing amounts. After 6 h of oxidation, *n*-octane, 1-octen-3-ol dimer, heptanal dimer, and (E)-2-hexanal were detected in region D, and their contents increased rapidly. After oxidation for 8 h, substances such as E-2 pentanal, ethyl propionate, 2-propanone, 1,8-eudesmol were detected in region E with relatively high content, and this region was mostly dimer of some substances, such as: pentanal, nonanal, *n*-hexanol, dimer of 2-heptene (E). Research had shown that nonanal had a strong rotting taste, which indicated that the yak meat has started to deteriorate and rot after 8 h of oxidation (García-González et al., 2013). As can be seen from the Fig. 2b that the characteristic volatile flavor compounds of yak meat had undergone complex changes during the oxidation process. The appearance of new small molecule volatile organic compounds such as aldehydes, ketones, alcohols, esters, and furanoids after 2 h of oxidation may be caused by sugar metabolism, amino acid metabolism, Maillard reaction and Strecker degradation of protein produced by lipid oxidation. The 1-octen-3-ol monomer was produced at 2 h of yak meat oxidation and gradually increased with the extension of oxidation time. At 6 h of oxidation, the dimer of 1-octen-3-ol appeared, and its content gradually improved with the increase of oxidation time. According to the analysis of the content change of 1-octene-3-ol, it was clear that meat products were gradually rancid during the oxidation process of yak meat, which was consistent with the results of the TVB-N value analysis. The saturated fatty acids such as octanal and nonanal were formed significantly later than the unsaturated fatty acids such as E-2-octenal and E-2-heptenal. This may be casued by the fact that the unsaturated aldehydes were more reactive than the saturated aldehydes and can participate in the Maillard Reaction (Wei et al., 2020).

3.4. Changes in metabolites during oxidation of yak meat

3.4.1. Qualitative results of metabolites in the oxidation of yak meat

The metabonomics data of yak meat were characterized by multi-dimensional and high correlation between different variables. Traditional univariate analysis cannot accurately, quickly and fully explore the potential information of the data. Thus, to analyze the metabolome data, it was necessary to reduce and classify the data by different stoichiometry principles and multivariate statistical methods, so as to obtain the detailed information of the metabolic differences between yak meat samples treated with different oxidation times (Cubero-Leon, Peñalver, & Maquet, 2014). For this purpose, We performed multivariate data analysis. The results showed that for PCA, the predictive power of cross-validation $Q^2 = 0.552$, PLS-DA ($Q^2 = 0.634$), OPLS-DA ($Q^2 = 0.816$) (Goodacre et al., 2007). For more information, please refer to the supplementary material (Fig. S1).

The variable importance for the projection (VIP) value was generally used to explain the importance of variables to the model. When VIP > 1, indicating that the characteristic peak was important, which was usually considered as one of the screening conditions for potential biomarkers. According to the OPLS-DA, VIP > 1 and $P < 0.05$ were used as criteria to search significantly different expressed metabolites. The above Table 2 shown that a total of 53 differential metabolites were screened. The types of differential metabolites include amino acids, nucleotides, purines, fatty acids, organic acids and carbohydrates. The differential metabolites identified from yak meat treated with different oxidation times contained some amino acids such as aspartic acid, tyrosine, alanine and phenylalanine, in addition to metabolites known to be high in meat (creatine, sarcosine, etc.), and also some sugars (glucose, maltotriose, etc.). Therefore, we analyzed that the metabolic pathways of yak meat during oxidation process may include amino acid metabolism, carbohydrate metabolism, lipid metabolism and so on. (X. Wang, Fang, He, Dai, & Fang, 2017).

3.4.2. Analysis of metabolite differences during oxidation of yak meat

In order to visualized the relationship between samples and the differences in expression of metabolites in different samples more comprehensively, the relative values of metabolites under different experimental conditions were used as metabolic levels to do hierarchical clustering analysis. The differences between data were visualized by different color gradients (Fig. 3).

