Liu *et al. Chem. Biol. Technol. Agric.* (2024) 11:22
https://doi.org/10.1186/s40538-024-00541-9

Chemical and Biological Technologies in Agriculture

RESEARCH

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Analysis of forage quality, volatile organic compounds and metabolic pathways in alfalfa (*Medicago sativa* L.) at different stages based on electronic nose and GC-MS

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Abstract

The odor of alfalfa (*Medicago sativa* L.), the most extensively cultivated forage, can interfere with livestock feeding. This study used an electronic nose in conjunction with gas chromatography–mass spectrometry (GC–MS) to examine the quality, volatile organic compounds (VOCs), and metabolic pathways of alfalfa at budding (X), early flowering (C), and full flowering (S) stages. Results showed that terpenoids increased first and then decreased with growth and development, heterocyclic substances decreased continuously, and alcohols and ketones increased. The crude protein and ether extract decreased and were positively correlated with terpenoids, heterocycles, and nitrogen and sulfur compounds, while the dry matter content, soluble carbohydrates, and neutral detergent fiber increased and were positively correlated with alcohols and ketones. The VOCs were most accumulated in the early flowering stages, which had more sweet and fruity flavors, and the main substances that differed from the budding stage and the full flowering stage were methyl heptanoate, butyl butyrate, β-ionone, and other esters and terpenoids. The monoterpene, sesquiterpene, and triterpene pathways were up-regulated in the early flowering stage, and the phenyl-propylene synthesis pathway was up-regulated in the full flowering stage. These substances and pathways were key to further improving alfalfa odor, grade and utilization.

Keywords Alfalfa, Electronic nose, GC–MS, Volatile organic compounds, Metabolic pathway

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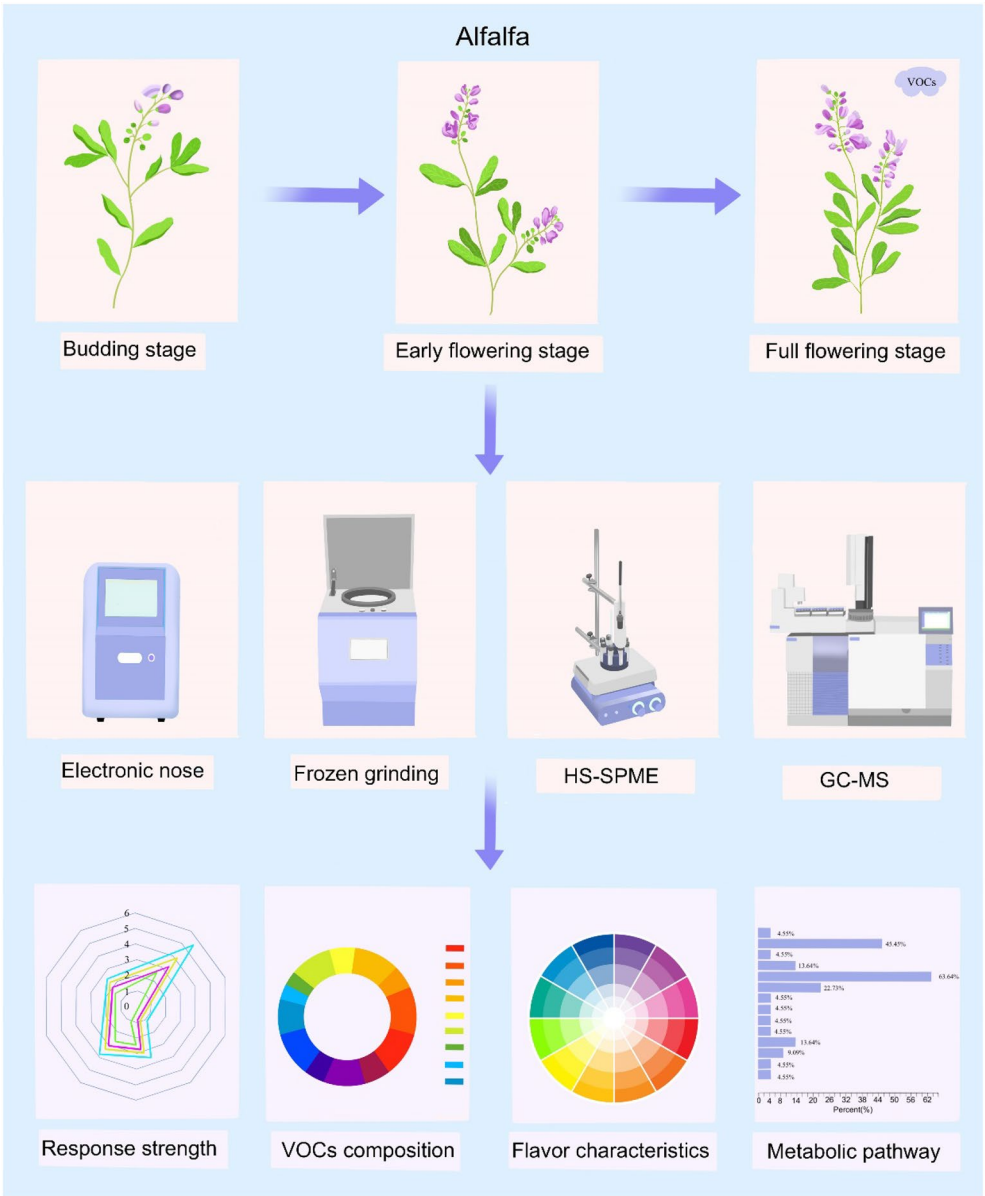
Graphical Abstract



Introduction

Alfalfa (*Medicago sativa* L.) is a perennial herbaceous plant in the legume family that is the most widely planted forage in the world. Alfalfa has strong adaptability and can grow in many types of climates and soil environments due to its well-developed root system. It also has the function of conserving water and soil and improving soils, and it has an irreplaceable role in agriculture and animal husbandry [1, 2]. Alfalfa has high grass production, excellent grass quality, and is rich in protein, dietary fiber, minerals, vitamins, and other nutrients [3], which can promote the lactation of cows and improve milk quality, as well as improve the reproduction and fertility rates of various cattle, making it an ideal food for cattle [4, 5].

China has the second largest area of alfalfa grass plantation in the world due to the adjustment of the agricultural planting structure and the vigorous promotion of

the development of the alfalfa industry, but at the same time, as the development of animal husbandry and the improvement of dairy cattle stock, alfalfa grass is still in great short supply, and there is an urgent need to improve the yield, quality, and rate of use of alfalfa [6]. The fresh smell of alfalfa is similar to the smell in the air after the lawn has been mowed, which cattle like to eat, but poor quality or old alfalfa has a fishy smell in the soil or a pungent moldy smell, which severely affects the quality and value of the feed [7, 8]. The current alfalfa quality assessment system sets olfactory indicators, but there are no specific types of volatile substances or grading standards, and in general, only the absence of an unpleasant odor is used to indicate qualified alfalfa quality, which will affect the reasonable judgment of alfalfa quality, because cattle are sensitive to the smell of different growth stages and the freshness of the alfalfa. Cattle usually prefer to eat fresh feed with a good smell, when the feed is moldy, deteriorated, or produces a bad smell, they will reduce the amount of intake or even refuse to eat, resulting in a waste of feed resources [9, 10].

Microbial growth, oxidation, and the presence of undesirable compounds and contaminants are risk factors for changes in feed flavor and loss of palatability. The electronic nose is fast and portable as an olfactory detection tool, allowing the identification of entire mixtures of organic samples without having to identify individual chemicals in the sample mixture [11]. Cheli et al. [12] showed that properly validated electronic nose analysis can differentiate between feeds contaminated with mycotoxins. The scent of forage is derived from volatile organic compounds (VOCs), which mainly refer to a variety of organic compounds with boiling temperatures of 50–260 °C at atmospheric pressure. VOCs are largely categorized into terpenes, benzene ring type and benzene propane type, fatty acid derivatives, and amino acid derivatives, depending on their sources of biosynthesis [13]. Zhang et al. [14] showed that the major volatile organic compounds that make up the fresh odor of cucumber include hydrocarbons, aldehydes, and ketones. Rasekh et al. [15] classified chili peppers and sweet peppers on the basis of odor by combining an electronic nose with chemometric methods and analyzing them to provide a better prediction of capsaicin content with high accuracy. Li et al. [16] found that as passion fruit ripens, it releases VOCs such as esters, alcohols, ketones, hydrocarbons, alkanes, and aldehydes. The main way these VOCs are made is through ester and amino acid metabolism. Rapisarda et al. [7] showed that volatile organic compounds in feed would affect the feed intake of ruminants, among which aldehydes, sulfides, lipids, and terpenes had a greater effect on palatability. These studies have shown that plants undergo significantly different odor changes as they grow or mature and that there may be a link between odor and quality.

The quality, odor, and structure are included in the grade evaluation of forage. There are many studies on the quality of forage, but there is a gap in the odor component, and the changes of VOCs at different stages of alfalfa growth and their correlation with quality are not known. Therefore, to gain insight into alfalfa VOCs, we identified and analyzed alfalfa VOCs at three periods using an electronic nose, headspace solid phase microextraction, coupled with gas chromatography–mass spectrometry (GC–MS) analysis. Our findings elucidated substances related to alfalfa odor, analyzed changes in volatiles during alfalfa growth and related metabolic pathways, and served as a baseline for improving alfalfa quality and utilization.

