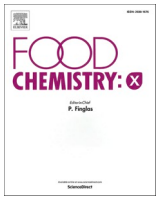
Food Chemistry: X 21 (2024) 101173

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Study on peanut protein oxidation and metabolomics/proteomics analysis of peanut response under hypoxic/re-aeration storage

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ARTICLE INFO

Keywords:
Peanut proteins
Oxidation
Peanut response
Hypoxic/re-aeration storage
Metabolomics
Proteomics

ABSTRACT

To better understand the effect of oxygen levels in the storage environment on peanut protein oxidation and explore the mechanism, the functional properties and the oxidation degree of peanut proteins extracted from peanuts under conventional storage (CS), nitrogen modified atmosphere storage (NS, hypoxic) and re-aeration storage (RS) were investigated. Metabolomics and proteomics were employed to analyze peanut's response to hypoxic/re-aeration storage environment. The results showed that NS retarded the decline of the functional properties and the oxidation of peanut proteins, while the process were accelerated after re-aeration. That was the result of the metabolic changes of peanuts under different storage environments. The omics results presented the decreased (NS)/increased (RS) levels of the antioxidant-related proteins acetaldehyde dehydrogenase and glutathione S-transferase, and the inhibition (NS)/activation (RS) of metabolic pathways such as the TCA cycle and the pentose phosphate pathway. This study provided a reference for the re-aeration storage of other agricultural products.

1. Introduction

Plant protein have been a good substitute for animal protein due to its properties in traceability, environmental protection and quality control reliability (Poore & Nemecek, 2019). As one of the most important sources of plant protein, peanut protein is nutrient-rich and its nutritional value is comparable to that of meat. Moreover, peanut proteins are cholesterol-free and also contains many additional biologically active components (e.g., vitamins and phytosterols) (Ying Chen et al., 2023).

The properties of peanut proteins are significantly affected by the quality of peanuts. Peanuts are highly hygroscopic and susceptible to oxidative deterioration during storage (Yu, Chen, Zhang, Zheng, Jiang, Ji et al., 2021). The oxygen levels in the storage environment could affect the oxidation and deterioration of peanuts, while the quality loss of peanuts could be greatly reduced by storage and transportation under low oxygen environment (Groot, Van Litsenburg, Kodde, Hall, de Vos, & Mumm, 2022). The investigation of Wu showed that nitrogen modified atmosphere storage (NS) could protect the integrity of cell membranes of fresh edible peanut kernels by reducing the accumulation of reactive oxygen species (ROS) and maintaining the high activity of antioxidant enzymes (Wu, Li, Zhang, Tian, Tao, Luo et al., 2022). However, it was not clear about the response of peanuts and the oxidation degree of peanut proteins to re-aeration after NS.

The changes of oxygen level in the storage environment may affect the internal metabolism of peanuts and the oxidation degree of peanut proteins. These impacts could be studied by metabolomics, proteomics and protein oxidization indicators. Metabolomics is a powerful and useful tool for analysis of all the small-molecule metabolites in peanuts (Yu, Chen, Wang, Chen, Liu, Tian et al., 2022). Proteomics is a method of studying metabolic pathways and protein composition (Yuan, Chen, Benjakul, Sun, & Zhang, 2022). Metabolomics studies showed that cinnamic acid and coumaric acid played a significantly positive role in peanut resistance to *Aspergillus flavus* (Wang, Liu, Yin, Wang, Cao, Wang et al., 2023).

In this paper, the response of peanuts and the oxidation degree of peanut proteins in NS and RS were investigated, using conventional storage (CS) as control. The free sulfhydryl content, carbonyl content, conformation and particle size of peanut proteins were determined to explore the oxidation degree. Nitrogen solubility index (NSI), water holding capacity (WHC), oil binding capacity (OBC), emulsifying activity index (EAI), emulsion stability (ES), foaming capacity (FC) and foaming stability (FS) of peanut proteins during peanut storage were also evaluated. Furthermore, metabolomics and proteomics were introduced to study the changes of metabolites and pathways of peanuts

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https://doi.org/10.1016/j.fochx.2024.101173
Received 7 December 2023; Received in revised form 23 January 2024; Accepted 1 February 2024
Available online 6 February 2024

under hypoxic/re-aeration storage.

The aim of this study was to investigate the effects of oxygen levels on the oxidation of peanut proteins and internal metabolism of peanuts. To the best of our knowledge, there have been few studies in combining proteomics and metabolomics to explain the mechanisms of oxidative denaturation of peanut proteins and elucidate the internal metabolism of peanuts, especially re-aeration storage, which contributed to the understanding of peanut quality changes under different storage environments at the molecular level.

Materials and methods

Peanut materials and reagents

Peanut Wanhua 2, harvested from Fangcheng County, Henan Province, China (Latitude 113.0°, and longitude 33.3°). Then the peanuts were dried and shelled, used as raw materials.

Petroleum ether, sodium dodecyl sulfate (SDS), urea and glycine chosen for this experiment were analytical grade reagents; ammonium acetate, acetic acid, acetonitrile, and methanol were chromatographic grade reagents.

Peanut storage

Three storage conditions, CS, NS and RS were used for peanut kernel storage. All samples were incubated in an incubator at 35 ℃ ± 1 ℃ and 65 % ± 5 % relative humidity (RH) for 300 days, and peanut kernels were taken every 60 days for the subsequent experiments.

CS: Peanut kernels were stored in transparent bags made of polyamide and polyethylene (PA/PE). NS: Peanut kernels were stored in PA/PE bags with nitrogen concentration of 98.5 ± 0.4 %. RS: Peanut kernels were first stored in NS for 120 days and then re-aerated in CS for 180 days.

Peanut protein extraction

Peanut isolate protein (PPI) was prepared by isoelectric precipitation method (Gong, Shi, Liu, Liu, Hu, Adhikari et al., 2016). Peanut flour obtained by Soxhlet defatting was mixed with water in the ratio of 1/10 (w/v), and the pH was adjusted to 9.0 using sodium hydroxide (1.0 mol/L). The mixture was shaken in a water bath at 45 ℃ for 45 min, and centrifuged for 10 min (11,000*g*). The supernatant was adjusted with hydrochloric acid to pH 4.5, and centrifuged at 11,000*g* for 10 min. The precipitate was properly dissolved with water, and freeze-dried to obtain peanut isolate protein (PPI).

