Base on metabolomics:analysis of the internal causes of nutrient changes in *Leymus chinensis* at different harvest stage

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Abstract

Background: *Leymus chinensis* is one of most valuable forage on the native grassland for its higher nutrition content. But the local farmer often gained the worse *Leymus chinensis* hay due to the unfavourable harvest stage, the nutrition of *Leymus chinensis* was decreasing from jointing stage to solid stage for its growth characteristics , and the results showed that the crude protein (CP) decreased and the fiber content increased which will caused the nutrition of forage reduced. However, the reason for the nutrient changes in *Leymus chinensis* has not yet explained at the metabolic level.

Results: In this research, the *Leymus chinensis* was harvested at two growth stage(jointing stage and solid stage). Metabolomics was used to analyze the expression changes of metabolites in the whole plant of *Leymus chinensis* at different harvest stage, and the metabolites were analyzed based on LC-MS/MS untargeted metabolomics. A total of 55 metabolic pathways and 119 metabolites were related to the nutritional change in *Leymus chinensis*. Most of the metabolites are involved in metabolic processes, including amino acid metabolism, carbohydrate metabolism and acid metabolism biosynthesis. Under the action of EBF1/2 and EIN3, cysteine participates in the ubiquitin-mediated protein degradation pathway. In solid stage, the increasing content of cysteine directly promotes the ubiquitin-mediated protein degradation rate, which is manifested reduce the synthesis of crude protein (CP) in *Leymus chinensis*, resulting in the decreasing of *Leymus chinensis* nutrient quality. Carbohydrate metabolism provides raw materials for the synthesis of hemicellulose, leading to the increasing of hemicellulose. In addition, phenylalanine provides the necessary conditions for the synthesis of lignin. These are the internal factors that lead to the decline of quality of *Leymus chinensis.*

Conclusions: This study analyzed the elucidate the relationship between the reduction in the nutritional value of *Leymus chinensis* and complex biological processes by the metabolomic,This study will provides a theoretical basis for producing high-quality *Leymus chinensi*s hay and sets the stage for further research.

Background:

Leymus chinensis is a high quality grass of rhizome type belonging to the family of grasses[1]. Making hay is the main way of *Leymus chinensi*s, which grows on the natural grassland. Because of its high nutritional value the fact that animals like to eat it when it is green as hay [2]. However, the value of *Leymus chinensi*s hay is affected by lots of factors, the one was the harvest stage[3]. *Leymus chinensi*s harvested at the jointing stage which has a greater nutrition than solid stage. At the tine of solid stage, the stem fraction of *Leymus chinensi*s is greater than jointing stage. Many reporters have showed that decreased crude protein (CP) and increased fibre contents in *Leymus chinensi*s will show different results at different stages[4]. *Leymus chinensi*s harvest at the optimum growth stage can improve both hay yield and quality. The current view was harvested at the jointing stage is thought to cause a high yields and nutrient concentrations in *Leymus chinensi*s. However, it is a difficult case in China especially in prairie areas, where *Leymus chinensi*s is always harvested at solid stage due to the local farmer thought that

more grass could be harvested at that time.However, due to the growth characteristics of plants, the *Leymus chinensi*s harvested at solid stage had severe fibrosis and low nutritional value[5]. Lot of nutrients and relative metabolites, especially proteins, may decrease a lot during this time, which is regarded as a main index for evaluating the nutritional value of forage, indicating a large impact on the production of high-quality hay and the stable and sustainable development of grass husbandry[6].

We find out the specific changes in nutrients during this period by metabolomics, and metabolomics provide an useful methods for identifying metabolites and associated pathways that are crucial for understanding the nutrient contents of *Leymus chinensi*s and the mechanisms underlying nutrient metabolite changes during different harvest stages[7]. At present, the research of metabolomics mainly focuses on the resistance,yield and nutrition of forage.There are few studies on the inner mechanism of using metabolomics to study the difference of nutrient quality of forage at different harvest stage. Metabolomics can promote the expression and identification of forage and its related metabolites. Metabolomics is an effective method for the analysis and identification of metabolites and metabolic pathways that may lead to decreased CP content in *Leymus chinensis* by LC-MS /MS [8].

At present, the evaluation methods of the nutritional value of forage mainly focuses on the index of CP and RFV[9]. The nutritional value of forage is mainly expressed by CP, and the amount of CP will directly affect the nutritional value of forage[10]. Amino acids are important precursors in protein synthesis and have important significance for protein synthesis. Using metabolomics to study the specific pathways and metabolites of CP synthesis plays an important role in establishing a new grade of forage nutrient quality. In this study, using metabolomics to study changes in the nutritional value of forage will set a precedent for re-evaluating the nutritional value of forage.

In this study, We will use metabolic to fully studied protein synthesis pathways and metabolites, and our goal is to fully understand the nutrient changes, metabolites and metabolic pathways of *Leymus chinensis*. We also want to identify the key regulatory pathways and metabolites that influence changes in metabolites and nutrient content in *Leymus chinensis*.

Statistical analysis

Three biological replicates were performed for each experiment, and nutritional data were analyzed using SAS 9.1.The mean differences were compared using Duncan's multiple range T-test. Comparisons with $P < 0.05$ were considered significantly significant, and the data are presented as the mean ± SE, three independent replicates at least [11] .

Results

Leymus chinensis nutrient content analysis

To assess the composition of the *Leymus chinensis* at different harvest stage,the main nutritional indexes were determined and recorded (Table 1). According to analyze of CP, it show the highest content

was existed the jointing stage, at 12.31%DM, and the lowest in the solid stage, at 8.27% DM. These results revealed that the CP content of *Leymus chinensis* continues to decreased from the jointing stage to solid stage. In contrast, the ADF content was 37.36% DM in jointing stage, which was significantly lower than the solid stage ($P < 0.05$). The content of NDF at jointing stage was 56.04% DM, which was significantly lower than at the solid stage ($P < 0.05$). The data showed that the fiber content in *Leymus chinensis* was increased gradually with the prolongation of growth period.

Metabolic pathways associated with changes in protein content in Leymus chinensis

To assess the metabolic pathways of the *Leymus chinensis* at different harvest stage,the main and secondary metabolism related to the change of CP in *Leymus chinensis* were analyzed and listed (Fig. 1, Fig. 2). we can find that the main metabolism contained glycan biosynthesis and metabolism, metabolism of terpenoids and polyketides, xenobiotics biodegradation and metabolism, lipid metabolism, carbohydrate metabolism, amino acid metabolism, enrgy metabolism, metabolism of other amino acid, nucleotide metabolism, metabolism of cafactours and vitamins, biosynthesis of secondary metabolism.meanwhile, the secondary metabolism concluded metabolism of terpenoids and polyketides, biosynthesis of cofactor and vitamins, and biosynthesis of other secondary metabolites.