The results of the heat map of differential metabolite analysis showed that with increasing oxidation time, the content of metabolites such as L-tyrosine, pyridoxine, phenylacetylglutamine, pelargonic acid, adenine, maltotriose, pantothenic acid and spermidine gradually decreased, and 3,4-dihydroxyphenylglycol, L-lysine, 9(S)-HPOOE, hydroxypyruvic acid, galactose, oleic acid, stearidonnic acid, sarcosine, 3-dehydroshikimate, L-asparagine, diaminopimelic acid, *N*-acetylleucine, glucose 6-phosphate and other metabolites were significantly enriched.

According to the metabolomics results, basically most amino acids showed higher levels after 8 h of oxidation compared to the control group. In particular, leucine and acetylleucine, which can react with the products of the Melad reaction to form aldehydes, had a great impact on the volatile flavor of the meat. Aromatic amino acids were precursors of yak meat volatile flavor, mainly including tyrosine, phenylalanine and tryptophan, which were mainly produced by oxidative hydrolysis of protein. Among them, 3-dehydroshikimate was an important intermediate product in the biosynthesis of aromatic amino acids; 3,4-dihydroxyphenylglycol was a synthetic product of tyrosine metabolism, both of their content increased gradually with the metabolism of amino acids in the oxidation process. Phenylalanine was a necessary amino acid for humans and animals. Most of them were oxidized to tyrosine by phenylalanine hydroxylase in body. It also synthesized neurotransmitters and hormones together with tyrosine, and participated in the metabolism of collective sugar and fat. Tryptophan can be involved in

Table 2
Differential metabolites of yak meat during the course of oxidation.