Materials and methods

Plant sample preparation

The variety of Alfalfa was WL319, planted in the experimental site of Tumetzuo Banner, Hohhot City, Inner Mongolia Autonomous Region (E40°64′, N111°17′). Alfalfa plants were sown on July 15, 2021, and 500 g of fresh samples of the whole plant were collected at the budding (X), early flowering (C), and full flowering (S) stages of the first harvest, with six replications per group.

Forage quality analysis

For quality analyses, 300 g of samples were collected from each group and oven dried at 65 °C for 72 h to maintain a constant weight. Samples were crushed after drying, passed through a 1-mm sieve, and stored in a sealed bag. Dry matter content (DM) was determined after drying at 65 °C for 48 h [17]. Crude protein (CP) was determined by determination of the nitrogen content of Dumatherm (Model: Dumatherm-01; Gerhardt Analytical Instruments Co. LTD, Germany) [18], acid detergent fiber (ADF) and neutral detergent fiber (NDF) were measured using an ANKOM fiber analyzer (Model: A2000i; Beijing ANKOM Technology Co., Ltd., China) [19], ether extract (EE) was measured using an ANKOM adipose tissue analyzer (Model: XT15i; Beijing Anke Borui Technology Co., Ltd., China) [20], and soluble carbohydrate (WSC) was measured by colorimetric analysis of anthrone [21].

Odor response intensity

The intensity of the odor response of the samples was determined using an electronic nose (Model: AIRSENSE/PEN3; Ensoul Technology Ltd., Germany), and the performance of the sensor is described in Table 2. The volatiles were measured by accurately measuring 2 g of sample into a 20 mL sample vial, leaving it for 10 min, and then inserting the electronic nose probe to suck up

the air at the tip and measure the volatiles. The setup parameters of the electronic nose were: carrier gas flow rate and injection flow rate of 300 mL/min, cleaning time of 95 s, zeroing time of 5 s, sample preparation time of 5 s, measurement time of 100 s, sample interval time of 1 s, and the signals from each sensor were recorded once every 2 s, and the data were analyzed using the Win Muster software that comes with the electronic nose. All samples were averaged after six parallel measurements, and the relative odor intensity was reflected by the ratio of the resistance of the volatile gas in the sample (G) to the resistance of the corresponding carrier gas (G0) as a function of time.

Volatile organic compounds

The materials were harvested, weighed, and immediately frozen in liquid nitrogen before being stored at -80 °C until they were needed. Subsequently, the samples were ground to a powder using liquid nitrogen. For each analysis, 500 mg (1 mL) of the powder was promptly transferred to a 20 mL headspace vial (Agilent, Palo Alto, CA, USA) containing a NaCl-saturated solution to inhibit any enzyme reaction. To seal the vials, crimp-top caps with TFE–silicone headspace septa (Agilent) were employed. During the solid-phase microextraction (SPME) analysis, each vial was placed at 60 °C for 5 min, after which a 120 μm DVB/CWR/PDMS fiber (Agilent) was exposed to the headspace of the sample for 15 min at 60 °C.

After sampling, the volatile organic compounds (VOCs) were desorbed from the fiber coating in the injection port of the GC apparatus (Agilent Model 8890) using the splitless mode at a temperature of 250 °C for 5 min. The identification and quantification of VOCs were performed using an Agilent Model 8890 GC and a 7000D mass spectrometer (Agilent). A 30 m × 0.25 mm × 0.25 μm DB-5MS (5% phenyl-polymethylsiloxane) capillary column was utilized. Helium gas was employed as the carrier gas at a linear velocity of 1.2 mL/min. The injector temperature was maintained at 250 °C, while the detector temperature was set at 280 °C. The oven temperature was programmed as follows: starting at 40 °C for 3.5 min, increasing at a rate of 10 °C/min up to 100 °C, followed by an increase at a rate of 7 °C/min up to 180 °C, then a further increase at a rate of 25 °C/min up to 280 °C, and finally holding for 5 min. Mass spectra were recorded using electron impact (EI) ionization mode at 70 eV. The temperatures of the quadrupole mass detector, ion source, and transfer line were set at 150 °C, 230 °C, and 280 °C, respectively. The identification and quantification of analytes were conducted using the selected ion monitoring (SIM) mode [22]. The metabolites of the samples were analyzed qualitatively and quantitatively by mass spectrometry based on a self-constructed database. MassHunter quantitative software was used to perform the integration and correction work, and the internal standard was 3-hexanone CAS: 24588-54-3.

Statistical analysis

The DM, CP, NDF, ADF, WSC, and EE contents of alfalfa were analyzed by ANOVA using SAS version 9.2 statistical software and were statistically significant at $P < 0.05$. Tables were produced using Excel 2010, and images were generated using GraphPad Prism 8.0.2 and R version 3.6.3. The Pearson correlation coefficient was calculated to draw a correlation heat map.

The identified metabolites were annotated using the KEGG Compound Database (http://www.kegg.jp/kegg/compound/), and then the annotated metabolites were mapped to the KEGG Pathway Database (http://www.kegg.jp/kegg/pathway.html). Metabolite set enrichment analysis was then performed, and significance was determined by the P values of hypergeometric tests.

Results

Chemical composition of alfalfa

The chemical composition of alfalfa is shown in Table 1. Different growth periods had significant effects on DM, CP, NDF, WSC, and EE ($P < 0.05$), but not on ADF ($P > 0.05$). Among them, the DM and NDF contents of alfalfa in the bud stage were significantly lower than in other periods ($P < 0.05$), the CP and EE contents were significantly higher than in other periods ($P < 0.05$), and the WSC contents were not significantly different from the first flowering stage ($P > 0.05$). The contents of DM, NDF, and WSC were significantly higher in the

Table 1 Differences in chemical composition of alfalfa during growth

Treatments	DM (%FM)	CP (%DM)	NDF (%DM)	ADF (%DM)	WSC (%DM)	EE (%DM)
X	14.78 ± 0.61c	24.13 ± 1.80a	34.11 ± 3.15c	30.94 ± 1.88a	3.09 ± 0.29b	1.92 ± 0.08a
C	19.23 ± 0.97b	20.85 ± 1.34b	39.16 ± 3.39b	32.74 ± 2.14a	3.26 ± 0.18b	1.56 ± 0.06b
S	25.02 ± 1.00a	16.83 ± 1.06c	43.32 ± 2.72a	32.78 ± 1.80a	3.77 ± 0.13a	1.46 ± 0.18b

Data were expressed as mean ± standard deviation (SD). Different letters indicate significant differences among different treatments ($P < 0.05$)

DM dry matter, *CP* crude protein, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *WSC* soluble carbohydrates, *EE* ether extract, *X* budding stage, *C* early flowering stage, *S* full flowering stage

flowering stage than in the other periods ($P < 0.05$), the contents of CP were significantly lower than in the other periods ($P < 0.05$), and the contents of EE were not significantly different from those in the first flowering stage ($P > 0.05$).

Odor response intensity of alfalfa

The PEN3 nose electronics are highly sensitive to sample odors and volatile compounds within the measurement range, and small changes may result in differences in sensor response values. From the radar plot of the aroma distribution in Fig. 1A, it can be seen that the sensors with higher response intensities for the alfalfa samples of different growth periods are W5S, W1W, W1S, W2S, and W2W, which are sensitive to the oxides of nitrogen, terpenes, inorganic sulfides, methyl groups, alcohols, aldehydes, and ketones, as well as the organic sulfides, respectively. Differences between samples were most evident in W5S, W1W, and W1S, indicating that the period of growth had a greater effect on the nitrogen oxides, terpenes, inorganic sulfides, and methyl groups of the alfalfa plant. Among them, the response intensity of nitrogen oxides was the largest at the budding stage and the smallest at the full flowering stage. The response intensity of terpenes, inorganic sulfides, and methyl was the largest at the early flowering stage and the smallest at the budding stage. The response intensity to alcohols, aldehydes, and ketones was the largest at the full flowering stage and the smallest at the budding stage.

Orthogonal partial least squares discriminant analysis (OPLS-DA) is a method of statistical analysis that shows differences between groups as well as within groups, and the Q2 value of the model was above 0.9 (Additional file 1: Fig. S1). Where the horizontal coordinate indicates the predicted principal component and the horizontal coordinate direction shows the discrepancy between the groups, where the vertical coordinate indicates the orthogonal principal component and the vertical coordinate direction shows the discontinuity within the groups, and percent indicates the rate of explanation of that component for the entire data set. According to the OPLS-DA plot (Fig. 1B), the samples of each group were separated significantly, indicating that the flavors of the alfalfa samples from the different growth periods were independent of one another and that overall differentiation was good. Among them, the budding stage and early flowering stage were distributed far away, and the full flowering stage was located between the budding stage and early flowering stage, indicating that alfalfa volatile components at the early flowering stage and budding stage were more distinct, and alfalfa in the full flowering stage had some differences from those in the budding stage and early flowering stage (Table 2).

Volatile organic compounds of alfalfa

A total of 782 volatile organic compounds were identified by GC/MS in alfalfa at the budding (X), early flowering (C), and full flowering (S) stages of the experiment (Additional file 2: Table S1). Volatile organic compounds in alfalfa contain acids, alcohols, aldehydes, amines, aromatics, esters, ethers, halogenated hydrocarbons, heterocyclic compounds, hydrocarbons, ketones, phenols, nitrogenous compounds, sulfurous compounds, terpenoids, and other compounds (Fig. 2A). Of these, terpenoids made up the largest percentage (18.18%), followed by esters (17.29%), heterocyclic compounds (15.11%), and ketones (9.35%), which were the major VOCs in the alfalfa crop over the three periods.