Characterization of the oxidation degree of peanut proteins

The determination of free sulfhydryl content and carbonyl content

Ellman's reagent was prepared by dissolving 4 mg of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) in 1 mL of Tris-glycine buffer (pH 8.0). 1 mL of Tris-glycine buffer containing 8 mol/L urea was mixed with a PPI solution (2 mg/mL). Then Ellman reagent was added to 50 μL, incubated at room temperature for 30 min, and centrifuged at 8000*g* for 10 min. The absorbance of the supernatant was measured at 412 nm.

The carbonyl content of the PPI was determined by the protein carbonyl content assay kit (Beijing Solebao Technology Co., Ltd.). The principle was that the carbonyl group could react with 2, 4-dinitrophenylhydrazine to form 2,4-dinitrobenzene with a characteristic absorption at 370 nm.

Endogenous fluorescence spectroscopy

0.1 mg/mL PPI solution (pH 7.0, 10 mmol/L PBS buffer) was determined by the method of Wang et al. (Wang, Li, Liu, Liu, Tan, Guo, Wang et al., 2022). The fluorescence emission ranges of the PPI solution were observed by fluorescence spectroscopy (Hitachi F-7100 Fluorescence Spectrophotometer, Hitachi, Japan) with an excitation wavelength of 290 nm, and scanning range from 320 to 400 nm, determined in triplicate.

Particle size of peanut proteins

According to the approach of Liu, Liu, and Zhao (2022), the PPI above was dissolved in phosphate buffer to a concentration of 2 %, stirred at room temperature for 1 h, and centrifuged at 10,000*g* for 15 min before collecting the supernatant for determination. The average particle size was determined by using a Malvern static light scattering instrument (ZS 90, Malvern Company, USA). The data analysis was performed by Malvern Zetasizer software.

Functional properties of peanut proteins

WHC and OBC of peanut proteins were determined referred to Saptashish (Saptashish, Yogesh, & Saxena, 2022). The water solubility, emulsifying and foaming properties of peanut proteins were evaluated by the method of Qu (Qu, Wang, Wang, Yu, & Wang, 2020).

Metabolomics experiment

25 mg of sample was weighed, and 500 μL extract solution (methanol:water = 3:1, with isotopically-labelled internal standard mixture) was added. Then the samples were homogenized at 35 Hz for 4 min and sonicated for 5 min in an ice-water bath. The homogenization and sonication cycle were repeated 3 times. Then the samples were incubated for 1 h at −40 ℃ and centrifuged at 12,000 g for 15 min at 4 ℃. The resulting supernatant was transferred to a fresh glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all the samples.

LC-MS/MS analysis were performed using an UPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo). The mobile phase consisted of 5 mmol/L ammonium acetate and 5 mmol/L acetic acid in water (A) and acetonitrile (B). The auto-sampler temperature was 4 ℃, and the injection volume was 2 μL. The Orbitrap Exploris 120 mass spectrometer was used to acquire MS/MS spectra in information-dependent acquisition (IDA) mode under the control of the acquisition software (Xcalibur, Thermo). The ESI source conditions were set as follows: sheath gas flow rate as 50 Arb, aux gas flow rate as 15 Arb, capillary temperature 320 ℃, full MS resolution as 60,000, MS/MS resolution as 15,000, collision energy as 10/30/60 in NCE mode, spray voltage as 3.8 kV (positive) or −3.4 kV (negative), respectively.

The raw data were converted to the mzXML format using ProteoWizard and processed for peak detection, extraction, alignment, and integration. MS2 database (Biotree DB) was applied in metabolite annotation.

TMT quantitative proteomics experiment

Protein extraction, digestion and LC-MS/MS analysis were referred to Ma' study (Ma, Ling, Su, Jiang, Nian, Chen et al., 2021).

Peanut proteins were extracted by Tris-HCl buffer and precipitated by ethanol solution of phenol. Then the mixture was incubated overnight at −20℃ and centrifugated at 12,000*g* for 15 min at 4 ℃. The protein pellets were washed with cold acetone, air-dried and solubilized with lysis buffer (8 mol L^{-1} urea, 20 mmol L^{-1} Tris-HCl, pH 8.5). The supernatants were collected for later use.

Peanut proteins (200 μg) were digested with trypsin according to the Filter Aided Sample Preparation protocol. For each sample, 2 μL of total peptides were separated and analyzed with a nano UPLC (EASY-nLC1200) coupled with a reversed-phase column (100 μm ID × 15 cm, Reprosil Pur 120 C18 AQ, 1.9 μm, Dr. Maisch). Mobile phases were H_2O with 0.1 % formic acid, 2 % acetonitrile (phase A) and 80 % acetonitrile, 0.1 % formic acid (phase B). Separation of sample was executed with a

90 min gradient at 300 nL/min flow rate. Gradient B: 2-5% for 2 min, 5–22 % for 68 min, 22–45 % for 16 min, 45–95 % for 2 min, 95 % for 2 min.

The separated substances were detected by a Q Exactive HFX Orbitrap instrument (Thermo Fisher Scientific) with a nano-electrospray ion source. Data dependent acquisition (DDA) was performed in profile and positive mode with Orbitrap analyzer for MS1. For MS2, the resolution was set to 45 k with a fixed first mass of 110 *m/z*. The top 20 most intense ions were fragmented with collision energy of 32 %, and isolation window of 0.7 *m/z*. The dynamic exclusion time window was 45 s, single charged peaks and peaks with charge exceeding 6 were excluded from the DDA procedure. Vendor's raw MS files were processed using Proteome Discoverer (PD) software (Version 2.4.0.305) and the built-in Sequest HT search engine.

Statistical analysis

The significance analysis of peanut oxidation indicators was carried out by SPSS (version 20.0, SPSS Inc., USA). ANOVA analysis with the Duncan's multiple range test was used to differentiate the significant differences at $p < 0.05$. Origin (version 9.1, Origin Lab, USA) was employed for chart drawing.

The MA website (https://www.metaboanalyst.ca/) was used to statistically analyze raw data on metabolites and proteins and to find significantly different substances. Significantly different metabolites were searched and annotated by database in Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) to analyze differences between KEGG metabolic pathways. Significantly different proteins were functionally annotated through the NCBI database (https://www.ncbi.nlm.nih.gov/), and protein metabolic pathways and KOG analysis were performed through the DAVID (https://david.ncifcrf.gov/summary.jsp) website. And all significantly different proteins were name-matched using the unipot database (https://www.uniprot.org/).