No.	Compounds	Specific charge	Retention time(s)	Molecular weight	Molecular formula	VIP	p value
1	Pantothenic acid	220.117	356.601	219.111	C9H17NO5	1.563	0.024
2	Pelargonic acid	159.065	275.572	158.131	C9H18O2	1.829	0.021
3	Adenine	136.061	101.751	135.055	C5H5N5	1.738	0.035
4	Guanidinosuccinic acid	176.013	60.524	175.059	C5H9N3O4	1.582	0.025
5	Glucose 6-phosphate	261.036	89.198	260.030	C6H13O9P	2.119	0.038
6	Hydroxykynurenine	223.987	55.280	224.080	C10H12N2O4	1.885	0.047
7	4-Hydroxycinnamic acid	146.980	768.008	164.047	C9H8O3	1.725	0.032
8	Pyrrole-2-carboxylic acid	111.020	707.268	111.032	C5H5NO2	2.152	0.019
9	Pyridoxine	170.037	720.105	169.074	C8H11NO3	1.990	0.029
10	3-Dehydroshikimate	171.993	62.018	172.037	C7H8O5	1.828	0.049
11	3,4-Dihydroxyphenylglycol	169.978	564.043	170.058	C8H10O4	1.091	0.037
12	Genistein	271.188	765.180	270.053	C15H10O5	1.503	0.028
13	3-Methyladenine	149.023	782.597	149.070	C6H7N5	1.851	0.015
14	Hydroxypyruvic acid	104.107	44.608	104.011	C3H4O4	1.294	0.024
15	L-Asparagine	133.101	741.636	132.054	C4H8N2O3	1.884	0.034
16	L-Aspartic acid	134.060	522.061	133.038	C4H7NO4	1.850	0.030
17	Anabasine	163.039	527.029	162.116	C10H14N2	1.160	0.029
18	3-Hydroxymethylglutaric acid	145.049	399.659	162.053	C6H10O5	1.218	0.043
19	13-L-Hydroperoxylinoleic acid	295.226	807.530	312.230	C18H32O4	1.067	0.048
20	2-trans,6-*trans*-Farnesal	221.190	801.163	220.183	C15H24O	2.080	0.014
21	β-Alanyl-L-lysine	218.150	76.134	217.143	C9H19N3O3	1.150	0.026
22	Triacetate lactone	127.038	222.208	126.032	C6H6O3	1.857	0.044
23	γ-Glutamyl-β-aminopropiononitrile	199.108	330.034	199.096	C8H13N3O3	1.642	0.028
24	Antiarol	184.072	775.443	184.074	C9H12O4	2.073	0.014
25	3-Methyl-L-tyrosine	195.101	822.857	195.090	C10H13NO3	1.269	0.036
26	Scopoline	156.102	107.199	155.095	C8H13NO2	1.470	0.040
27	Aspartame	277.117	404.459	294.122	C14H18N2O5	1.913	0.039
28	β-Tyrosine	182.081	316.903	181.074	C9H11NO3	1.959	0.028
29	Phenylacetylglutamine	265.104	771.953	264.111	C13H16N2O4	2.218	0.013
30	2-Hydroxy-3-oxoadipate	176.038	217.378	176.032	C6H8O6	1.583	0.029
31	Osthol	245.111	792.550	244.110	C15H16O3	1.441	0.038
32	Progesterone	314.232	664.832	314.225	C21H30O2	1.358	0.034
33	Diaminopimelic acid	190.107	36.698	190.095	C7H14N2O4	1.697	0.048
34	L-Homophenylalanine	180.102	425.497	179.095	C10H13NO2	1.592	0.046
35	Dehydroepiandrosterone	269.211	800.045	288.209	C19H28O2	2.067	0.015
36	Spermidine	143.914	57.322	145.158	C7H19N3	1.468	0.039
37	L-Tyrosine	180.065	127.233	181.074	C9H11NO3	1.621	0.035
38	Phenylacetic acid	134.864	31.901	136.052	C8H8O2	1.649	0.014
39	5-Hydroxyindoleacetic acid	191.107	765.738	191.058	C10H9NO3	1.762	0.038
40	Cytidine	242.078	149.162	243.086	C9H13N3O5	1.478	0.046
41	12-Hydroxydodecanoic acid	215.165	693.570	216.173	C12H24O3	1.527	0.023
42	(-)-Epigallocatechin	306.076	98.932	306.074	C15H14O7	1.419	0.027
43	5-Aminopentanoic acid	116.927	33.071	117.079	C5H11NO2	1.764	0.022
44	Stearidonic acid	275.201	804.458	276.209	C18H28O2	1.837	0.031
45	6-Deoxy-5-ketofructose 1-phosphate	223.000	77.110	242.019	C6H11O8P	1.769	0.035
46	Sarcosine	88.039	97.841	89.048	C3H7NO2	1.638	0.037
47	*N*-Acetylleucine	172.097	344.806	173.105	C8H15NO3	1.657	0.031
48	Methyl β-D-galactoside	194.082	807.497	194.079	C7H14O6	1.225	0.022
49	9,10-Epoxyoctadecenoic acid	295.231	837.036	296.235	C18H32O3	1.993	0.024
50	9(S)-HPODE	312.226	780.933	312.230	C18H32O4	1.733	0.027
51	D-Erythrose 4-phosphate	199.001	76.874	200.009	C4H9O7P	1.703	0.018
52	Maltotriose	503.163	103.708	504.169	C18H32O16	1.405	0.019
53	13-OxoODE	293.212	741.833	294.220	C18H30O3	1.657	0.027

the metabolism of some proteins in animals, and 5-hydroxyindole acetic acid was the final product of tryptophan metabolism (Zahari et al., 2021).

With the increase of oxidation time, the content of L-aspartic acid and glutamic acid decreased significantly and the content of L-aspartic acid increased significantly. It was shown that L-aspartic acid and glutamic acid were mainly responsible for the fresh volatile flavor of yak meat, and the most abundant free amino acids in meat and meat products were glutamic acid and aspartic acid, which account for 20.25% of all amino acids (Zhou et al., 2021) The significant decrease in their content indicated that the fresh volatile flavor of yak meat gradually disappeared and the quality deteriorates during the oxidation process. L-aspartic acid helped produce carbamyl phosphate-CP, an enzyme that helps process waste products of protein metabolism and form urea. The L-asparagine was the synthesis of aspartic acid and adenosine triphosphate (ATP) (Hansen & Behrman, 2016). Creatine was present in meat in the form of creatine phosphate, which promoted ATP synthesis. As the oxidation time increased, the amount of sarcosine first increased and then slightly decreased. This may be because sarcosine was an intermediate in the metabolism of choline to glycine, and as oxidation proceeds, sarcosine gradually changed to glycine, which played an important role in protein oxidation and biometabolism.