The distribution of volatile organic compounds in alfalfa was hierarchically clustered for the three growth periods, and the results are shown in the heat map (Fig. 2B). In alfalfa, the majority of volatile organic compounds accumulated significantly during the early flowering stage but accumulated less during the budding and full flowering stages. As can be seen from the OPLS-DA results, the gap between the groups is larger for samples of different growth periods, and the difference between the 6 samples within groups within the same growth period is less, indicating that alfalfa volatile organic compounds differed among growth periods (Fig. 2C). Among them, the difference in VOC composition between group C and group X was large, and group S was between the two groups.

The relative content analysis of different types of VOCs in each group is shown in Fig. 2D. The VOCs of alfalfa in the three stages were mainly composed of heterocyclic compounds, terpenes, ketones, and alcohols. Among them, the heterocyclic compound content decreased gradually, and the full flowering stage was significantly lower than the bud stage ($P < 0.05$). The contents of terpenoids increased at first and then decreased, with the highest in the early flowering stage and the lowest in the full flowering stage ($P < 0.05$). Ketones and alcohols did not change much in the budding stage and early flowering stage but increased significantly in full flowering stage ($P < 0.05$).

Correlation between chemical composition and volatile organic compounds

The correlation between the chemical composition of alfalfa and volatile organic compounds is shown in Fig. 3. CP was extremely significantly positively correlated with terpenoids, heterocyclic compounds, nitrogen

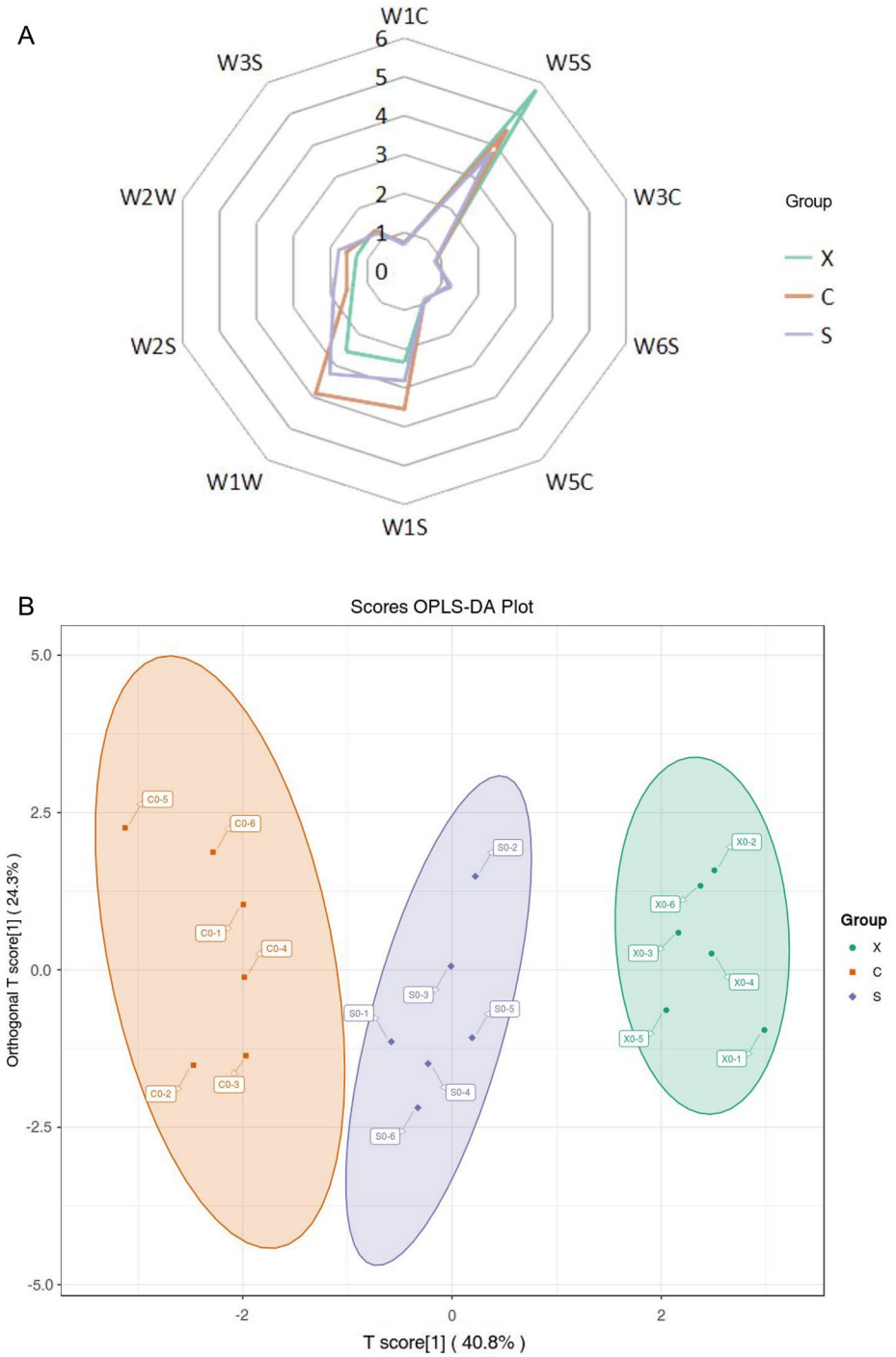


Fig. 1 Radar plot of odor response intensity of alfalfa (**A**). OPLS-DA analysis of odor response intensity of alfalfa (**B**) $Q^2 = 0.923$, $R^2 = 0.969$, $R^2X = 0.748$. X: budding stage. C: early flowering stage. S: full flowering stage. Radar plot demonstrate the response intensity of each group at different sensor channels, and the circles in the OPLS-DA score plots indicate 95% confidence intervals

compounds, and sulfur compounds ($P < 0.01$) and significantly positively correlated with aromatics ($P < 0.05$), but extremely significantly negatively correlated with ketone, alcohol, and halogenated hydrocarbons ($P < 0.01$). EE was extremely significantly positively correlated with heterocyclic compounds, nitrogen compounds, and sulfur compounds ($P < 0.01$), but extremely significantly negatively correlated with halogenated hydrocarbons ($P < 0.01$)

Table 2 Electronic nose sensor name and corresponding substance

Serial number	Sensor name	Performance description
1	W1C	Aromatic component
2	W5S	High sensitivity, very sensitive to nitrogen oxides
3	W3C	Ammonia, sensitive to aromatic components
4	W6S	Mainly selective for hydrogen
5	W5C	Aromatic component of alkane
6	W1S	Sensitive to methyl groups
7	W1W	Sensitive to terpenes and inorganic sulfides
8	W2S	Sensitive to alcohols, aldehydes and ketones
9	W2W	Aromatic component sensitive to organic sulfides
10	W3S	Sensitive to alkanes

and significantly negatively correlated with aldehyde ($P<0.05$). In contrast, WSC was extremely significantly negatively correlated with terpenoids, heterocyclic compounds, and nitrogen compounds ($P<0.01$) and significantly negatively correlated with aromatics ($P<0.05$), but extremely significantly positively correlated with ketone, alcohol, and halogenated hydrocarbons ($P<0.01$). DM was extremely significantly negatively correlated with terpenoids, heterocyclic compounds, nitrogen compounds, sulfur compounds, and aromatics ($P<0.01$), but extremely significantly positively correlated with ketone, alcohol, and halogenated hydrocarbons ($P<0.01$). NDF was extremely significantly negatively correlated with heterocyclic compounds and nitrogen compounds ($P<0.01$), and significantly negatively correlated with terpenoids ($P<0.05$), but extremely significantly positively correlated with halogenated hydrocarbons ($P<0.01$) and significantly positively correlated with ketone ($P<0.05$). ADF was significantly negatively correlated with nitrogen compounds ($P<0.05$).

Differential volatile organic compounds in alfalfa

The Q^2 value of the model set by OPLS-DA was above 0.9 (Additional file 1: Figure S1), indicating the high stability of the model, and alfalfa volatile organic compounds were found to differ significantly during the different growth periods and were analyzed for either Fold Changes ≥ 2 or ≤ 0.5, VIP ≥ 1, and P values < 0.05. Figure 2B shows that most VOCs showed an increasing and then decreasing trend during alfalfa growth. Compared to the budding stage, 239 VOCs were upregulated and 12 VOCs were decreased at the early flowering stage (Fig. 4A). Compared to the early flowering stage, 64 VOCs were upregulated and 123 VOCs were decreased at the full flowering stage (Fig. 4B). Compared to the budding stage, 165 VOC were up-regulated and 8 VOC were down-regulated at the full flowering stage (Fig. 4C). This indicates that VOCs accumulated strongly in alfalfa during the early bloom stage and gradually declined through the bloom stage, and these significantly differentially expressed VOCs could be important factors in the formation of aromas during alfalfa growth. 105 VOCs were selected by screening for VOCs that were initially upregulated and then decreased for alfalfa growth (Additional file 3: Table S2). Of these, esters (24) were the main VOCs that were differentially expressed, followed by terpenoids (23), heterocyclic compounds (13), and ketones (11). These substances were the main enriched substances in alfalfa during the early flowering period, distinguishing it from other periods.