Metabolic pathways associated with protein change in Leymus chinensis **at different harvest stage**

The *Leymus chinensis* were assessed by metabolomics from two harvest stages. A total of 119 metabolites of *leymus chinensis* were screened and identified, among which 64 metabolites were significantly up-regulated and 55 were significantly down-regulated(Table 2). After further study, we found that out of 55 down-regulated metabolic pathways were associated with protein reduction included 13 amino acids, 1 pyrimidine, 1 purine, 5 carbohydrate metabolism, 12 acid metabolism, and 23 other metabolism were involved(Table 3).13 amino acids metabolic pathways included amino sugar and nucleotide sugar metabolism,cysteine and methionine metabolism, phenylalanine,tyrosine and tryptophan biosynthesis, phenylalanine metabolism, asparate and glutamate metabolism, clycine, serine and threonine metabolism, arginine and proline metabolism, arginine metabolism, lysine biosynthesis, lysine degradation, tyrosine metabolism, tryptophan metabolism, histidine metabolism; 5 carbohydrate metabolism included pentose phosphate pathway, galactose metabolism, fructose and mannose metabolism, glycolysis/gluconeogenesis, pentose and glucuronate interconversions;12 acid metabolism included a-linolenic acid metabolism, C5-branched dibasic acid metabolism, 2-oxocarboxylic acid metabolism, biosynthesis of unsturated fatty acids, arachidonic acid metabolism, linoleic acid metabolism, nicotinate and nicotinamide metabolism, folate biosynthesis, glyoxylate and dicarboxylate metabolism, fatty acid metabolism, fatty acid biosynthesis, fatty acid elongation, fatty acid degradation. 23 other metabolism included terpenoid backbone biosynthesis, vancomycin resistance, and vitamin B6 and so on.

Protein profiles of Leymus chinensis **at different harvest stages**

The changes in the metabolic profiles of the whole *Leymus chinensis* samples between two groups were analyzed using the metabolomics method based on HILIC UHPLC-Q–TOF technology. The significantly different metabolites were selected based on the criteria of an OPLSDA model VIP > 1 and a P-value < 0.05. To evaluate the rationality of the candidate metabolites and more fully and intuitively illustrate the relationship between the samples and the metabolites in samples exhibiting differences in expression patterns, we conducted a hierarchical cluster analysis based on the expression of significantly different metabolites in each group of samples. This approach assisted in the accurate selection of marker metabolites and the investigation of changes in related metabolic processes (Fig. 3).Finally, 119 significant variations in metabolites were detected, which are shown in Table 2. These metabolites mainly included amino acids, organic acids, carbohydrates, purines, lipids and pyrimidines (Table 3). A total of 55 metabolites were up-regulated while 64 were down-regulated at two harvest stages. The expression levels of L-glutamic acid, L-asparagine, purine, pyrimidine and other protein synthesis-related metabolites were down-regulated, whereas L-phenylalanine and carbohydrates, lipids and other substances were significantly up-regulated. We submitted the differential metabolites to the

KEGG website for the analysis of relevant pathways, and we found that these differentially expressed metabolites were mainly involved in the biosynthesis of secondary metabolites, protein digestion and absorption, the bio-synthesis of amino acids and the biosynthesis of phenylpropanoids. The changes in these metabolites provide important information for our study of changes in the nutritional quality of *Leymus chinensis*.

Leymus chinensis hormone signal transduction

There were eight signal transductiones was associate with the CP reduce in the *leymus chinensis*. The change of protein content in herbage is directly related to the metabolic reaction of amino acid. We can find there were three animo acid hormone signal transductiones(Fig. 4), and they were tryptophan metabolism, cysteine and methonine metabolism, and phenyalalanine metabolism.There were two transduction in the the tryptophan metabolism, when the synthesis produce the AUXIAA, the one signal transduction reaction is ubiquitin mediated proteolysis, and the other signal transduction reaction was promote the cell enlargement, and it was helpful with the plant growth.Cysteine and methonine metabolism had two hormone signal transduction. After the EIN3 was existed, The one hormone signal transduction was to promote the ubiquitin mediated proteolysis, and the other signal transduction promote the fruit ripening senescence with the ERF1/2 and DNA.Phenyalalanine metabolism had one signal transduction, when the PR-1was existed, it was mainly used for disease resistance.

Discussion

Metabolomics study of *Leymus chinensis*

The CP content of forage is often different due to different species, harvest stage and other factors[12]. According to the chemical properties of forage, CP are mainly divided into two types[13]: true protein nitrogen and non-protein nitrogen. Non-protein nitrogen is mainly composed of free amino acids, amides,

purines, pyrimidines and alkaloids, accounting for about 30% of the total nitrogen content of forage[14] .Protein composition in forage mainly includes 20 amino acids, such as alanine, lysine, glutamic acid and aspartic acid. Amino acids, as the components of proteins, play a very important role in the nitrogen metabolism pathway in forage, and they are the preconditions for the composition of amino acids, purines and pyrimidines[15] . Studies have shown that glutamate plays a significant or extremely significant role in protein synthesis in wheat[16]. Study[17] showed that the content of glutamate in alfalfa in the early flowering stage was significantly higher than that in the mid-flowering stage(P<0.05), and the L-glutamic acid was the main aimo acid which affect the CP changed. The above studies indicate that amino acids are related to protein synthesis, and the amount of amino acid content will affect the amount of protein synthesis, according to the results of this study alanine content in jointing stage is lower than that in solid stage. In addition, the levels of purine and pyrimidineare was lower in solid stage. Alanine, purines and pyrimidines content was reduced before the protein synthesis, which could lead to protein synthesis decreased, which was the reason of CP content in solid stage is lower than the jointing stage. The content of phenylalanine increased, but the protein content did not increase in the related metabolic pathway[18]. It can be considered that phenylalanine mainly comes from the hydrolysis of protein, which may also be one of the reasons for the decrease of protein content. Ttyrosine, after hydroxylation of phenylalanine, is involved in the process of glucose metabolism of plants was together with phenylalanine to promote the synthesis of carbohydrate[19]. The results of this study showed that the expression levels of sugars and other substances in solid stage were significantly up-regulated. Forage signaling figure of this study show that cysteine metabolic pathways in the generated EIN3, under the action of EBF1/2 and u, products are mainly used for ubiquitin protein degradation mediated, this is the only one in the plant in the metabolic pathway of protein degradation pathway, which may be CP in *Leymus chinensis* in jointing stage processed to the solid is the main reason, which will be our main research direction in the future.