Results

Effects of different storage conditions on the oxidation of peanut proteins

The change of oxygen levels in the storage environment may lead to the oxidation of peanut proteins to different degrees. Fig. 1(a) and (b) showed the changes of the free sulfhydryl content and carbonyl content in peanut proteins. The results showed that the free sulfhydryl content decreased and the carbonyl content increased with time, indicating that peanut proteins were oxidized during storage. It could be seen from Fig. 1(a) and (b) that NS could retard the oxidation of peanut proteins. After re-aeration, the oxidation increased rapidly, and the oxidation degree of the peanut proteins was comparable to CS at the end of storage. Fig. 1(c) showed the changes in endogenous fluorescence spectra of peanut proteins extracted from peanuts under different storage conditions after 10 months of storage. The maximum emission wavelength of peanut proteins varied from 344 nm in the beginning to 335 nm in NS, 335 nm in CS and 333 nm in RS at the end of the storage. In addition, the fluorescence intensities of peanut proteins were reduced from 637.5 AU (0 day) to 550.8 AU in NS, 527.8 AU in CS and 487.2 AU in RS. Fig. 1 (d) demonstrated the particle size variation of peanut proteins during storage. The particle size of peanut proteins tended to increase, indicating that peanut protein aggregation occurred during storage. Similarly, NS slowed the oxidative aggregation of peanut proteins, and re-aeration accelerated the aggregation of peanut proteins.

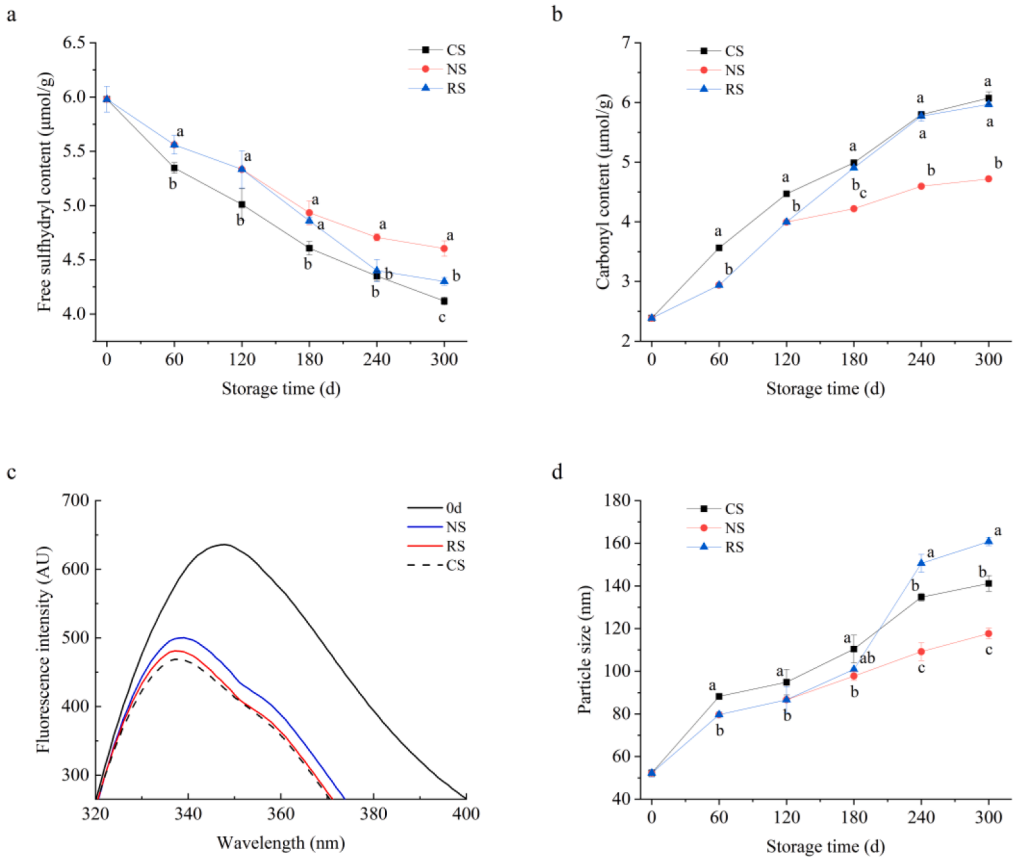

Fig. 1. Changes of the (a) free sulfhydryl content, (b) carbonyl content, (c) endogenous fluorescence spectra and (d) particle size of peanut proteins during peanut kernels' storage. CS denoted conventional storage, NS denoted nitrogen modified atmosphere storage, RS denoted re-aeration storage, The different letter in each storage method indicates significant difference ($p < 0.05$).

Effects of different storage conditions on functional properties of peanut proteins

Fig. 2 displayed the changes of the NSI, WHC, OBC, EAI, ES, FC and FS for peanut proteins extracted from peanuts under different storage conditions.

Protein solubility could characterize the denaturation of peanut proteins, usually exhibited by NSI. Fig. 2(a) presented the variation of NSI of peanut proteins extracted from peanuts during storage under different storage conditions. The data showed that after 10 months of storage, the peanut protein solubility decreased from 140.7 ± 1.2 mg N/100 g to 121.0 ± 0.8 mg N/100 g, 134.0 ± 0.8 mg N/100 g, and 126.0 ± 0.8 mg N/100 g in CS, NS, and RS, respectively.

The changes of WHC and OBC of peanut proteins were shown in Fig. 2(b) and (c). The decreasing rate of WHC and the increasing rate of OBC under CS were always greater than that under NS along with peanut storage. However, the change rate of the two functional properties was accelerated after re-aeration.

The emulsification properties of the peanut proteins were characterized by EAI and ES (Fig. 2d and e). It could be seen from Fig. 2(d) and (e) that the EAI and ES both decreased along with peanut storage in the three storage conditions. Similarly, NS was more effective in maintaining the emulsification properties of peanut proteins. And the emulsification properties of peanut proteins decreased rapidly after re-aeration.

FA and FS were two important interfacial properties of protein. The changes of FC and FS during storage could be referred to Fig. 2(f) and (g). They all showed decreasing trends under different storage conditions. NS could retard the decrease of foaming properties, while RS accelerated it.

Overview of differential metabolites

A total of 2258 metabolites were identified in peanuts. These substances were subject to further statistical analysis to screen the differential metabolites. Principal component analysis (PCA) was first used to analyze the data. PCA was a statistical method that converted a potentially relevant set of variables into a linearly uncorrelated set of variables by orthogonal transformation, and the converted set of variables was called principal components. The PCA results were shown in Fig. 3(a), where the horizontal and vertical coordinates PC[1] and PC[2] indicated the first and second principal components, respectively. Each scatter in Fig. 3(a) represented one sample, and the colors of the scatters referred to different sample groups.