The differences in volatile organic compounds at different growth times are important for the formation of alfalfa odors; therefore, the details of these different metabolites were further analyzed in the present study. For the flavor wheel plot, the top 10 sensory flavors with the highest number of annotations were selected based on the differential metabolites and the annotated sensory flavor profiles obtained from filtering and identification. Figure 5 shows the flavor profiles of the differential metabolites. Compared to the budding and full flowering stages, the early flowering stage contains more sweet, fruity, woody, green, waxy, herbaceous, floral, tropical, citrus, and honey. In the case of the sweet flavor, it was primarily due to the increased content of heptanoic acid, methyl ester, benzaldehyde, 3-methyl- and 2-hexenoic acid, (E)-. In the case of fruit flavor, this is primarily due to the increased content of β-ionone, butanoic acid, butyl ester, and hexanoic acid, methyl ester. For the woody flavor, is mainly caused by the increase of acetic acid, phenyl ester, 3-octanol, and 2, 6, 6-trimethyl-2-cyclohexene-1, 4-dione. In the case of the green flavor, it was primarily caused by the increase in heptane, 1,1-dimethoxy-, butanoic acid, butyl ester, and heptanoic acid, methyl ester. The green flavor, it is mainly caused by the increase of

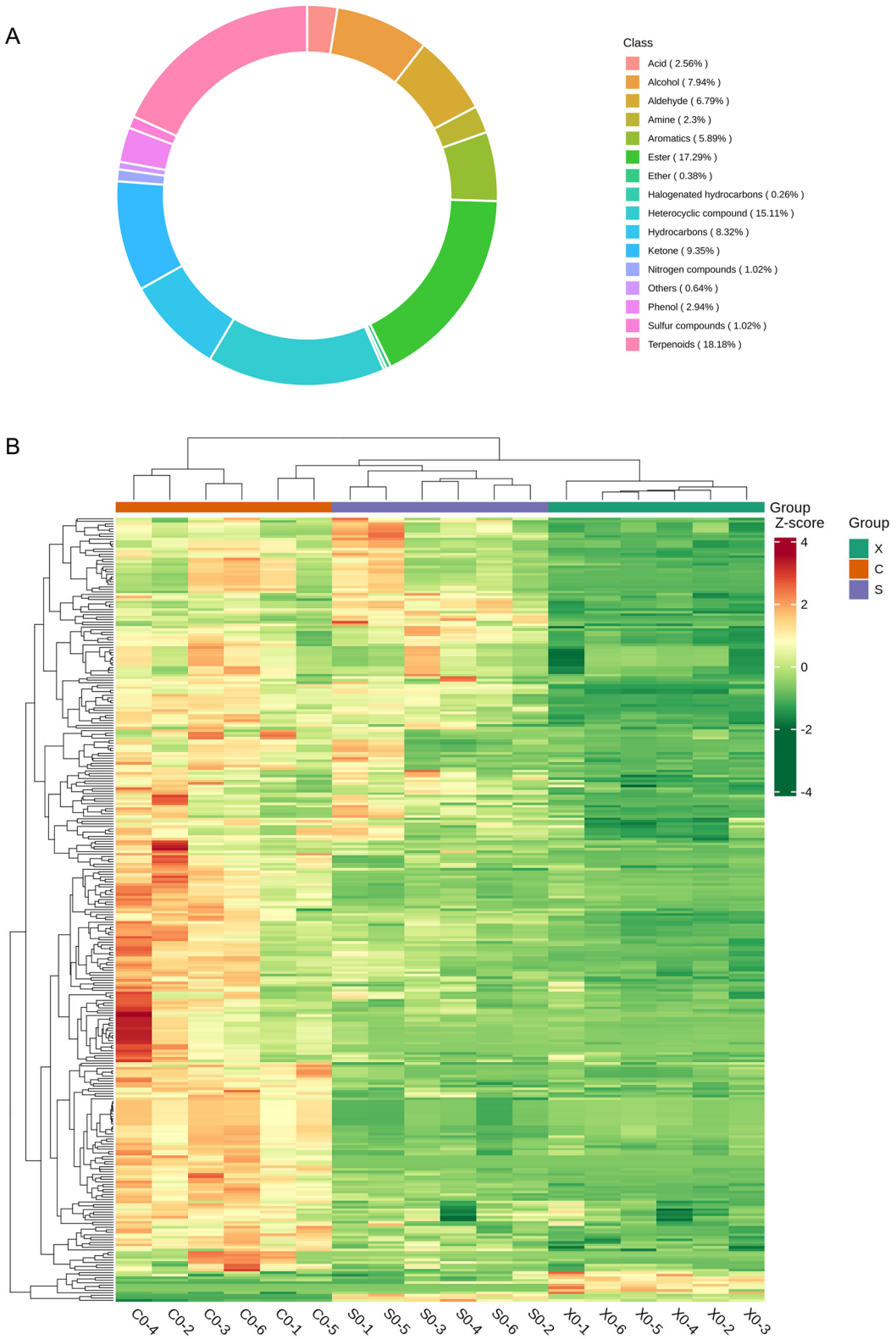


Fig. 2 Species and proportion of 782 volatile organic compounds in alfalfa (**A**). Different accumulation patterns of volatile organic compounds in alfalfa (**B**). X: budding stage. C: early flowering stage. S: full flowering stage. OPLS-DA analysis of volatile organic compounds in alfalfa (**C**). The relative contents of VOCs in alfalfa at different periods (**D**) $Q^2 = 0.909$, $R^2 = 0.978$, $R^2X = 0.440$

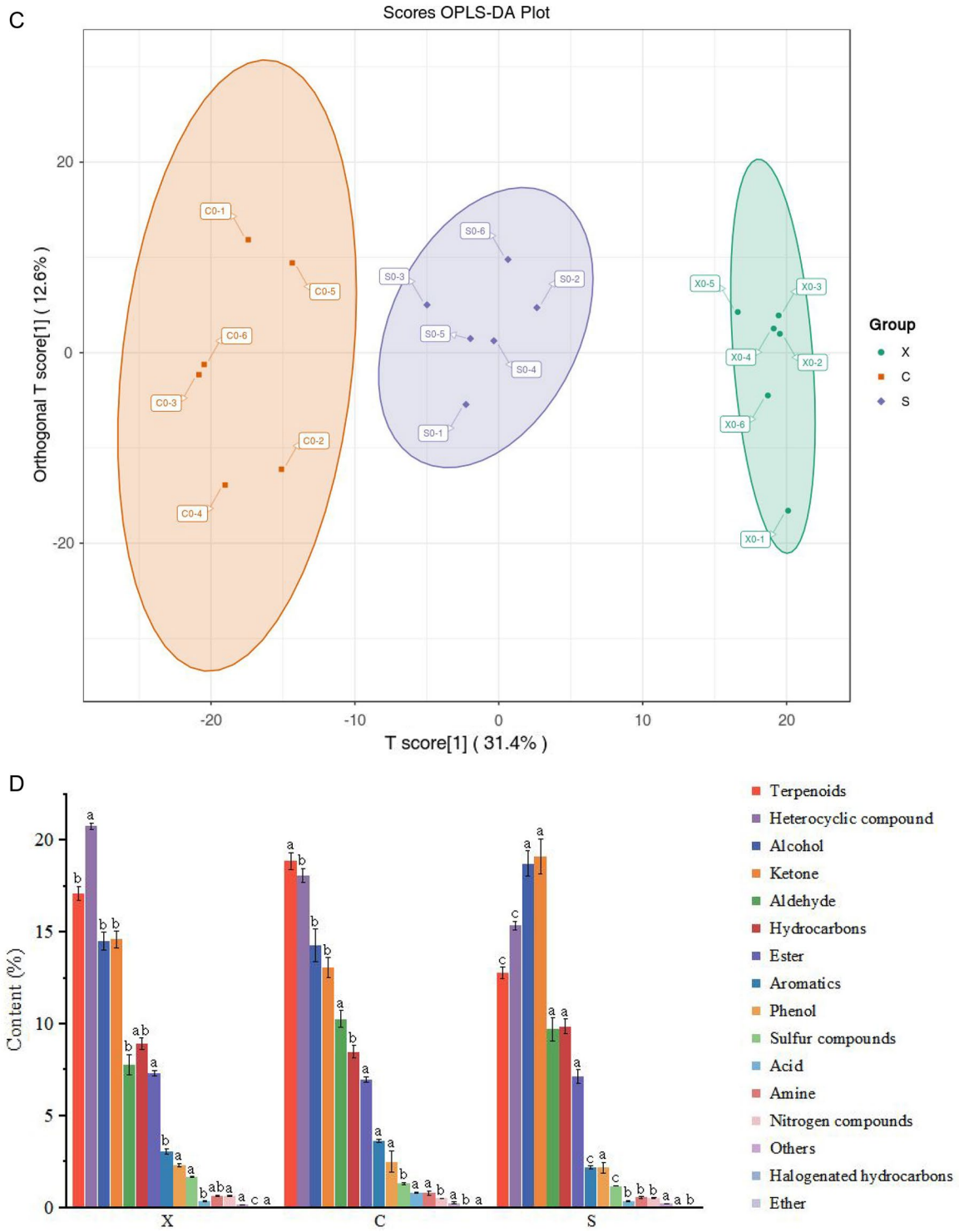


Fig. 2 continued

heptanoic acid, methyl ester, nonanoic acid, methyl ester, and formic acid, octyl ester. These substances may be the key substances that distinguish alfalfa odor composition in the flowering period from other periods.