The growth and development process of *Leymus chinensis* is mainly dominated by amino acid metabolism[20]. Amino acid, as the main mode of existence and transportation of nitrogen compounds in *Leymus chinensis*, is also regarded as the principle of maintaining the balance of nitrogen and the synthesis of enzymes and hormones in vivo[21]. The nutritional quality of *Leymus chinensis* relies mainly on the protein content, amino acid composition and its equilibrium state, amino acid content that in the condition with higher and lower which will affect the amount of protein synthesis, and the essential amino acid was considered as one of *Leymus chinensis* nutrition qualitative decision factors, on how to improve protein content at the same time, and the amino acid related research is a top priority in the current scholars research [22-24]. Studies have shown that the improvement of crop quality cannot rely solely on the simple accumulation of protein and amino acid content, but it should be comprehensively evaluated in combination with the yield of crops, especially the proportion of essential amino acids[25] . In the nitrogen metabolism pathway, the first is the assimilation of ammonia, followed by the absorption of amino acid nitrogen to participate in the nitrogen metabolism[26]. The assimilation of ammonia first forms glutamine and glutamic acid, and then forms other amino acids and proteins[27]. Glutamic acid plays an important role in the nitrogen metabolism pathway , because glutamic acid is the initial

precursor of protein synthesis, which is mainly co-synthesized by alpha-ketoglutaric acid and amino acid transaminase[28]. In this study, the reduced content of glutamic acid and protein synthesis precursor decreased, which reduces the amount of protein synthesis in *Leymus chinensis*, performance for protein content is reduced, the main reason is that glutamate content directly to reduce the influence to the *Leymus chinensis* metabolism, influenced the late protein synthesis, and made an impact on the quality of the *Leymus chinensis*.

Nutrition changes

Changes in CP,ADF and NDF contents changes

In this study, harvest time affected CP of *Leymus chinensis*. Significant (P< 0.05), CP of *Leymus chinensis* decreased with the delay of harvest time. The decrease of CP in *Leymus chinensis*may was related to its growth and development, with the delay of harvest time, the ratio of stem and leaf of *Leymus chinensis* increased, while a large amount of CP in *Leymus chinensis* is stored in leaves, so the CP decreases. On the other hand, harvest time delayed will cause *Leymus chinensis* consume a large amount of CP for its own growth and the formation of awn needles, leading to the reduction of CP[29]. The content of ADF and NDF has an important influence on its nutritional quality, among which ADF affects the digestibility[30] . NDF affects grazing rate of livestock, while high NDF content leads to low grazing rate[31]. In this study, harvest stage had significant effect on ADF and NDF of *Leymus chinensis* (P< 0.05). The content of ADF and NDF in *Leymus chinensis* increased with the delay of harvest stage, which may lead to the decrease of quality of *Leymus chinensis* with the delay of harvest stage.

In recent years, the proper harvesting of forage has been paid more and more attention by researchers at home and abroad. At present, the researches mainly focuses on the influence of harvest time on grass yield, quality and regeneration rate [32]. Some scholars pointed out that the proper harvest of forage should meet the following basic conditions[33]: first, the harvest stage should be conducive to the growth of forage; Secondly, it is necessary to maintain high nutritional quality of forage after harvest. Finally, harvesting is beneficial to the enrichment of nutrients in the roots of forage, so as to ensure the safe wintering of forage. Some scholars found that dry matter yield increased by 18% when alfalfa was cut at the peak of flowering, but dry matter digestibility and CP content decreased by 5.4% and 8.0% respectively[34]. With the prolongation of alfalfa growth period, the grass yield and dry matter content have been increasing, but the nutrients show a change trend of first increasing and then decreasing, and the budding stage is the critical point for the rise and fall of nutrients in forage[35]. When the forage was harvested at the flowering stage, the CP content remained at the highest level, and a higher yield could be obtained[36]. The above research results indicate that the harvest stage has an important influence on the preservation of forage's nutritional quality. During the whole growth period of native grass, with the continuous extension of the growth period of forage, the forage yield gradually increases, but the quality gradually decreases. The longer the harvest period, the higher fiber. Therefore, grass yield and nutrient quality should be taken into account in the harvest of forage. Harvest stage has a great impact on the

yield, quality and rejuvenation of forage, and proper harvest can ensure the yield and nutritional value of forage.

Establishment of forage nutrition evaluation methods

At present, the CP, NDF and ADF are the common indicators to evaluate nutritive value while the CP is the main index, but the studies about the quality evaluation of forage from the level of metabolism has not mentioned. Due to the physiological characteristics of forage heterogeneity, the different harvest stage will cause the different CP . In order to get a higher CP , we need to choose the suitable harvest stage[37] . CP was mainly expressed on the physiological and biochemical reaction synthesis of amino acids, and alanine as an important precursor of protein synthesis, which has important influence on the formation of a protein, and we can say alanine content on the forage quality has important influence, and this experiment showed alanine was important on the influence of CP, to explained the amino acids from molecular level for the importance of forage quality evaluation[38]. Therefore, based on the analysis results of this experiment, it is necessary to continue to clarify the changes of forage quality through amino acid molecules, such as glutamic acid, strengthen the research on the relationship between protein synthesis precursor and forage quality, and discuss the evaluation index of hay quality from the molecular level.

Conclusions

Our data analyzed and interpretated the *Leymus chinensis* metabolites, the change of metabolic pathways, and the aspects of nutritional quality changes on *Leymus chinensis* metabonomicson from jointing stage to the solid stage. A large number of different expressed metabolites are mainly involved in carbohydrate expression metabolism, starch and sucrose metabolism, cysteine biosynthesis and amino acid biosynthesis. *Leymus chinensis* has low CP in solid stage, mainly due to glutamic acid content decreased. The metabolism of carbohydrate synthesis of hemicellulose do not receive the raw material, cause the grass hemicellulose content increases, further lead to increased NDF content. In addition, the increase of phenylalanine content of lignin synthesis provides the necessary conditions. Together, these results provided the internal causes of the content of CP decreased and NDF and ADF increased in *Leymus chinensis* from jointing stage to the solid stage. This study provides the result of the innovation and further clarified on the quality difference of *Leymus chinensis* at two important stages. In addition, it offers a new methods to explain and evaluate forage nutrition, and our results also show the reasons for CP changes. Therefore, this study illustrates the nutritional value of *Leymus chinensis*reduce relation with complex metabolic biological process, and provide the theoretical basis for the future production and technical support of *Leymus chinensis*.