Since PCA was an unsupervised classification model, the data could be affected by variables unrelated to the grouping information, resulting in the differences between samples from different groups that were not better emphasized. Therefore, a supervised statistical method of discriminant analysis was introduced, orthogonal partial least squares discriminant analysis (OPLS-DA). The results of the OPLS-DA were shown in Fig. 3(b and c), where the horizontal coordinate t[1]P denoted the predicted principal component scores of the first principal component, demonstrating the differences between sample groups, and the vertical coordinate t[1]O denoted the orthogonal principal component scores, representing the differences within sample groups. As could be seen from Fig. 3(b and c), the samples were all within the 95 % confidence interval.

Based on the above analysis, the variable importance in the projection (VIP) > 1 (the first principal component variable of the OPLS-DA model) and $p < 0.05$ (Student's t test) were adopted as the screening criteria for significantly different metabolites. The screened significantly differential metabolites were displayed in the volcano plots Fig. 3. Red, blue and gray in Fig. 3(d and e) indicated significantly up-regulated, greatly down-regulated and non-significantly differential metabolites, respectively. After screening, 447 significantly differentiated metabolites were identified within the NS vs. CS, with 266 substances up-regulated and 184 metabolites down-regulated. In the RS vs. CS, 592 differential metabolites were identified, with 391 up-regulated metabolites and 201 down-regulated metabolites (Table S1).

Differentially expressed proteins analysis

The results of the protein assays were also analyzed by PCA and OPLS-DA, and the results were presented in Fig. 4(a–c).

To better understand the mechanism of peanut quality changing under different storage conditions and the conversion relationships between metabolites, a proteomic analysis was performed by LC-MS/MS. In group NS vs. CS, 180 differentially expressed proteins ($p < 0.05$) were identified, of which 112 were highly expressed proteins and 68 were lowly expressed proteins. A total of 42 differentially expressed proteins were quantified in group RS vs. CS, with 37 highly expressed proteins and 5 lowly expressed proteins (Table S2). Differentially expressed proteins of peanuts in different storage conditions were more intuitively illustrated by volcano plots (shown in Fig. 3d and e).

Eukaryotic Clusters of Orthologous Groups (KOG) was a database that categorized proteins into orthologous groups. The proteins in each KOG were derived from an ancestral protein and represented either orthologs or paralogs. The detected proteins in our study were compared to the KOG database, and probable functions were anticipated by using a functional categorization statistical analysis. Fig. 5(a) and (b) showed the results of the KOG analysis, where the vertical axis represented the number of proteins and the horizontal axis represented the annotated KOG entries. The differential proteins had a higher concentration of KOG annotations in posttranslational modifications, protein turnover, chaperones; translation, ribosomal structure and biogenesis; general function prediction only etc. for NS vs. CS, and posttranslational modifications, protein turnover, chaperones for RS vs. CS.

Overview of differential metabolic pathways

To gain a further understanding of the mechanism of peanut quality changing under different storage conditions, differential metabolites (VIP > 1 and $p < 0.05$) in each group were mapped into KEGG database. The results of differential metabolic pathways were summarized in Table S3. The metabolic pathway analysis was illustrated in the bubble diagrams (shown in Fig. S1), where each bubble represented a metabolic pathway. Fig. S1 suggested that the differential metabolic pathways in NS vs. CS and RS vs. CS were mainly carbohydrate metabolism and amino acid metabolism. However, the pathways in different groups were not exactly the same. In NS vs. CS, differential metabolites were enriched to 11 differential metabolic pathways including pentose phosphate pathway; galactose metabolism; arginine biosynthesis and alanine, aspartate and glutamate metabolism. In RS vs. CS, differential metabolites were enriched to 14 differential metabolic pathways including glycolysis/gluconeogenesis; valine, leucine and isoleucine degradation; pyruvate metabolism; propanoate metabolism; one carbon pool by folate; aminoacyl-tRNA biosynthesis and fatty acid biosynthesis.

Differential Abundance Score (DA score) demonstrated the overall variation of differential metabolites in metabolic pathways. The results obtained were visualized by using DA score plots (Fig. 5c and d).

The transformational relationships between metabolites and the roles of enzymes in the metabolic pathways were visualized in Fig. 6.

Discussion

Effects of different storage conditions on the oxidation and functional properties of peanut proteins

NS was an efficient method for grain storage, which can slow down the deterioration of grain quality. It was reported that NS could retard rice deterioration, protect rice cell from oxidative damage, and maintain cellular integrity and stability (Qu, Li, Yang, Xia, Lu, & Hu, 2022). Huang et al. demonstrated that NS could maintain the content of