Oxidation also caused significant changes in other metabolites. The content of hydroxyurine significantly increased, while the content of pyridoxine significantly decreased. This may be because hydroxykynurenine was an intermediate product of tryptophan metabolism. During the oxidation process, the tryptophan produced by proteolysis was oxidized to hydroxyuranine, which was converted to niacin with the assistance of pyridoxine (Fan et al., 2020).

In addition to protein metabolites, we also found more nucleotide sequences in the differential metabolites of yak meat. 3-Methyladenine was an inhibitor of phosphatidylinositol 3-kinase and specifically blocked the synthesis of autophagy in lysozyme (Zahari et al., 2021). Cytosine was one of the pyrimidine nucleosides that made up nucleic

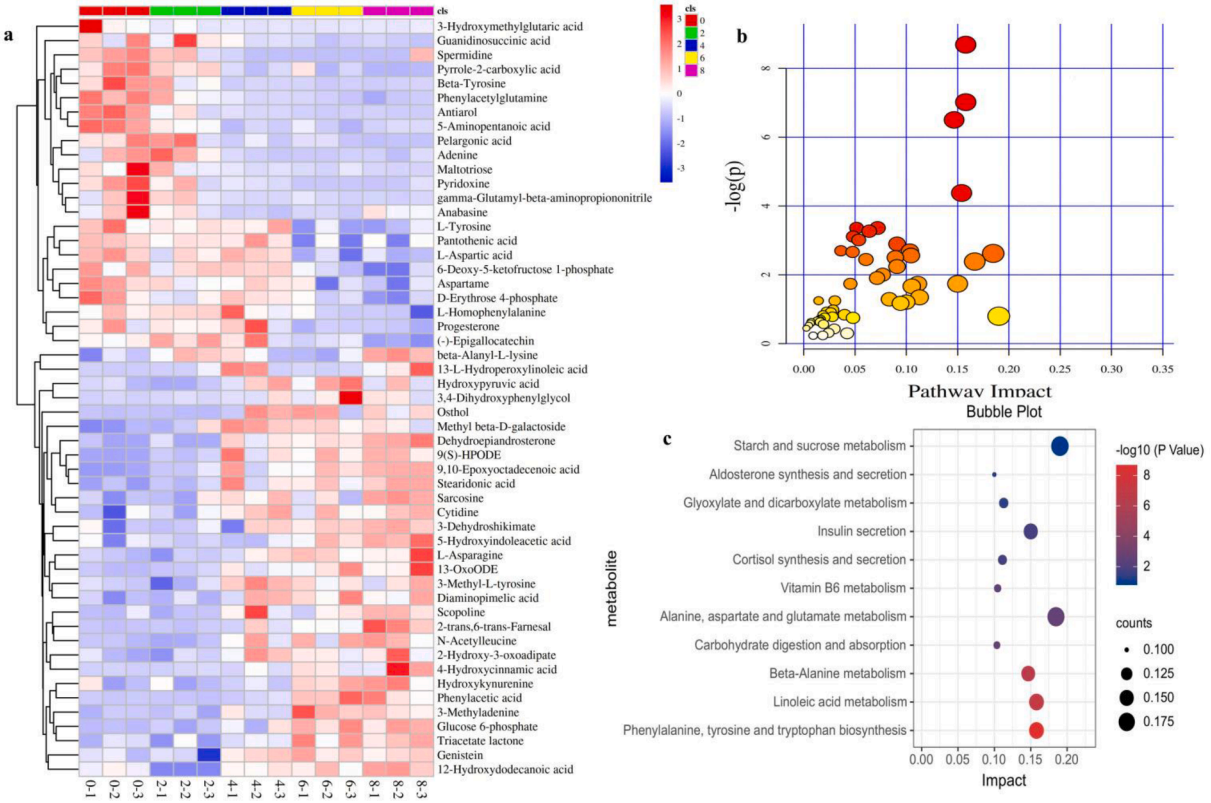

Fig. 3. Changes in metabolites of yak meat during the course of oxidation. (a: heatmap of differential metabolites, b: pathway prediction of differential metabolites based on KEGG analysis. c: pathway enrichment of differential metabolites).

acids and was produced by the hydrolysis of ribose.