Methods

Forage material

Leymus chinensis was grown at Inner Mongolia Agricultural University field experimental site in Chifen(41°17'10"–45°24'15" N–116°21'07"–120°58'52"E),Inner Mongolia, China, in 2018.We are licensed by the local agriculture and pastoral bureau and certified by grass industry experts, and we are conducting experiments in accordance with the local grassland protection law. The selected *Leymus chinensis* is naturally grown on native grassland. When taking samples of *Leymus chinensis*, the staff followed the local grassland protection law and the regulations on sampling wild species in the convention on trade in endangered species of wild fauna and do as the :https://www.cites.org/ requested. The identification of *Leymus chinensis* samples was conducted after consulting with Mr Zhang weiguo, a well-known expert in plant classification in China. Zhang weiguo devoted himself to the identification and classification of forage grass species in the field all the year around, with rich field work experience and scientific research foundation.We took *Leymus chinensis* as the experimental material and started the experiment on August 20, 2017. When the forage was harvested, the growth periods were jointing stage and solid stage. The jointing stage is a period which has a rapid growth of stem on *Leymus chinensis*. Generally, the first stem node of more than 50% plants in the whole field is exposed to the ground by 1.5–2.5cm, and solid stage refers to the period from flowering to maturity of forage seeds. We took samples with sterilized gloves and cut them with sterilized scissors to gained two stage samples . Each sample was 200g, and one sample was placed in an envelope bag, then the samples were dried in a 105℃ oven for 30 min. Washing the *Leymus chinensis* samples with PBS buffer, wraping them with tin foil, marking them and immediately freeze them in liquid nitrogen, and then transfer them to -80℃ refrigerator for later use.

Nutrition Analyze

The contents of crude protein (CP) was tested in accordance with the reference of the AOAC international [39]. The content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the Ankom A200 fiber analyzer (Ankom Technology, Macedon, NY) by the method of Van Soest[40].

Metabolite Analyze

Metabolite extraction

80 mg samples were added to liquid nitrogen for grinding, and 1 ml of methanol/acetonitrile/water (2:2:1, v/v) was poured into the mixture. Then, the samples were rotated and mixed well. After ultrasonic crushing at low temperature for 0.5 h, the samples were repeated three times.

Liquid mass spectrometry

In order to obtain reliable data, this study added QC sample analysis to monitor the entire experimental process. The metabolites of samples were extracted by precipitation protein method, and the same amount of samples were mixed to prepare quality control (QC) samples. The samples were randomly sorted by computer, and QC samples were inserted before.

Description of metabolite extraction

Samples were defrosted on ice and metabolites were extracted with 50% methanol buffer. Temporarily shelved, 20 ml samples were pre-cooled with 120 ml 50% methanol, vortex for 1 min, incubate at room temperature for 10 min. Set -20°C for 24 h. After centrifugation for 20 min, the supernatant was transferred to the new 96-well plate. Pre-storage qualitative analysis at -80°C.

Description of liquid phase parameters

All samples were collected by using instructions on the LC-MS/MS system. All the chromatograms were collected by UPLC system (SCIEX, UK). ACQUITY UPLC T3 column (100 mm x 2.1 mm, 1.8 m (including waters, UK) is mainly responsible for the phase separation. Gradient elution specification was set as follows: 0–0.5 min, 5% B; 0.5–7 min, 5% to 100% B; 7–8 min, 100% B; 8–8.1 min, 100% to 5% B; 8.1 –10 min, 5% b.

Mass spectrum parameter description

Metabolites were analyzed by a high resolution tandem mass spectrometer TripleTOF5600plus (SCIEX, UK). Q-TOF works in positive and negative ion modes. Curtain gas is 30 PSI, ion source gas1 is 60 PSI, ion source gas2 is 60 PSI, and the temperature of an interface heater is close to 650℃. For positive ion mode, the floating adjustment of ion spray voltage is 5000 V. For negative ion mode, the floating adjustment of ion spray voltage is -4500v. The mass spectrometry data were collected using IDA mode. The TOF quality range is 60 ~ 1200 Da. Scanning can be obtained in a time of 150 ms, when more than 100 counts per second (count /s), with a 1+ charge quantity, up to 12 ions can be collected. The total cycle time was set at 0.56s.

Information analysis process

In order to obtain the accuracy of data, it is necessary to analyze the offline data strictly according to the steps of information analysis. Through MSConvert software of Proteowizard, plain data is parsed and converted into readable data mzXML. Peak value is extracted by XCMS software and quality control is carried out on peak value. The substances after quality control were annotated by CAMERA and ion, and were identified by metaX software. The first level and second level information of mass spectrum were identified and matched with in-house standard product database. Metabolites were annotated through HMDB, KEGG and other databases to clarify the physicochemical properties, biological functions and characteristics of metabolites. Differential metabolites were quantitatively screened by metaX software.

Information analysis description

Peak extraction, peak grouping, correction, secondary peak grouping, isotope and adder annotation and other preprocessing of MS data were carried out by XCMS software. The raw data is converted to mzXML format and processed by XCMS. Retention time (RT) and molecular data were fused, and each ion was identified. Different peak strengths were recorded to generate a three-dimensional matrix composed of peak index, sample name and ion strength information. The exact molecular weight (m/z) and molecular weight data of samples were accurately compared through online KEGG and HMDB databases, and

metabolites were annotated. If the observed value is compared with the database value and the quality difference is less than 10ppm, then the standard metabolite. In addition, we can identify and verify metabolites through an internal metabolite fragment library. The peak strength was pretreated by METAX. After eliminating the features detected in QC samples less than 1/2 or biological samples 4/5, k-nearest neighbor algorithm was used to inject the residual peak of the missing value, so as to further improve the data quality. The evaluation of PCA outlier detection and batch processing were performed on the data was obtained by preprocessing. Based on the robust loess signal correction method to control the quality is applied to the data of injection sequence, so as to weaken the change of signal strength and enhance the reliability of the experiment.

Metabolomics assay

Metabolites of *Leymus chinensis* at different harvest times in typical grassland were analyzed by high performance liquid chromatography-mass spectrometry (HILIC UPLC-Q-TOF/MS). The analysis method is stable and the metabolomics data obtained are reliable. In the process of sample analysis, in order to verify the performance of the system, representative samples are processed after averaging by collecting QC samples. The analysis process includes all samples. QC samples are treated with actual samples and each 5 samples are added to ESI positive or negative to analyze the stability of the monitoring instrument in different batches. The similarity of QC includes peak shape, separation degree, retention time and intensity distribution of metabolites involved in configuration files. It will get the QC samples to analysis 5 times in total ion to flow chart after comparing spectral overlap, as shown in figure 5, the results show that the response intensity and retention time of chromatographic peak of basic overlap, the method is stable and reliable in the whole experiment process, good repeatability and stability , and it does not exist the technical error caused by man-made factors, the sample is suitable for this study.