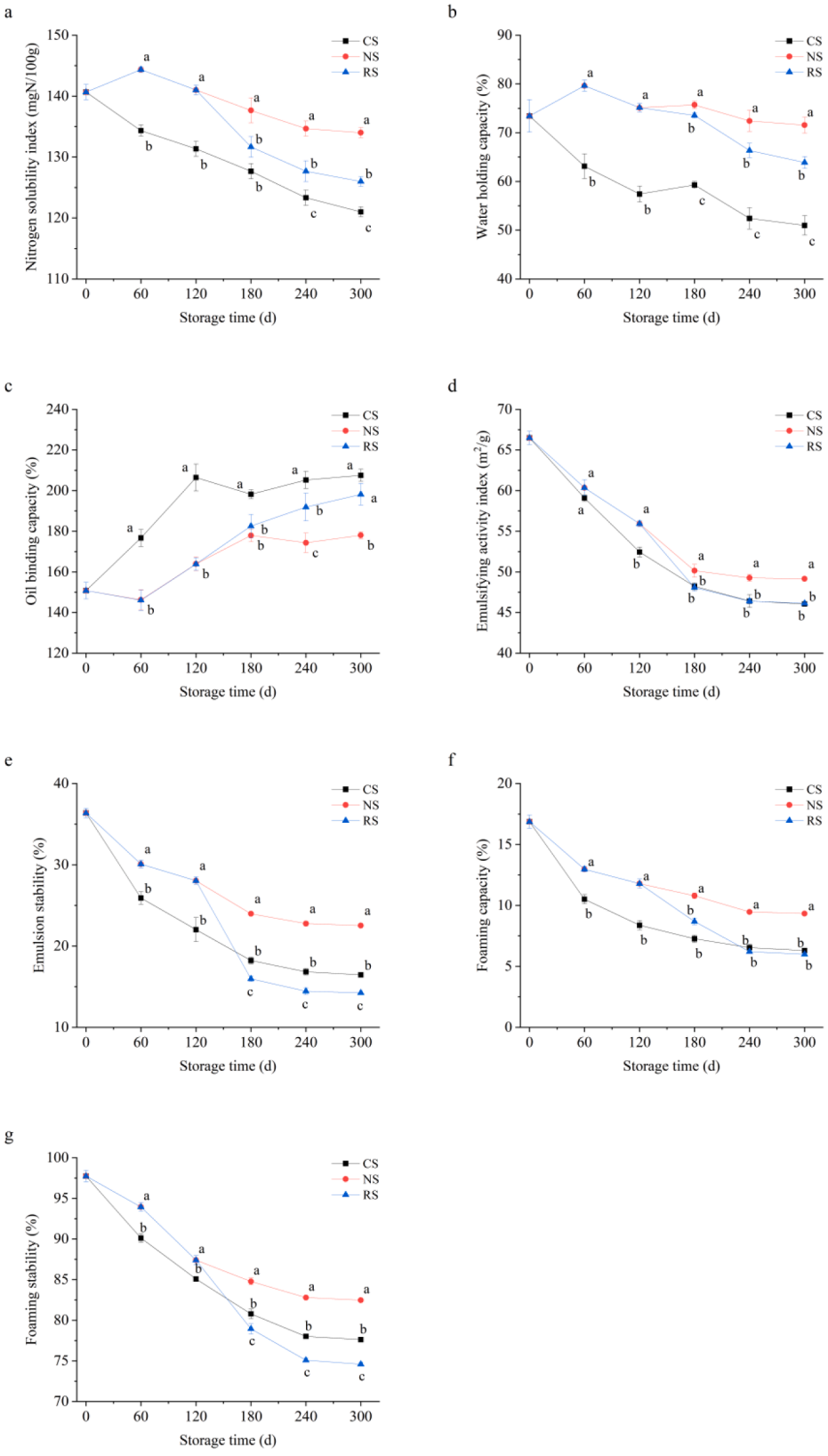

Fig. 2. The changes of (a) nitrogen solubility index, (b) water holding capacity, (c) oil binding capacity, (d, e) emulsifying properties and (f, g) foaming properties of peanut proteins during peanut kernels' storage. CS denoted conventional storage, NS denoted nitrogen modified atmosphere storage, RS denoted re-aeration storage, The different letter in each storage method indicates significant difference ($p < 0.05$).