Functional annotation and enrichment analysis of differential volatile organic compounds in alfalfa

Volatile organic compounds interact with one another in the body to form different pathways, and results were

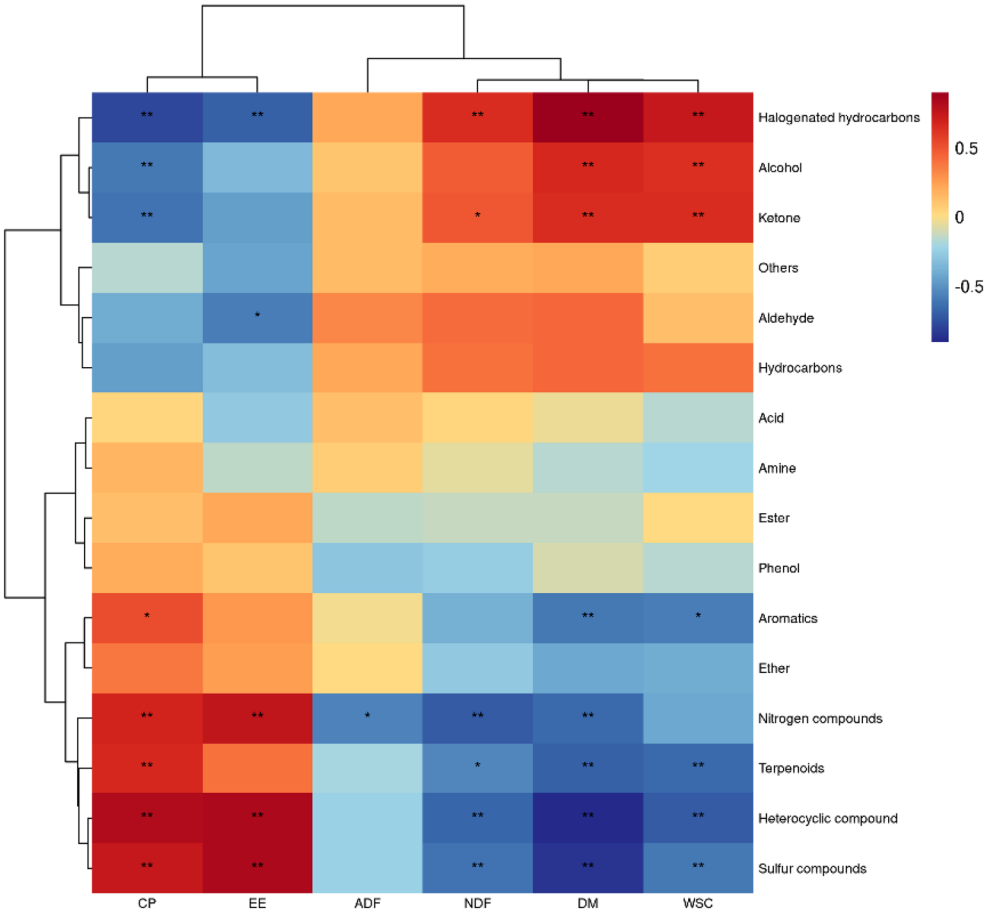


Fig. 3 Correlation between chemical composition and volatile organic compounds of alfalfa. Red means positive correlation, blue means negative correlation. *: $P < 0.05$, **: $P < 0.01$

annotated and ranked using the KEGG database (Kyoto Encyclopedia of Genes and Genomes) for metabolites and differential pathways, as indicated in Fig. 6. The metabolic pathway types of the differential VOCs between group X and group C were primarily the metabolic pathway (63.64%), biosynthesis of secondary metabolites (45.45%), and monoterpenoid biosynthesis (22.73%). The types of metabolic pathways of the differential VOCs between groups C and S were mainly metabolic pathways (50%), biosynthesis of secondary metabolites (50%), and sesquiterpenoid and triterpenoid biosynthesis (25%). The metabolic pathway types of the differential VOCs between group X and group S were primarily the metabolic pathway (50%), biosynthesis of secondary metabolites (33.33%), and phenylpropanoid biosynthesis (22.22%).

The Differential Abundance Score (DA Score) is a pathway-based analysis of changes in metabolism, and the Differential Abundance Score captures the global change of all metabolites within a pathway. In addition, the 20 paths in terms of the *P* value are chosen from smallest to largest in Fig. 7. The pattern of expression of metabolite metabolism in terpene backbone biosynthesis, metabolic pathway, and monoterpene biosynthesis pathway was significantly upregulated in group C compared to group X. This was followed by an upregulated trend in the expression of metabolites in the nicotinate and nicotinamide metabolism, N-glycan biosynthesis, fatty acid biosynthesis, and biosynthesis of cofactors pathways. The trend of metabolite metabolism expression in the trpenoid backbone biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, and propane, piperidine, and pyridine alkaloid biosynthesis pathways was significantly downregulated in group S compared to group C. This was followed by a downregulated trend in the expression of metabolites in the N-glycan biosynthesis and fatty acid biosynthesis pathways. The trend of metabolite metabolism expression in the phenylpropanoid biosynthesis pathway was significantly upregulated in group S compared to group X. This was followed by an upregulated trend in the expression of metabolites in the nicotinate and nicotinamide

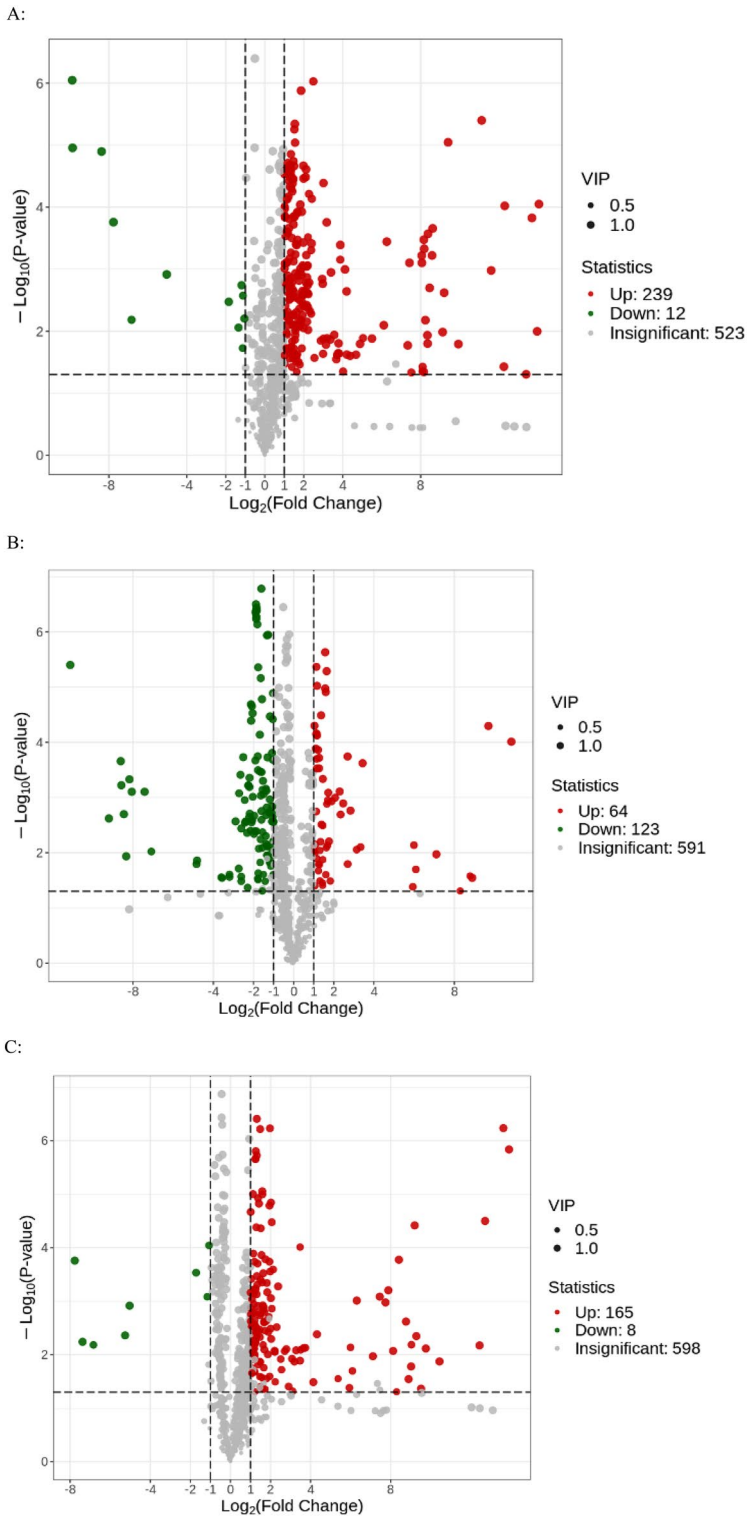


Fig. 4 Volcanic map of volatile organic compounds differences in alfalfa. X to C (**A**) $Q^2 = 0.952$, $R^2 = 0.990$, $R^2X = 0.670$, C to S (**B**) $Q^2 = 0.977$, $R^2 = 0.997$, $R^2X = 0.609$, X to S (**C**) $Q^2 = 0.972$, $R^2 = 0.997$, $R^2X = 0.559$. The green dots represent the downward accumulation of VOCs and the red dots represent the upward accumulation of VOCs between different ratios