We can see that the equipment of this experiment is stable, the data is reliable, and the stability is good from the figure 6. The metabolic spectrum differences obtained in the experiment can well reflect the biological differences between samples.

Leymus chinensis in typical steppe was regarded as research materials, based on metabonomics analysis, using high performance liquid chromatography tandem high-resolution mass spectrometer Triple TOF 5600 in positive and negative ions to metabolic group detection mode, combined with biological information analysis mass spectrometry data interpretation of biological information analysis mainly using XCMS software substance detection using METAX software for quantitative differences in material selection, respectively using METAX software for material level of mass spectrogram Using the in - house atlas for material secondary metabolites annotation mass spectrogram, to study the the internal causes of nutrition in *Leymus chinensis* at different harvest stage from the molecular level and provide a reasonable and efficient theoretical basis, to gained high quality forage production in the future research and provide the new direction and new ideas.

Abbreviations

CP
crude protein
NDF
neutral detergent fiber
ADF
acid detergent fiber
QC
quality control

Declarations

Ethics approval and consent to participate

This is to certify that the paper entitled Base on metabolomics:analysis of the internal causes of nutrient changes in *Leymus chinensis* at different harvest stage was approved by Inner Mongolia Agricultural University Animal Ethics Committee(12150000460029509N).

Consent to publish

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing Interest

The authors declare that they have no conflicts of interest to disclose.

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Authors’ contributions

WW, GGT and YSJ designed the experiments; WW and TRZ conducted the experiments; WW, CC and QY analyzed the data; WW and TRZ wrote the paper. All authors read and approved the final manuscript.

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Tables

Table 1 Nutrients of *Leymus chinensis* at different harvest stage

Harvest stage	CP （% DM）	ADF（% DM）	NDF（% DM）
Jointing stage	12.31±0.14 a	37.36±0.36 b	56.04±1.01 b
Solid stage	8.27±0.77 b	43.65±0.33 a	72.36±1.63 a

a, b Means ± SD within columns with different letters indicating significant differences ($P < 0.05$)

Table2 The changes of metabolites in *Leymus chinensis*

Number	Model up	Metabolites	Model down	Metabolites
1	ESI(+)	(R)-3-Amino-2-methylpropanoate	ESI(−)	L-2-Aminoadipate
2	ESI(+)	L-3-Aminoisobutanoate	ESI(−)	LL-2,6-Diaminoheptanedioate
3	ESI(+)	3-Methyl-2-oxobutanoic acid	ESI(−)	meso-2,6-Diaminoheptanedioate
4	ESI(+)	L-Valine	ESI(−)	(2S,4S)-4-Hydroxy-2,3,4,5-tetrahydrodipicolinate
5	ESI(+)	Methylmalonate	ESI(−)	N-Acetyl-L-2-amino-6-oxopimelate
6	ESI(+)	2-Methyl-1-hydroxypropyl-ThPP	ESI(−)	N-Succinyl-LL-2,6-diaminoheptanedioate
7	ESI(+)	3-Methyl-1-hydroxybutyl-ThPP	ESI(−)	N6-(L-1,3-Dicarboxypropyl)-L-lysine
8	ESI(+)	D-Xylose	ESI(−)	L-Glutamate
9	ESI(+)	L-Arabinose	ESI(−)	L-Arginine
10	ESI(+)	D-Ribulose	ESI(−)	N-Acetyl-L-glutamate 5-semialdehyde
11	ESI(+)	D-Xylulose	ESI(−)	L-Leucine
12	ESI(+)	L-Xylulose	ESI(−)	L-Isoleucine
13	ESI(+)	D-Lyxose	ESI(−)	L-Glutamate
14	ESI(+)	L-Ribulose	ESI(−)	O-Acetyl-L-serine
15	ESI(+)	L-Lyxose	ESI(−)	L-2-Aminoadipate
16	ESI(+)	Xylitol	ESI(−)	L-Arginine
17	ESI(+)	Ribitol	ESI(−)	L-Phenylalanine
18	ESI(+)	L-Arabitol	ESI(−)	LL-2,6-Diaminoheptanedioate
19	ESI(+)	D-Arabitol	ESI(−)	meso-2,6-Diaminoheptanedioate
20	ESI(+)	alpha'-Trehalose 6-phosphate	ESI(−)	N-Acetyl-L-glutamate 5-semialdehyde
21	ESI(+)	Sucrose 6'-phosphate	ESI(−)	L-Histidine
22	ESI(+)	Maltose 6'-phosphate	ESI(−)	(2S□4S)-4-Hydroxy-2,3,4,5-tetrahydrodipicolinate
23	ESI(+)	6-Phospho-beta-D-glucosyl-(1,4)-D-glucose	ESI(−)	O-Phospho-L-serine
24	ESI(+)	Sucrose 6-phosphate	ESI(−)	L-Tryptophan
25	ESI(+)	alpha-Maltose 1-phosphate	ESI(−)	L-Arginine
26	ESI(+)	Anthranilate	ESI(−)	D-Arginine
27	ESI(+)	3-Hydroxybenzoate	ESI(−)	L-Phenylalanine
28	ESI(+)	3,4-Dihydroxybenzoate	ESI(−)	2-Oxo-4-phenylbutyric acid
29	ESI(+)	2-Oxo-4-phenylbutyric acid	ESI(−)	L-Tryptophan
30	ESI(+)	L-Arogenate	ESI(−)	2-Amino-3,7-dideoxy-D-threo-hept-6-ulosonic acid
31	ESI(+)	Shikimate 3-phosphate	ESI(−)	6-Deoxy-5-ketofructose 1-phosphate
32	ESI(+)	5-Amino-2-oxopentanoic acid	ESI(−)	L-Glutamate
33	ESI(+)	2-Amino-4-oxopentanoic acid	ESI(−)	5-Phosphoribosylamine
34	ESI(+)	Phenylacetaldehyde	ESI(−)	D-Glucosamine 6-phosphate
35	ESI(+)	Succinate	ESI(−)	N-Acetylaspartylglutamate
36	ESI(+)	Phenylacetic acid	ESI(−)	Ectoine
37	ESI(+)	4-Hydroxybenzoate	ESI(−)	O-Phospho-L-serine
38	ESI(+)	Salicylate	ESI(−)	L-Tryptophan
39	ESI(+)	Phenylpropanoate	ESI(−)	Tetrahydrofolate
40	ESI(+)	alpha-Oxo-benzeneacetic acid	ESI(−)	2,6-Dihydroxyphenylacetate
41	ESI(+)	4-Hydroxy-2-oxopentanoate	ESI(−)	L-Phenylalanine□
42	ESI(+)	cis-3-(Carboxy-ethyl)-3,5-cyclo-hexadiene-1,2-diol	ESI(−)	D-Phenylalanine
43	ESI(+)	Lactose 6'-phosphate	ESI(−)	Phenylacetylglutamine
44	ESI(+)	2-Dehydro-3-deoxy-6-phospho-D-galactonate	ESI(−)	Hypoxanthine
45	ESI(+)	3-beta-D-Galactosyl-sn-glycerol	ESI(−)	5-Phosphoribosylamine
46	ESI(+)	Melibiitol	ESI(−)	Inosine
47	ESI(+)	Raffinose	ESI(−)	2-(Formamido)-N1-(5'-phosphoribosyl)acetamidine
48	ESI(+)	D-Gal alpha 1->6D-Gal alpha 1->6D-Glucose	ESI(−)	Urate-3-ribonucleoside
49	ESI(+)	Stachyose	ESI(−)	Mannitol
50	ESI(+)	Arbutin	ESI(−)	D-Sorbitol
51	ESI(+)	(R)-3-Amino-2-methylpropanoate	ESI(−)	2-O-(alpha-D-Mannosyl)-D-glycerate
52	ESI(+)	5-Methylcytosine	ESI(−)	D-Sorbitol
53	ESI(+)	Thymine	ESI(−)	Galactitol
54	ESI(+)	Methylmalonate	ESI(−)	D-Galactosamine 6-phosphate
55	ESI(+)	Thymidine	ESI(−)	N-Acetyl-D-galactosamine
56	ESI(+)	Adenine		
57	ESI(+)	Secologanin		
58	ESI(+)	4,21-Dehydrogeissoschizine		
59	ESI(+)	4,21-Dehydrocorynantheine aldehyde		
60	ESI(+)	Strictosidine aglycone		