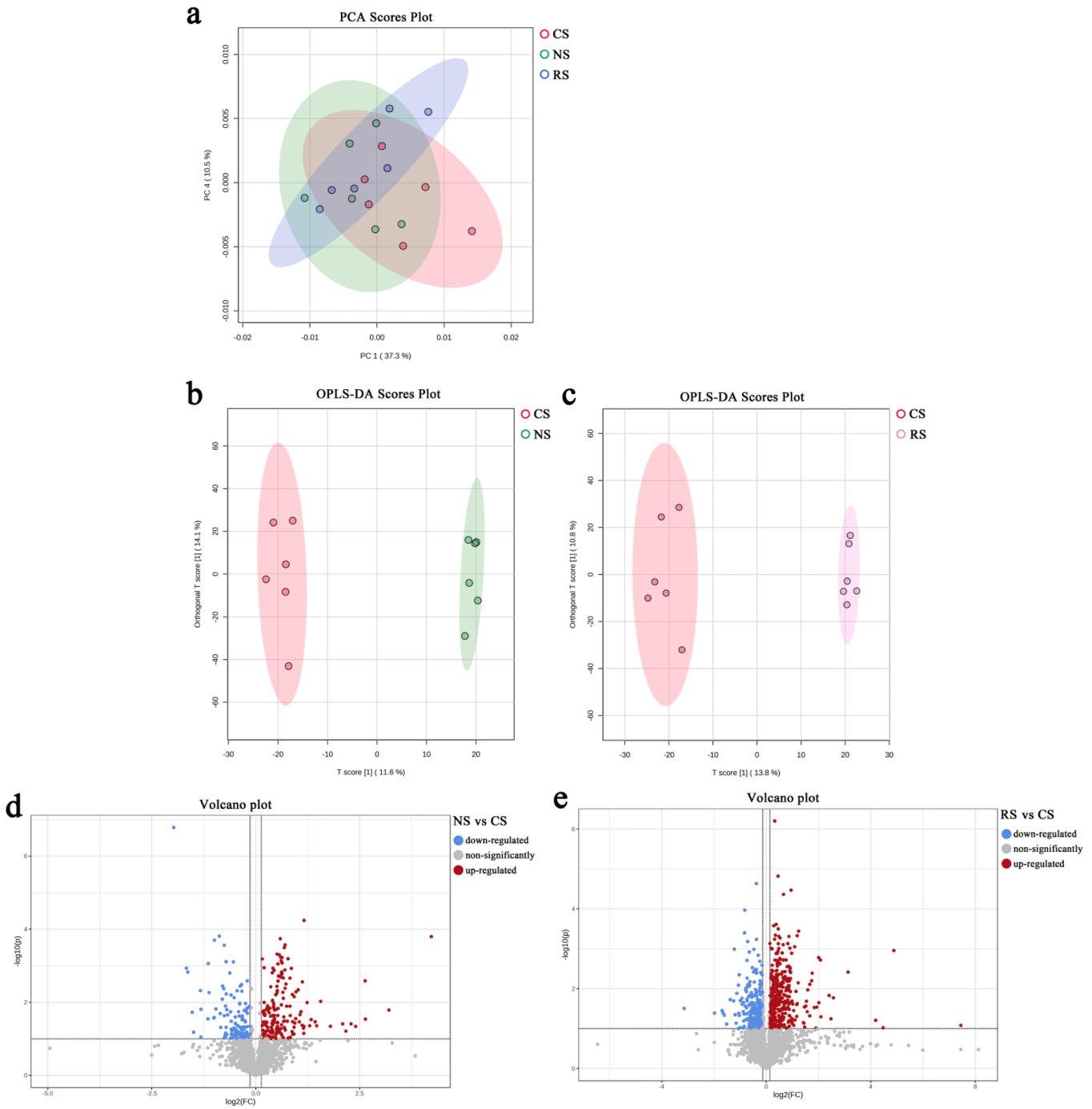

Fig. 3. (a) The PCA score plot of metabolomics. *Each scatter referred to one determination. Different scatter shapes and colors indicated different groups. And the larger the interval between sample points or group circles, the greater the difference in overall metabolic level. (b and c) The OPLS-DA score plot of metabolomics in NS vs. CS and RS vs. CS. *Each dot represented one determination, scatter shapes and colors indicated different group. (d and e) The volcano plot of metabolomics in NS vs. CS and RS vs. CS. *Each dot represented one identified metabolite. The significantly up-regulated metabolites, down-regulated metabolites and non-significantly regulated metabolites were displayed in red, blue and gray, respectively. CS denoted conventional storage, NS denoted nitrogen modified atmosphere storage, RS denoted re-aeration storage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phytosterols (campesterol, stigmasterol, beta-sitosterol) and phenolic compounds (gallic acid, beta-coumaric acid, ferulic acid, caffeic acid, syringic acid, catechin, quercetin) in brown rice (Huang, Belwal, Li, Wang, Aalim, & Luo, 2020). However, there were few reports on quality changes of agricultural products under RS conditions. Research showed that apple fruit quality was negatively affected by RS, particularly fruit firmness and flavor (Wood, Thewes, Reynaud, Kittemann, Sautter, Wünsche et al., 2022). Our research on peanuts also illustrated that RS accelerated the oxidation of peanut proteins. These results could provide a reference for RS of other agricultural products.

The free sulfhydryl content and carbonyl content were good indicators of peanut protein oxidation level. The sulfhydryl of proteins could be oxidized to disulfide bonds, causing the destruction of protein space structure. Carbonylation was an irreducible and non-enzymatic modification of proteins and was often used to determine the degree of oxidation in proteins (He, Wu, Li, Zheng, Tian, Jiang et al., 2021). The

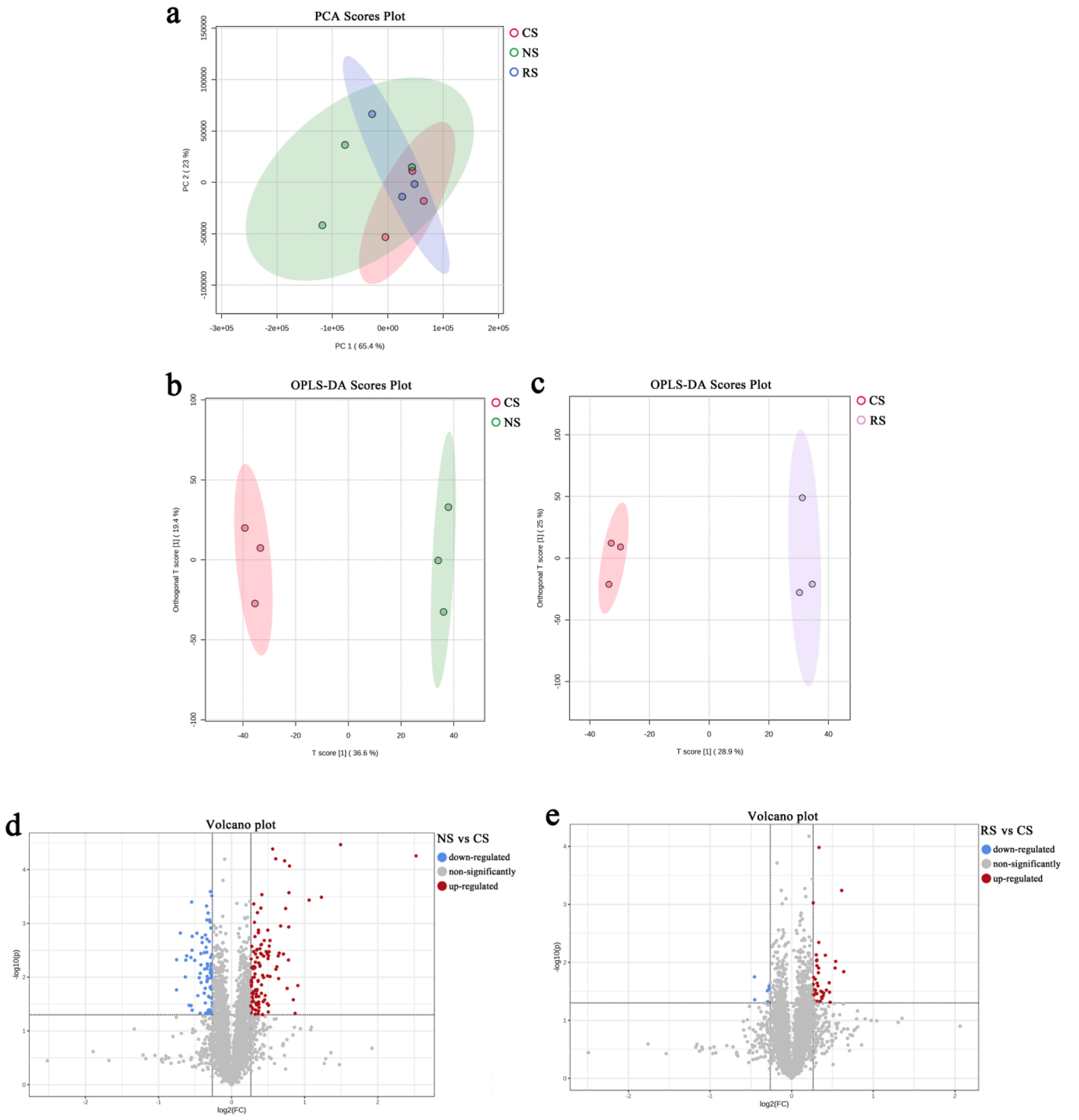

Fig. 4. (a) The PCA score plot of proteomics. *Each scatter referred to one determination. Different scatter shapes and colors indicated different groups. And the larger the interval between sample points or group circles, the greater the difference in overall metabolic level. (b and c) The OPLS-DA score plot of proteomics in NS vs. CS and RS vs. CS. *Each dot represented one determination, scatter shapes and colors indicated different group. (d and e) The volcano plot of proteomics in NS vs. CS and RS vs. CS. *Each dot represented one identified metabolite. The significantly up-regulated metabolites, down-regulated metabolites and non-significantly regulated metabolites were displayed in red, blue and gray, respectively. CS denoted conventional storage, NS denoted nitrogen modified atmosphere storage, RS denoted re-aeration storage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher the carbonyl content, the higher the degree of protein oxidation. In our study, less oxidation of peanut proteins in NS was observed, which was due to the hypoxic condition that slowed down the oxidation rate of the peanut proteins. However, the free sulfhydryl content decreased and carbonyl content increased rapidly after re-aeration. These results suggested that after re-aeration, peanut metabolism was changed due to elevated oxygen levels in the storage environment, leading to an accelerated oxidation rate.