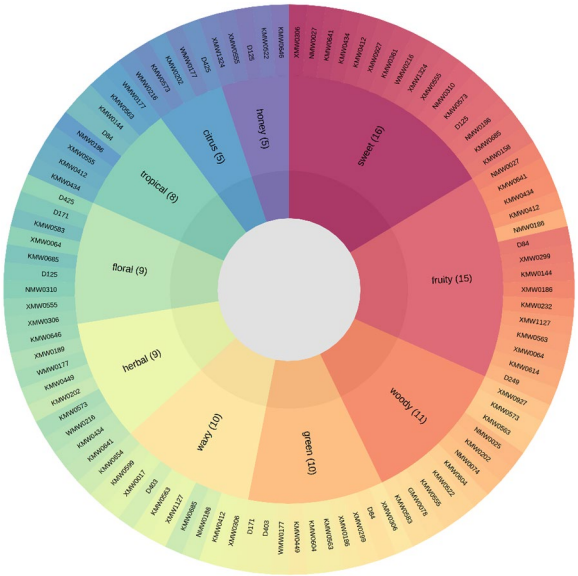


Fig. 5 Top 10 sensory flavor wheel plots of different volatile organic compounds in alfalfa. The inner circle is the top 10 sensory flavor features annotated by differential metabolites in the comparison group with the highest number. The numbers in parentheses represent the number of differential metabolites annotated to this sensory flavor feature, and the outer circle represents differential metabolites, corresponding substance number is shown in Schedule 1

metabolisms and limonene and pinene degradation pathways.

Discussion

The chemical composition analysis revealed that the duration of the growth period exerted a substantial impact on the nutritional characteristics of alfalfa. Specifically, the DM, WSC, and NDF content of alfalfa exhibited a notable increase as the growth period was extended. During the initial phase of growth, the stems and leaves of alfalfa exhibited characteristics of youth and tenderness, accompanied by a notable abundance of water and relatively low levels of DM and WSC [23]. During the period between budding and flowering stages, there was an accumulation of DM and WSC. However, the content of NDF increased due to the activation of metabolic pathways associated with lignin synthesis. This led to an increase in the lignification of peduncles, resulting in a decrease in the nutritive quality and palatability of alfalfa. The NDF is a crucial constituent of the cell wall, playing a significant role in bolstering the structural integrity and stability of the cell wall. In addition, an elevated concentration of NDF can facilitate the proper development and blooming of flowers. Furthermore, it is worth noting that the metabolic processes of plants experience an increase in intensity throughout the stages of budding and flowering. As a result, a portion of the metabolites produced must be utilized by cellulose breakdown metabolism to provide the necessary energy for the growth and development of the plant. The NDF is a kind of cellulose that exhibits limited susceptibility to degradation. Consequently, plants use regulation of photosynthesis rate, carbon allocation and transport, and related metabolic pathways to adjust energy storage and metabolite accumulation during this phase to promote their optimal growth and development. Furthermore, the alterations in DM, WSC, and NDF content in plants are influenced by environmental conditions and the physiological state of the plant. Plants fortify the structural integrity of their cell walls by augmenting the NDF content, enhancing their capacity to adapt to environmental stressors and mitigate potential harm [24]. The decrease in amounts of crude protein and ether extract in alfalfa throughout the reproductive period can be linked to the increased metabolic activity observed from the emergence of buds to the flowering stage. The CP is distinguished by the swift proliferation of alfalfa vegetation and the emergence of reproductive structures, which require a significant amount of nitrogen absorption to facilitate the production of proteins and other essential nutrients. However, the limited availability of nitrogen sources poses a constraint on the plant's capacity to regularly assimilate a sufficient quantity of nitrogen to meet its significant nitrogen demands. As a result, there is a reduction in the protein composition of the alfalfa plant. Concurrently, the period during which buds form and flowers bloom is a pivotal stage for alfalfa plants as they undergo the transition into the reproductive phase. During this particular temporal interval, alfalfa plants exhibit a higher allocation of energy and resources towards the reproductive process rather than growth. Moreover, this occurrence prompts the plant to dedicate a higher proportion of its resources towards augmenting the growth of reproductive structures, such as flowering organs and seeds while diminishing the allocation towards sustaining other tissues and organs [25]. Simultaneously, it is noteworthy that the nutritional composition of alfalfa is mostly concentrated within the chloroplasts of its leaves. Consequently, a higher proportion of leaves within the plant corresponds to superior quality. However, as the plant matures and its leaves age, there is a continuous decline in both crude protein and ether extract levels, resulting in a deterioration of quality [26].

The findings from the e-nose analysis indicated a notable disparity in the response intensity of alfalfa during the budding and early flowering phases. This discrepancy can be attributed to the elevated levels of crude protein in alfalfa during the budding stage, as well as the alterations in protein composition and molecular structure that

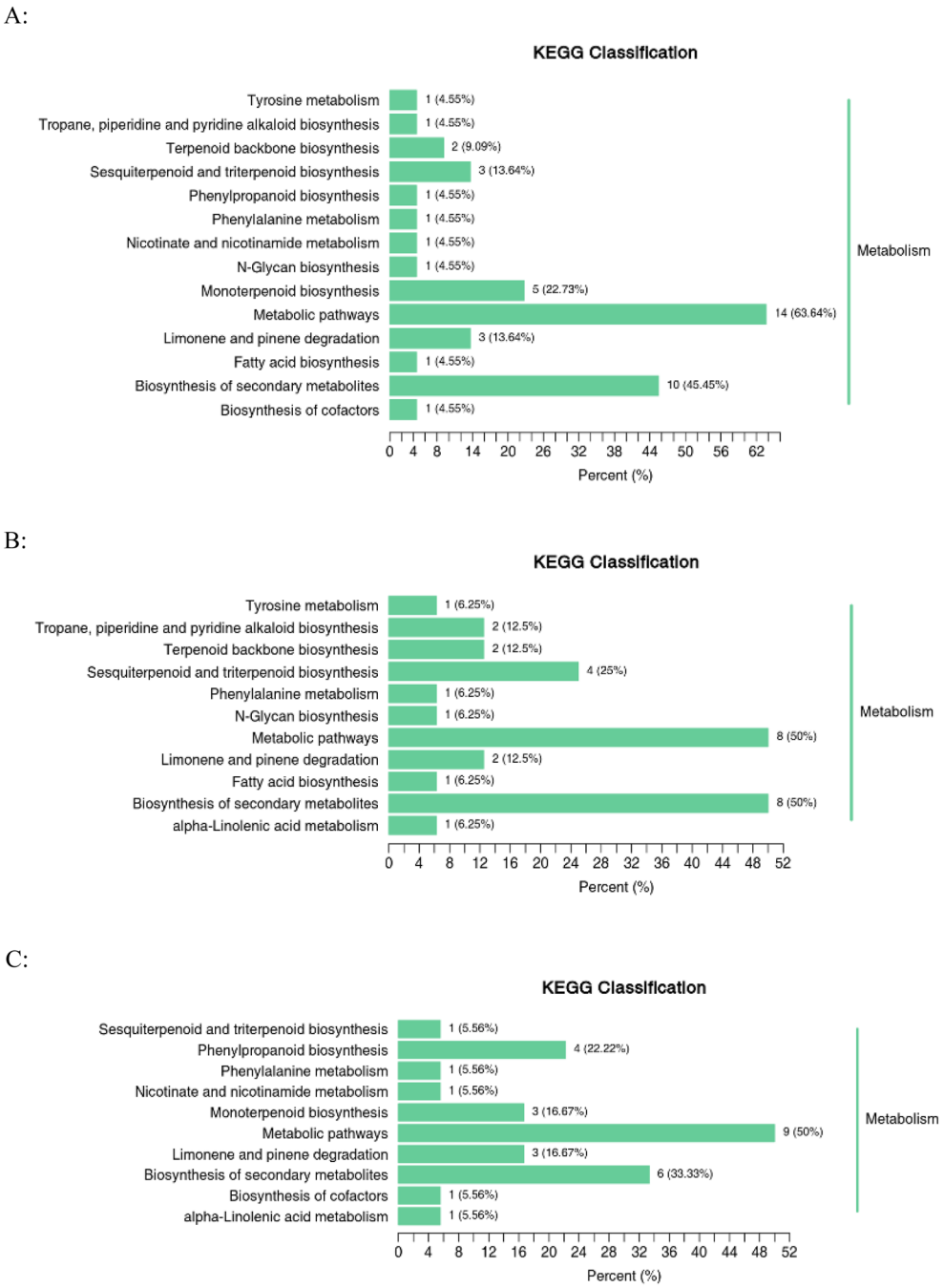
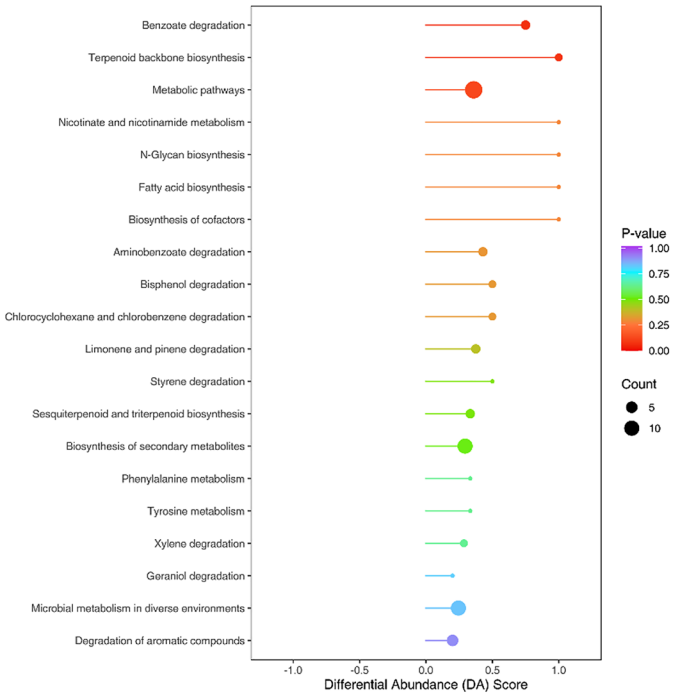