61	ESI(+)	Horhammericine
62	ESI(+)	Dialdehyde
63	ESI(+)	Vindoline
64	ESI(+)	Vinblastine

Table 3 Metabolic pathways associated with protein decline in *Leymus chinensis*

Metabolic pathway items	Number	Metabolism
Amino acids	13	Amino sugar and nucleotide sugar metabolism
		Cysteine and methionine metabolism
		Phenylalanine, tyrosine and tryptophan biosynthesis Phenylalanine Metabolism
		Asparate and glutamate metabolism
		Clycine, serine and threonine metabolism
		Arginine and proline metabolism,
		Arginine metabolism
		Lysine biosynthesis
		Lysine degradation
		Tyrosine metabolism
		Tryptophan metabolism
		Histidine metabolism
Pyrimidine	1	Pyrimidine metabolism
Purine	1	Purine metabolism
Carbohydrate metabolism	5	Pentose phosphate pathway
		Galactose metabolism
		Fructose and mannose metabolism
		Glycolysis/gluconeogenesis
		Pentose and glucuronate interconversions
Acid metabolism	12	
		1. linolenic acid metabolism
		C5-branched dibasic acid metabolism
		2-Oxocarboxylic acid metabolism
		Biosynthesis of unsturated fatty acids
		Arachidonic acid metabolism
		Linoleic acid metabolism
		Nicotinate and nicotinamide metabolism
		Folate biosynthesis
		Glyoxylate and dicarboxylate metabolism
		Fatty acid metabolism
		Fatty acid biosynthesis
		Fatty acid elongation, fatty acid degradation
Other metabolism	23	Terpenoid backbone biosynthesis
		Vancomycin resistance
		Vitamin B6 ...