The endogenous fluorescence of proteins originated from the emission of tryptophan residues was commonly used to indicate changes in the conformation of proteins (Guo, Qiu, Deng, Mao, Guo, Xu et al., 2019). Fig. 1(c) could help us understand more intuitively the effect of different storage conditions on the conformation transformation of peanut proteins. Along with storage, the fluorescence intensity

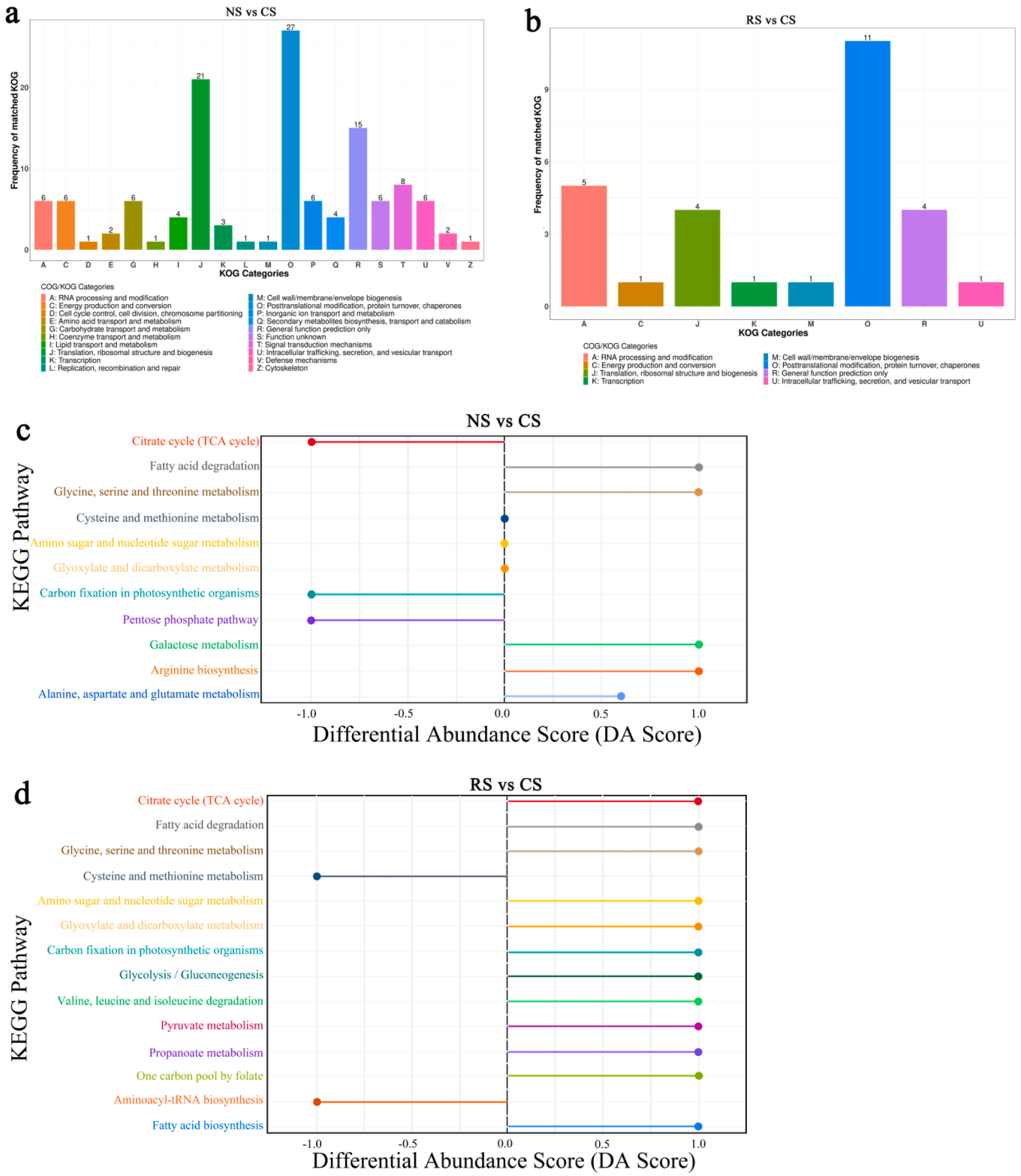

Fig. 5. (a and b) The enrichment analysis of Eukaryotic Clusters of Orthologous Groups (KOG) classes of proteins in peanuts under different storage conditions. * The bars represent the number of proteins per KOG category (y-axis) found in proteomics. (c and d) Differential abundance score of differential metabolic pathways in NS vs. CS and RS vs. CS. *The differential abundance score reflects the average total change in all metabolites in a pathway. A score of 1 indicates that all metabolites measured in the pathway have increased, and −1 indicates that all metabolites measured in the pathway have decreased.

decreased and the maximum emission wavelength displayed a blue-shift. Compared to the three storage conditions, NS had less effect on the conformation of peanut proteins than CS, whereas RS performed the worst. This suggested that the oxidation of the peanut proteins led to the micro-environment alteration of tryptophan residues.