The unique volatile flavor of meat was mainly derived from lipids. In our study, the metabolites of lipids such as stearic acid and linoleic acid all increased gradually with increasing oxidation time. Stearic acid was generally produced by the hydrolysis of fats and oils, and linoleic acid was found in animal and vegetable fats and oils as glycerides, along with other fatty acids. Oxidized linoleic and oleic acids were metabolized to produced octanal, nonanal and other carbonyl compounds that were considered odorous in sensory tests. The gradual increase in linoleic acid indicated that the yak meat has gradually spoiled. Glycerophospholipid metabolism was identified to be associated with the spoilage process of meat products (Cheng et al., 2015). In addition, 2-Hydroxy-3-oxoadipate also found in the differential metabolites, and it was an intermediate product of gluconeogenesis and played an important role in the process of gluconeogenesis. Meanwhile, 2-Hydroxy-3-oxoadipate could be combined with pyruvate to greatly increase the metabolic rate of the body and promote fatty acid oxidation.

3.4.3. *Differential metabolite pathway analysis*

In order to further analyze the potential metabolic pathways of the effect of oxidation on the volatile flavor of yak meat, KEGG was used to analyze the function of differential metabolite related pathways, and 53 differential metabolites were enriched into 46 metabolic pathways (Table S1). Significantly enriched pathways with pathway impact values greater than 0.1 were selected for analysis. As shown in Fig. 3c, the metabolism of starch and sucrose metabolism had the most significant effect on the volatile flavor compounds in yak meat. According to KEGG database analysis, it was mainly caused by the metabolism of D-Glucose-6-phosphate. D-glucose-6-phosphate was a molecule produced after phosphorylation of glucose, and it was also a common molecule in biological cells and was involved in biochemical pathways such as pentose phosphate pathway and glycolysis (Zhang et al., 2021). In addition, metabolic pathways such as biosynthesis of phenylalanine, tyrosine and tryptophan, metabolism of linoleic acid, metabolism of vitamin B6, metabolism of β-alanine, metabolism of alanine, aspartic acid and glutamic acid, digestion and absorption of carbohydrates, metabolism of glyoxylate and dicarboxylic acids were significantly enriched.

11 significantly enriched metabolic pathways were identified in the KEGG enrichment results. All of these pathways involve amino acid metabolism, which suggested that amino acids were the main reason for the significant differences with volatile flavor compounds in yak meat samples after different oxidation times. At the same time, arachidonic acid produced by the metabolism of linoleic acid was also involved in fatty acid biosynthesis and other metabolic pathways. Adipaldehyde and benzaldehyde were the oxidation products of linoleic acid, which was also consistent with GC-IMS results. The glyoxylate cycle was a very important reaction in the conversion of fats to sugars in animals, and dicarboxylic acids were the products of fatty acid oxidation. Vitamin B6 was closely related to protein metabolism, it was involved in amino acid transamination reactions and decarboxylation, and also participated in the conversion of cysteine and methionine in the form of coenzymes. (Li et al., 2019) All of these metabolic pathways had a greater impact on the quality of yak meat, thereby affecting the formation of volatile flavor.