Figures

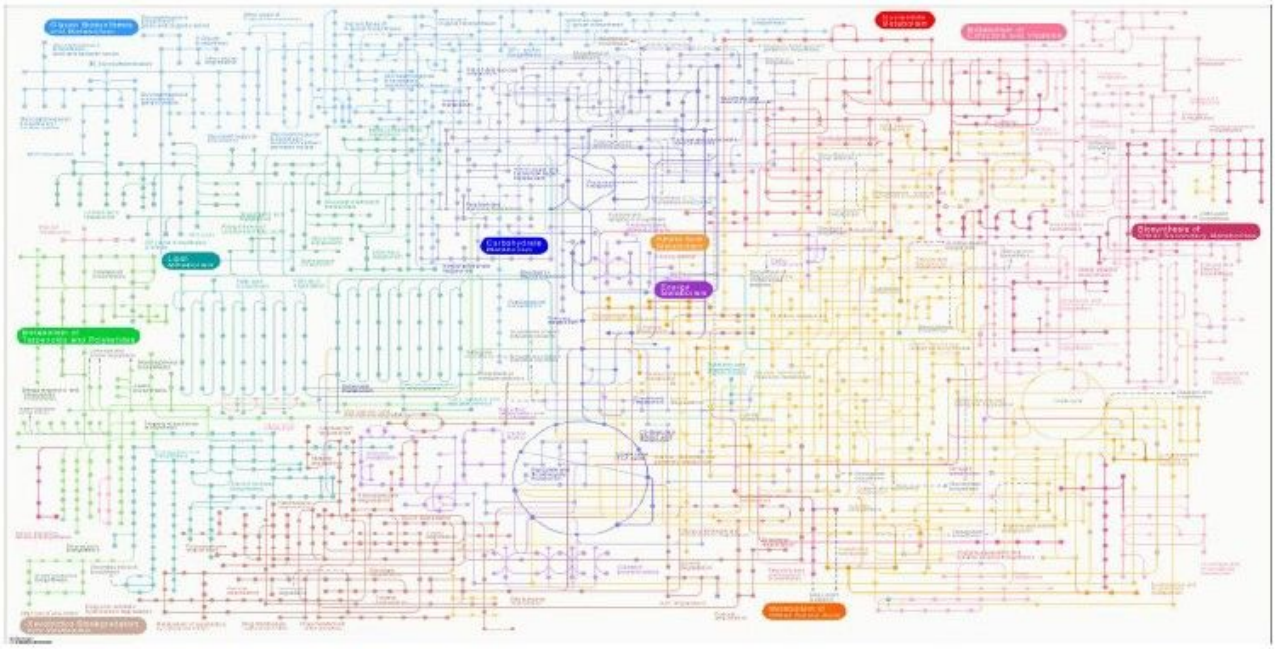

Figure 1

The main metabolism related to the change of CP content in Leymus chinensis

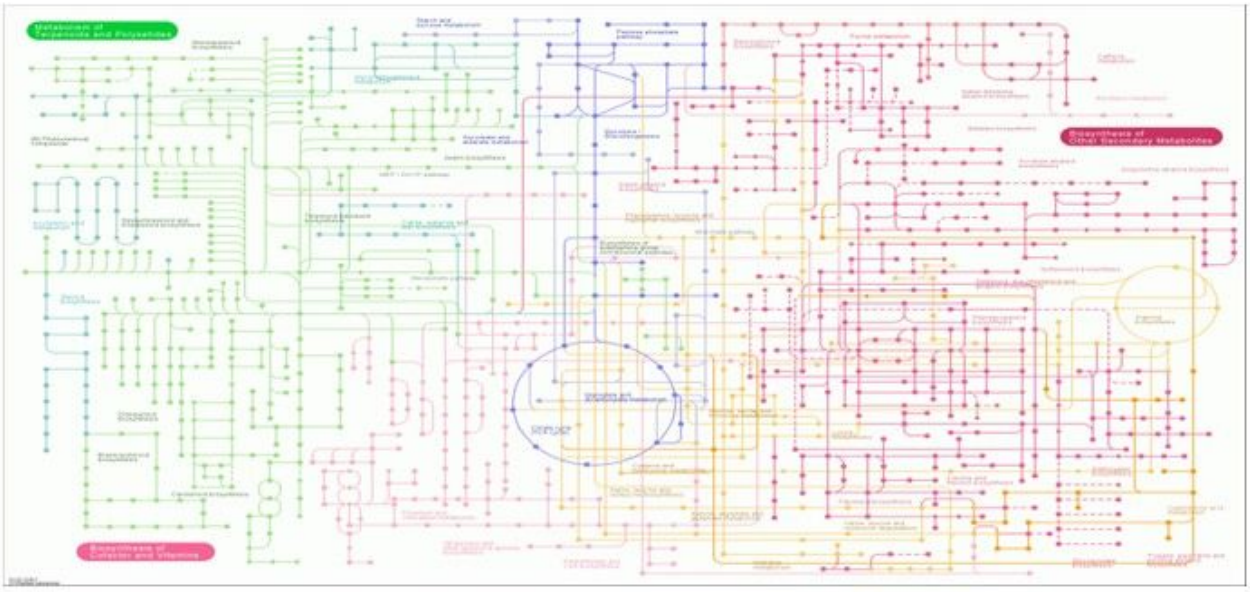

Figure 2

The secondary metabolism related to the change of CP content in Leymus chinensis

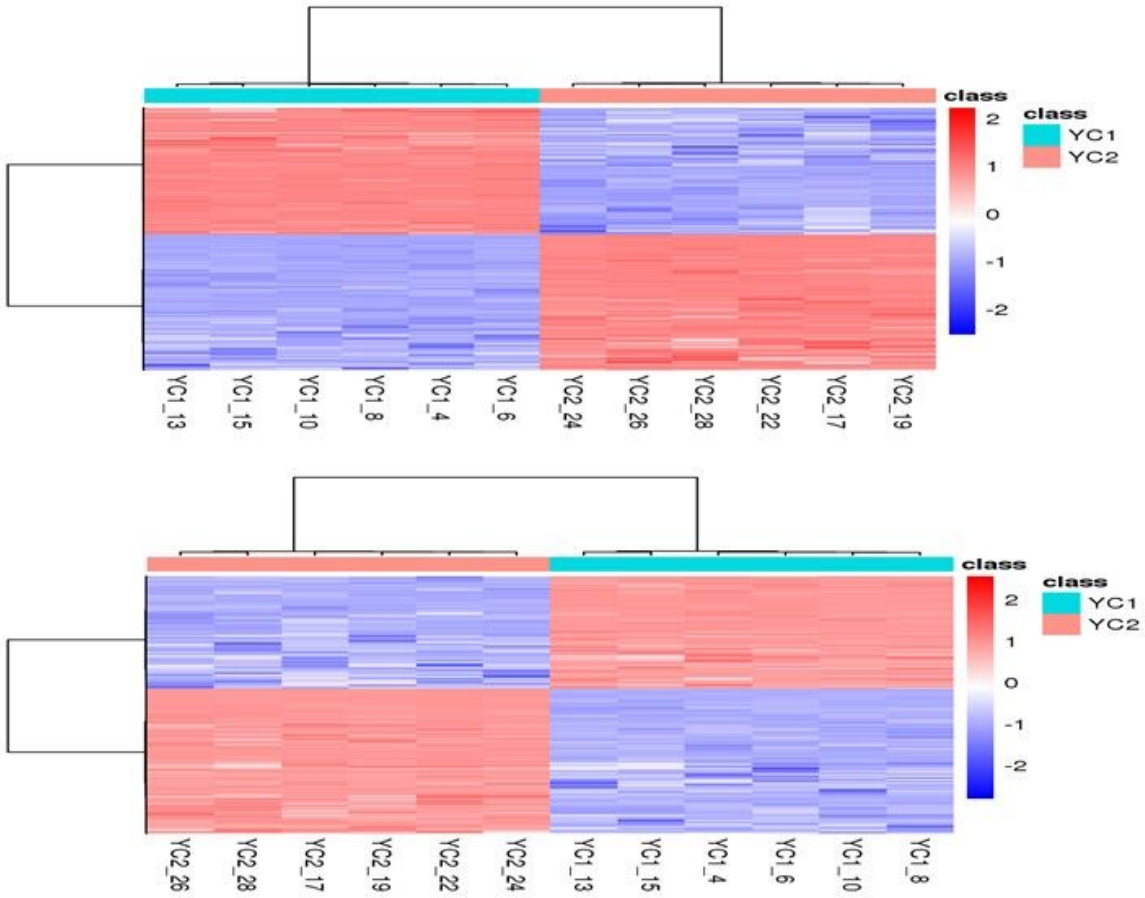

Figure 3

Hierarchical cluster heat map of differential metabolites. Hierarchical cluster heat map of significantly different metabolites during Leymus chinensis development in two harvest stages. a Positive mode, b negative mode.