Fig. 6 KEGG classification of different volatile organic compounds in alfalfa. X to C (**A**), C to S (**B**), X to S (**C**)

occur as the plants undergo growth, development, and metabolic processes. These processes result in the degradation of proteins, leading to the production of peptides, amino acids, and volatile nitrogen oxides [27]. The early flowering stage had the maximum response intensity for terpenoids, inorganic sulfides, and methyl groups, whereas the lowest response intensity was observed at the budding stage. The reason for this phenomenon can be attributed to the monoecious nature of alfalfa, which classifies it as a heterogamous pollinated plant. The reproductive process of alfalfa seeds relies heavily on insect pollination, as highlighted by Tucak et al. [28]. In addition, terpenoids and methyl groups assume significant functions in the growth, development, and

A:



B:

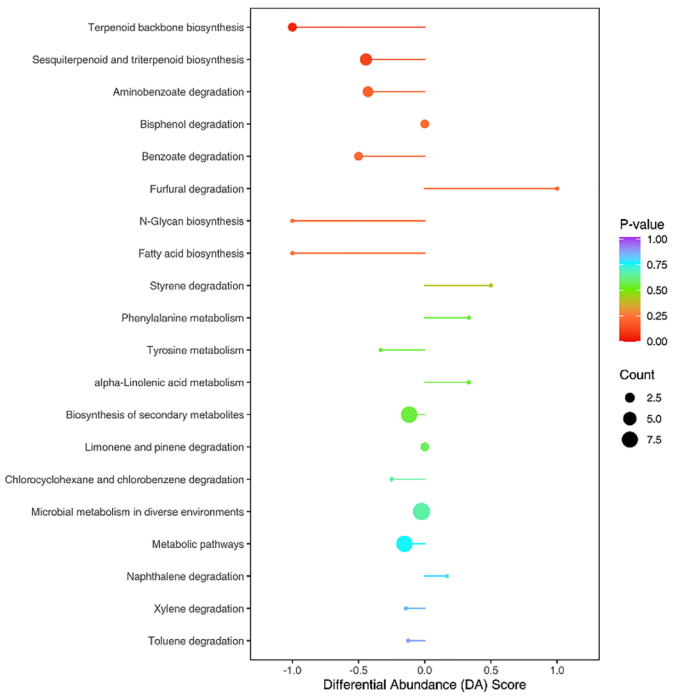


Fig. 7 Overall changes of KEGG metabolic pathway of volatile organic compounds in alfalfa. X to C (**A**), C to S (**B**), X to S (**C**). The dot size at the end of the line segment represents the number of differential metabolites in the pathway. The dot distribution on the left of the central axis and the longer the line segment indicates that the overall expression of the pathway is more inclined to be down-regulated; the dot distribution on the right of the central axis and the longer the line segment indicates that the overall expression of the pathway is more inclined to be up-regulated

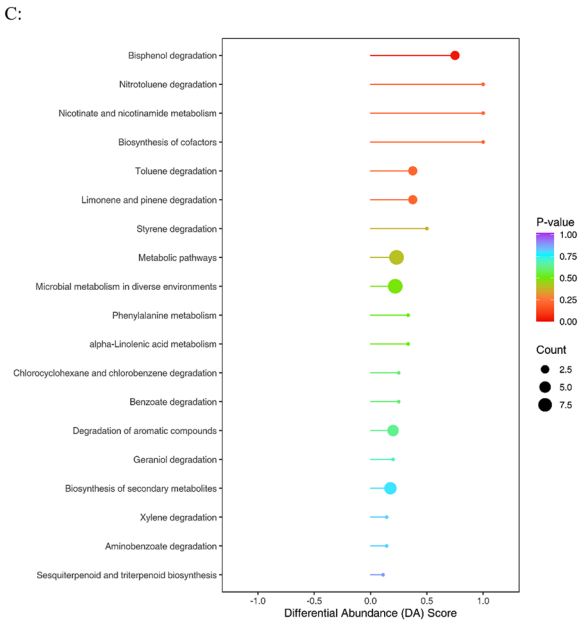


Fig. 7 continued

ecological interactions of plants. Numerous studies have demonstrated that plants possess a wide array of terpenoids, exhibiting remarkable structural and functional diversity, which influence the preference and selection of plants by certain insects to a certain degree. In addition, these factors also impact the efficiency of pollination and the overall reproductive success of plants. In addition, methyl groups can also exert influence in the interplay between plants and microbes. Certain plant species have the ability to attract or inhibit microorganisms through the release of methyl groups. This mechanism aids plants in acquiring nutrients and water in intricate environments, consequently enhancing their competitive advantage. As a result, a substantial quantity of terpenoids and volatile methyl groups are produced by these plants during the initial stage of flowering [29]. The results of the OPLS-DA analysis showed a significant difference in odor between the early flowering stage and the budding stage. This may be attributed to the combination of alfalfa stems, leaves, and flowers creating an overall difference in odor. The steady deterioration in the quality of alfalfa during its growing period may result in variations in the odor of alfalfa during the early flowering stage. The rapid detection and differentiation of alfalfa quality might potentially be achieved by conducting an analysis and building a data model that correlates the response strength of an electronic nose with the quality of the sample [30].

The findings from the analysis of volatile organic chemicals showed that terpenes, esters, heterocyclic compounds, and ketones were the main components affecting the odor of alfalfa. Terpenoids are abundantly found in many natural sources and play a significant role in alfalfa growth by primarily serving as antibiotics, agents for sterilization, and providing protection against desiccation. Simultaneously, they also serve as essential signaling molecules throughout the alfalfa pollination process, attracting pollinating insects and facilitating the dissemination and transmission of flowers [31]. The majority of esters are characterized by volatility and aromatic properties that provide aroma and flavor to the plant and also play a crucial role in regulating alfalfa odor, attracting insects, and promoting pollination [32].

The findings from the electronic nose tests revealed a significant variation in the VOCs present in alfalfa across its various growth stages. In their study on the ripening of navel orange fruit, Hou et al. [33] observed that samples collected from various months exhibited recognizable patterns in principal component analysis (PCA). This is the same result as in this study, where there were differences in VOCs from alfalfa at different times of the year, proving that the use of an electronic nose was able to differentiate between the odors of alfalfa. In a study conducted by Song et al. [34], it was shown that the ripening stage of jujube fruit had a notable impact on its volatile contents. Specifically, jujubes that were semi-red ripened exhibited superior overall flavor quality compared to other ripening stages. This is similar to the results of the present study, where alfalfa odor was better during the initial flowering stage, and some of the flavor

substances may gradually diminish during the later stages of flowering or ripening. In their study, Fan et al. [35] examined the olfactory characteristics of begonia at various stages and observed a pattern in the total quantity of volatile compounds. Specifically, they noted an initial increase followed by a subsequent decline from the bud stage until the conclusion of flowering. This trend can be attributed to the highest concentration of volatile compounds at the beginning of flowering, which is consistent with the results of this experiment in alfalfa. According to the studies conducted by Boachon et al. [36] and Zhou et al. [37], it has been observed that petunia plants possess the ability to synthesize terpenoids. These terpenoids play a crucial role in safeguarding the developing stigma against potential pathogens during the period spanning from the bud stage to the initial stage of flowering. This finding may explain the diversity of volatile organic compounds in alfalfa during early flowering.

Based on the findings of the correlation study, it was observed that there exists a positive association between CP and EE with terpenoids, heterocyclic compounds, nitrogen compounds, and sulfur compounds. Conversely, a negative correlation was observed between CP and EE with ketones and alcohols. However, the variables DM, WSC, NDF, and ADF exhibited a contrasting pattern. The findings of this study revealed a negative correlation between the levels of terpenes, sulfur, and nitrogen compounds and the concentrations of protein and fat. Conversely, there was a positive association between the levels of ketones and alcohols with the buildup of dry matter and soluble carbohydrates. The presence of ketones and alcohols in alfalfa can be deduced as indications of its aging process. This observation aligns with the findings of Boué et al. [38], who identified alcohols and ketones as suitable marker molecules for assessing the maturation of soybeans.