3.5. *Relevance analysis of key volatile flavor compounds and differential metabolites*

The yak meat was involved in a lot of reactions in the oxidation process, so the formation of volatile flavor compounds was a complicated process. Differential metabolic compounds may be potential factors affecting the volatile flavor of yak meat. Therefore, 18 differential volatile flavor compounds with high OAV values and 24 major differential metabolites with high corresponding and VIP values were selected. The correlation between key volatiles and differential metabolites (Fig. 4a) and the correlation among key volatiles, differential

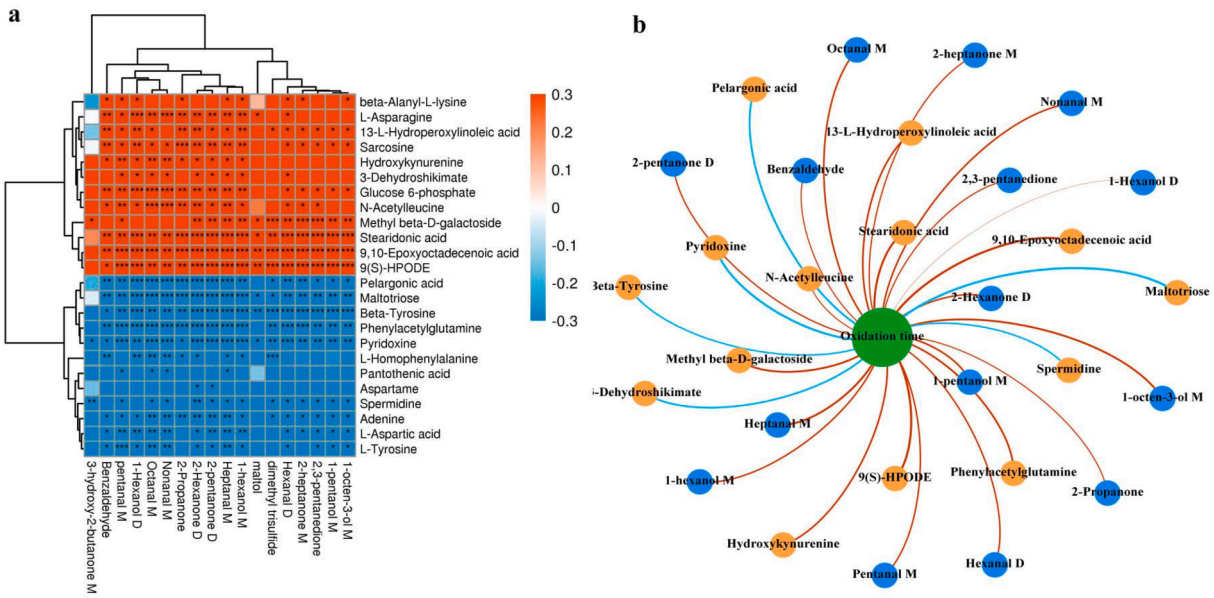

Fig. 4. Relationship between key volatiles and differential metabolites of yak meat during the course of oxidation. (a): correlation between key volatiles and differential metabolites; (b) correlation among key volatiles, differential metabolites and oxidation time. Redline: positive correlation. Blueline: negative correlation. Yellow circle: metabolites. Blue circle: volatiles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metabolites and oxidation time (Fig. 4b) were analyzed using the Parson's method. According to the results, the main volatile flavor precursor were fatty acids, amino acids and glycogen.

The results showed that stearic acid, oleic acid and linoleic acid had a strong positive correlation with volatile flavor compounds such as hexanal, heptanal, octanal, nonanal, 1-octen-3-ol and 2,3-pentanedione, and also with oxidation time. This is because most of the volatile flavor compounds in the oxidation process of yak meat were degradation products of fat. Arachidonic acid was the product of linoleic acid metabolism, while hexanal mainly originated from the automatic oxidation of arachidonic acid. Heptanal, octanal and nonal and some other aldehydes were derived from the oxidation of oleic acid. The alcohol compounds in yak meat were mainly produced by the action of specific fat oxidase on linoleic acid and other polyunsaturated fatty acids, and 1-octene-3 alcohol, as a representative substance, was produced by the reaction of alkoxy radicals with fat molecules during lipid oxidation (Xiang, Jin, Gouda, Jin, & Ma, 2019). Ketones, such as 2, 3-pentanedione, were produced mostly by the oxidation of an alkoxy group by another free alkane group during lipid oxidation. In the qualitative results of volatile flavor compounds, a certain amount of alkanes were also found, and the corresponding fatty acids were detected in the metabolites. The results showed that the oxidation products of fatty acids had a great influence on the volatile flavor compounds of yak meat.