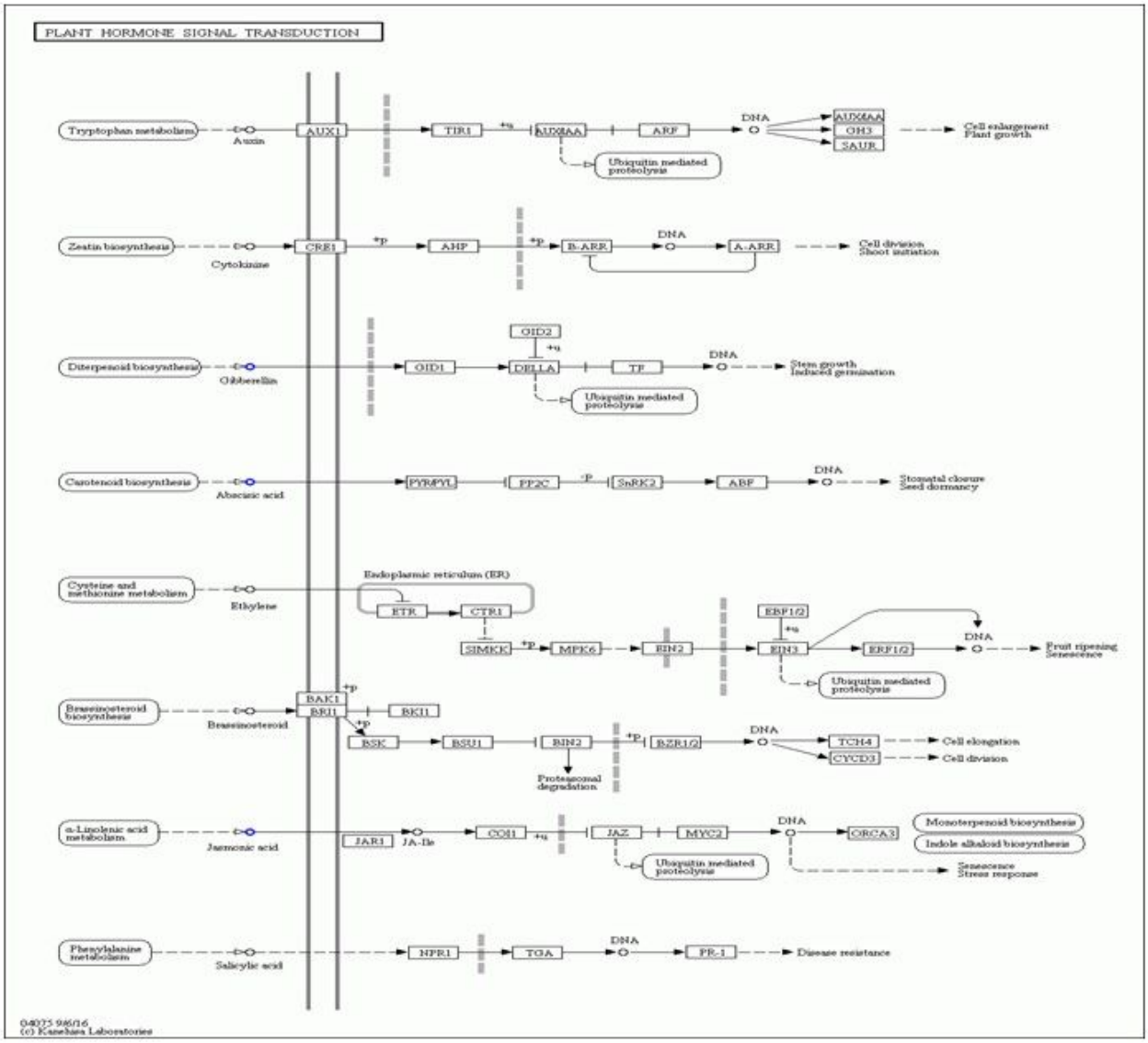

Figure 4

Leymus chinensis hormone signal transductiones

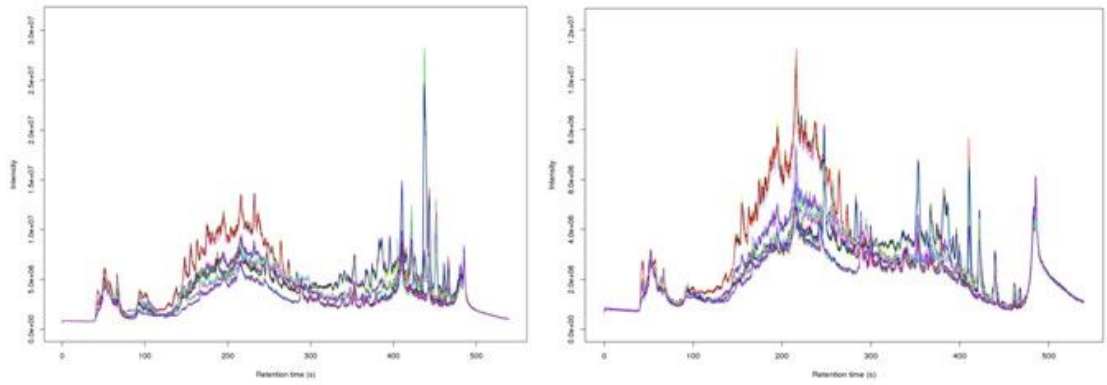

Figure 5

The TIC overlaps map of QC sample

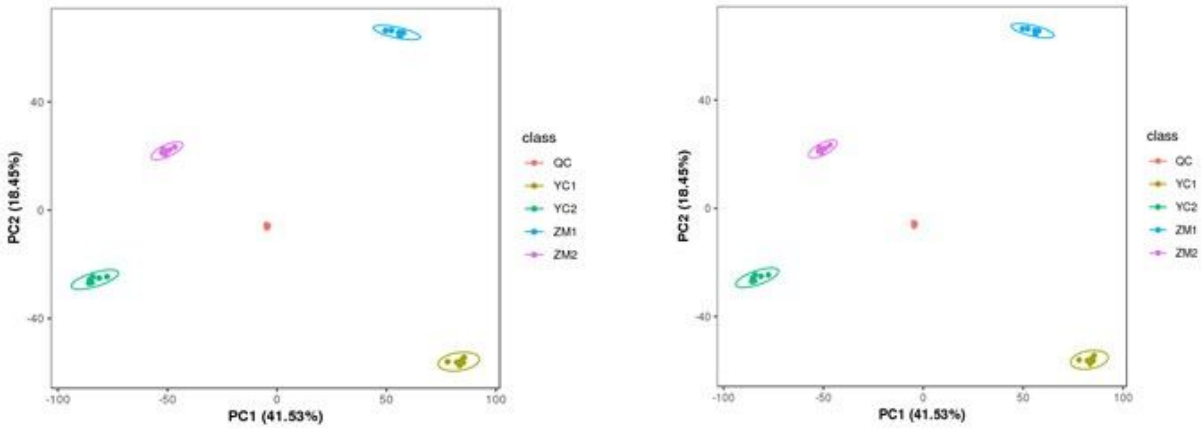

Figure 6

The PC map of total sample