The odor was found to be influenced by methyl heptanoate, β-ionone, benzaldehyde, and 3-octanol, as indicated by the outcomes of the taste wheel investigation. Methyl caproate, methyl heptane, methyl acetate, and several other methyl esters are characterized by a delicate sweetness and a mild acidic scent. These compounds exhibit pronounced fruity sweetness and aroma, and they can be used as feed additives in feeds to enhance the flavor and mouthfeel of the feed and to enhance the animal's appetite for the feed [24]. Benzaldehyde is found in hyacinth, lemongrass, cinnamon, and iris and has a bitter flavor similar to that of almonds and nuts. It is the most commonly used aromatic aldehyde in the industrial sector and is also used in agriculture and the feed industry [39]. β-Ionone has a floral, fruity, and woody flavor and is an intermediate in the production of vitamin A, which promotes cell growth, visual function, reproductive health, and the proper functioning of the immune system in animals. 3-Octanol has a strong greasy, nutty, and herbal aroma, a mushroom aroma, and a cheese aroma when diluted. It is often used as a spice in the processing industry to improve the odor of products [40].

The findings from the examination of metabolic pathways revealed that the primary differential metabolic pathways throughout the growth of alfalfa were secondary metabolite production, monoterpene biosynthesis, sesquiterpene and triterpene biosynthesis, and phenylpropanoid biosynthesis. Terpenoids and phenylpropanoids are prevalent volatile components found in plant aromas. Extensive research has demonstrated the existence of two distinct biosynthetic pathways for terpenes: the mevalonate pathway and the methylerythritol phosphate pathway [29]. The mevalonate route involves the utilization of three acetyl CoA molecules to synthesize mevalonate. Subsequently, mevalonate undergoes a series of enzymatic reactions, including pyrophosphorylation, decarboxylation, and dehydration, ultimately resulting in the production of isopentenyl pyrophosphate (IPP). The synthesis of methrythritol involves a sequential sequence of events initiated from pyruvate and 3-phosphoglyceraldehyde, which are intermediates derived by glycolysis or the C4 pathway. These processes ultimately lead to the formation of methrythritol phosphate, followed by the production of dimethylacrylate diphosphate (DMAPP) [41]. IPP and DMAPP synthesized by alfalfa through these pathways can further combine to form different types of terpenoids, such as monoterpenes, sesquiterpenes, and triterpenes, which are involved in phytohormone synthesis and signaling to regulate alfalfa's odor characteristics and physiological activities [42]. Phenylpropanoids are commonly categorized into three distinct groups of elements. Specifically, the phenylpropanoids encompass aromatic amino acids, such as phenylalanine and tyrosine, which play a crucial role in the biosynthesis of coumarins and lignans. Legumes contain high levels of coumarin, which gives off a sweet herbal aroma that attracts insects to spread pollen, acts as a natural fungicide to help plants fight pathogens, and acts as a feed to boost the immune function of animals [43]. Phenylalanine and tyrosine, which are aromatic amino acids, play a crucial role in the biosynthesis of phenylpropanoids. Phenylpropyl compounds possess a chemical structure characterized by a phenylpropylamine framework, which encompasses a benzene ring, a propane moiety, and amino groups. The presence of certain functional groups, such as acyl, methoxy, and chitosan groups, or the presence of a condensed ring structure, contributes to the distinctive scent exhibited by phenylpropyl compounds, which not only affect the flavor of the feed but also, enter

the body of the livestock through digestive and metabolic processes and may also have an impact on the appetite and health of the livestock [44].

The examination of pathway alterations revealed an upregulation of the terpene synthesis pathway during the initial phase of flowering, followed by a downregulation in the later stage of flowering. Conversely, the phenylpropanoid synthesis pathway exhibited an upregulation, specifically during the flowering stage. These observations suggest that terpenes are synthesized in substantial quantities from the onset of bud development until the early flowering stage, while phenylpropanoids are synthesized in significant amounts from the early flowering stage until the full flowering stage. Terpenoids include monoterpenoids and sesquiterpenoids, which play a significant role in contributing to the aromatic characteristics of plants. Monoterpenes encompass a variety of chemicals, such as limonene, that are frequently present in aromatic plants and herbs, imparting a pleasant and fragrant scent to these botanical specimens. Diterpenes encompass a variety of chemicals, including malondialdehyde and lauricene, which exhibit a pronounced pine aroma and are prevalent in numerous species of pine plants. These substances are known to stimulate appetite in livestock and also have antimicrobial, antioxidant, and anti-inflammatory properties that promote growth and improve immunity in livestock [45]. Terpenoids, being derivatives containing oxygen, can be categorized into many subgroups, including alcohols, acids, ketones, carboxylic acids, esters, and glycosides. Included in this group are several acids, such as citric acid, malic acid, and aromatic folic acid, among others. These chemicals have acidic, fruity, sweet, and floral characteristics. Citric acid is the main compound in acidic substances, it has the characteristics of food attraction, anti-stress, no residue, etc., and it has achieved good results in reducing the stress of weaned piglets and improving the production performance [46]. Alcohol terpenoids are characterized by the presence of hydroxyl functional groups within their chemical structure, which typically impart them with pleasant and refreshing scents. Ketones possess carbonyl functional groups within their chemical composition, and their olfactory characteristics predominantly exhibit herbaceous, aromatic, and minty notes. The addition of these substances to the feed suppresses unpleasant odors in the feed and gives it an aromatic smell, thus increasing the average daily feed intake of the animal [47]. The phenylalanine group represents the second category of VOCs found in plants. Within this group, phenylalanine lyase (PAL) serves as the primary regulatory enzyme responsible for facilitating the conversion of the principal substrate, L-phenylalanine, into this volatile molecule. This conversion occurs through a sequence of hydroxylation, acylation, and methylation processes. Aromatic amino acids play an important role as precursors in the synthesis of phenylalanine analogs. Adding amino acids to feed can improve the digestive function of animals and increase feed utilization [48]. The time from bud emergence to early blooming in alfalfa is characterized by concurrent nutritional and reproductive growth, which is closely associated with robust growth metabolism. This metabolic activity is perhaps connected to the increased expression of terpenoid production. During the period of flowering, alfalfa undergoes a significant uptake of nutrients, resulting in a substantial decrease in crude protein content. This fall in protein content may be attributed to the up-regulation of phenylpropanoid production. The process of N-glycan biosynthesis is intricately linked to intricate protein modifications and is indispensable for the survival of both unicellular and multicellular organisms. It assumes a crucial function in the growth and elongation of pollen tubes in plants, and is also closely associated with protein signaling mechanisms within the plant system, possibly related to the production of nitride compounds in odor [49]. Fatty acids play a crucial role as precursors in the biosynthesis of flavor compounds in various fruits, including peach fruit. Notably, the unsaturated fatty acid linolenic acid is implicated in the production of aromatic compounds, such as aldehydes and esters, which are important compounds in the formation of alfalfa odor [14, 50]. These metabolic pathways have been observed to influence the composition of volatile organic compounds in alfalfa, and further research is needed to identify the key enzymes involved in these processes to achieve regulation of alfalfa odor. The electronic nose and GC–MS techniques have the advantages of being fast, accurate, and reproducible compared to traditional odor sensory evaluation methods for feed. Currently, there are few studies on feed odors and no uniform evaluation methods, and it is recommended that future research focus on the wider application of this technology in the feed field.

Conclusion

In this study, the odor and quality of alfalfa were analyzed by combining an electronic nose with GC–MS. The results showed that terpenoids increased first and then decreased with growth and development, heterocyclic substances decreased continuously, and alcohols and ketones increased. The crude protein and ether extract decreased and were positively correlated with terpenoids, heterocycles, and nitrogen and sulfur compounds, while the dry matter content, soluble carbohydrates, and neutral detergent fiber increased and were positively correlated with alcohols and ketones. Volatile organic compounds accumulated the most in the early flowering

stages, which had more sweet and fruity flavors, and the main substances that differed from the other two groups were esters and terpenoids, such as methyl heptanoate, butyl butyrate, and β-ionone, and the differential metabolic pathways were mainly terpenoids and phenyl-propylene synthesis. The analysis of volatile organic compounds in alfalfa provided new ideas for odor formation, quality identification, production, and utilization of alfalfa.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00541-9.

Additional file 1: Figure S1. OPLS-DA evaluation model verification diagram.

Additional file 2: Table S1. A total of 782 volatile organic compounds.

Additional file 3: Table S2. Different volatile organic compounds and odor characteristics.

Acknowledgements
Thank to Wuhan Metware Biotechnology (Wuhan, China) for its constructive comments and technical support with metabolic data analysis in this manuscript.

Author contributions
Conceptualization, methodology, data curation, writing—original draft preparation and writing—review and editing: YCL. Methodology: JB, QS, MJL and PBS. Writing—original draft preparation, writing—review and editing, investigation and resources: ZJW, LS. Writing—review and editing: GTG. Project administration and funding acquisition: YSJ, YTL. All authors have read and agreed to the published version of the manuscript.

Funding
This research was financially supported by the National Key R&D Program of China (2022YFD1300801-02), the National Key R&D Program of China (2022YFE0111000), the National Natural Science Foundation of China (32260346), Development and demonstration of key technologies for high quality forage production and grass product processing (CCPTZX2023B07), and the Special Fund Project of Scientific and Technological Achievements Transformation of Inner Mongolia Autonomous Region (2021CG0006).

Data availability
The data that supports the findings of this study are available in the supplementary material of this article.

Declarations

Competing interests
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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Received: 23 October 2023 Accepted: 23 January 2024
Published online: 12 February 2024

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