FAAs also had a great influence on the volatile flavors of yak meat. Some of them were the precursor of Maillard reaction, and some were produced by Strecker amino acid degradation reaction. Asparagine, sarcosine and isoleucine were positively correlated with most of the volatile flavor compounds, while tyrosine, glutamine and phenylalanine were negatively correlated with most of the volatile flavor compounds; tyrosine was negatively correlated with oxidation time. Aspartic acid, alanine, and glutamate, known as umami amino acids, are important umami substances in yak meat (Kim, Kemp, & Samuelsson, 2016). Alanine was degraded by Strecker amino acid degradation reaction to form hexaldehyde, and aspartic acid in the action of transaminase to oxaloacetic acid; oxaloacetic acid then further reaction to give *n*-butanol, 2-butanone and other ketones. Leucine and isoleucine can be degraded to isovaleraldehyde, isobutyraldehyde, etc. These aldehydes can also further react to generate corresponding alcohols and acids. In addition, 2-methyl-butyric acid was also formed by the degradation of leucine. Phenylalanine can be degraded to benzaldehyde and so forth. Sulfur-containing amino acids such as methionine canl be degraded to produce mercaptan, and mercaptan further reaction to generate sulfur-containing compounds such as dimethyl trisulfide. A variety of free amino acids combined together to created the rich volatile flavor of yak meat, which made the volatile flavor compounds of yak meat more natural.

Glycogen was also an important volatile flavor precursor substance. Glucose and galactose were positively correlated with volatile flavor compounds and oxidation time; maltose was negatively correlated with volatile flavor compounds and oxidation time. After yak meat was slaughtered, blood flow in the body stopped, oxygen supply was interrupted, myogenic glycogen reacted anaerobically under the action of glycogen phosphatase, and the generated lactic acid accumulated, causing the pH of meat products to dropped, resulting in the ruptured of lysosomes in myogenic cells and the released of glycogen debranching enzymes. Under the action of glycogen debranching enzyme, glycogen was gradually broken down into glucose, galactose and other monosaccharides. Maltose, in turn, was hydrolyzed to two molecules of glucose, catalyzed by maltase. Glucose can then be used as a precursor for the Merad reaction, which has a definite effect on the volatile flavor compounds.

4. Conclusion

It was found that the cooking loss, TVB-N and TBARS of yak meat were significantly increased and the total sulfhydryl was significantly decreased after treatment with hydroxyl radical oxidation system ($P < 0.05$). The results indicated that the degree of oxidation of yak meat gradually increased with the increase of oxidation time; the quality of yak meat gradually deteriorated, which in turn also had some effects on the volatile flavor of yak meat.

With the increase of oxidation time, volatile flavor compounds such as heptanal, octanal, nonanal, 2,3-glutaraldehyde and 3-hydroxy-2-butanone were significantly increased and positively correlated with oleic acid, linoleic acid and stearic acid. It indicated that fatty acids were important flavor precursor compounds. Volatile flavor compounds such as octanal and nonanal may be related to the acidification of yak meat at

the later stage of oxidation, while 2,3-pentanedione and 3-hydroxy-2-butanone were the main flavor compounds in yak meat. The content of 3-dehydrocinchoninic acid and tyrosine decreased gradually with the increase of oxidation time, and 3-dehydromangiferous acid contributed to the generation of aromatic flavor compounds, while tyrosine was the precursor of 3-dehydromangiferous acid. Benzaldehyde produced by oxidation of phenylalanine can be used as an indicator compound for flavor deterioration of yak meat. However, more volatile flavor compounds in yak meat and their specific mechanisms of change need more work to be identified.

Ethical guidelines

Ethics approval was not required for this research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131103.

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