The particle size of proteins could reflect the aggregation degree of proteins caused by oxidation. Cao et al. observed that oxidation of proteins caused an increase in particle size (Cao, Ma, Huang, & Xiong,

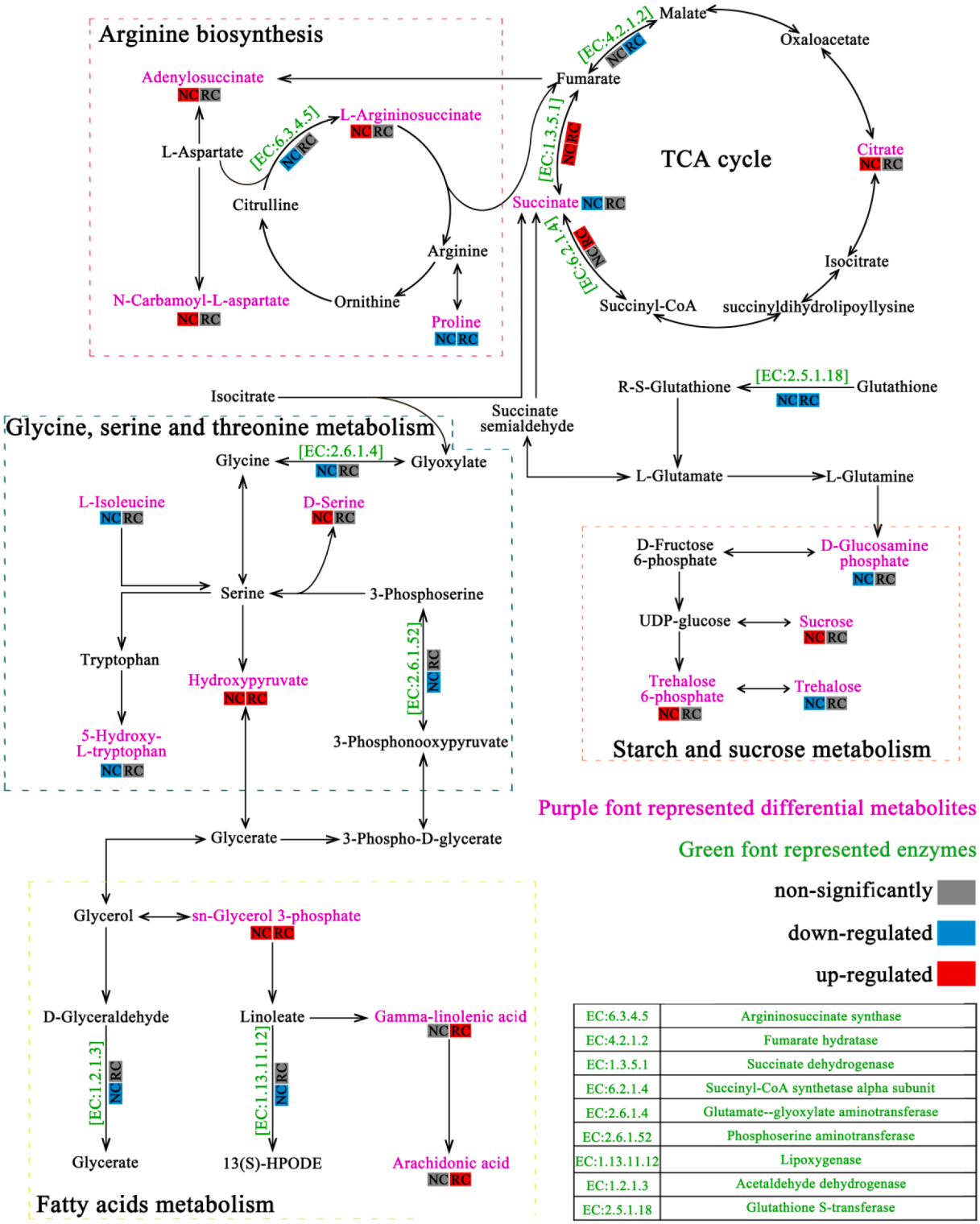

Fig. 6. The changes of metabolites and proteins in metabolic pathways of peanuts under different storage conditions. *Red plots, blue plots, and gray plots represent significant up-regulation, down-regulation, and no significant difference in a comparison group, respectively. Purple font represented differential metabolites, Green font represented enzymes, CS denoted conventional storage, NS denoted nitrogen modified atmosphere storage, RS denoted re-aeration storage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2020). In our study, the particle size of peanut proteins increased rapidly after reaeration, suggesting that peanuts in RS encountered more intense oxidative reactions.

Changes in the spatial structure and particle size of peanut proteins due to oxidation directly affected the functional properties of peanut proteins. It was reported that the dissolution rate of proteins in aqueous solutions would decrease by their larger particle size (Meena, Singh, Gupta, Borad, & Parmar, 2018). And oxidation-induced peanut protein denaturation could lead to the exposure of hydrophobic groups, and result in the increased WHC and decreased OBC. The results of Wang et al. indicated that changes in protein conformation induced by sonication reduced protein emulsification (Wang, Li, Guo, Liu, Liu, Tan

et al., 2022). Ma et al. suggested that higher free sulfhydryl content may help to stabilize the emulsion and improve the emulsifying properties of the protein (Ma, Xie, Zhang, Wang, Hu, Sun et al., 2018). Shi et al. noted that smaller protein sizes facilitated protein dispersion in emulsions (Shi, Zou, Sun, Lu, Zhang, Gao et al., 2019). In addition, there was a strong correlation between the foaming properties of proteins and their solubility, while solubility was influenced by protein particle size, suggesting that an increase in particle size due to protein aggregation triggered a decrease in foaming performance.

In our study, the oxidation of peanut proteins resulted in the alteration of the protein spatial structure, thereby leading to the aggregation of peanut proteins and affecting changes in the functional properties of peanut proteins. It could be seen from the results that the functional properties of peanut proteins declined along with the storage regardless of storage conditions. The functional properties of peanut proteins were better maintained under NS than CS. Interestingly, the functional properties of peanut proteins declined rapidly after re-aeration, showing the effect of oxygen level changes on the functional properties of peanut proteins.

Metabolomics analysis

Carbohydrate metabolism

The tricarboxylic acid cycle (TCA cycle) involved in plant growth and provided the carbon skeleton for the biosynthesis of some amino acids. Succinate was one of the key intermediates within the TCA cycle. Such intermediates of TCA had been proved to enhance plant stress tolerance (Xu, Chen, Wang, Jia, & Xu, 2020). It was reported that hypoxia stress made the TCA cycle of plants limited (Wang, Han, Luo, Rong, Song, Jiang et al., 2022). In our study, the results showed that succinate was significantly down-regulated in peanuts under NS vs. CS, with no significant difference in RS vs. CS. This suggested that the TCA cycle was inhibited and succinate synthesis was reduced in NS, and that the TCA cycle was activated to synthesize succinate when peanuts were re-exposed to oxygen in RS.

In the starch and sucrose metabolism, sucrose produced trehalose 6-phosphate (T6P), which was a precursor substance of trehalose. Sucrose, as a major energy substrate, acting as a signal regulator, was involved in a wide range of biological processes. Sucrose could enhance plant tolerance and sensitivity to abiotic stresses. T6P acted as a signaling molecule regulating carbon metabolism, photosynthesis and homeostasis. It also engaged in plant-pathogen interactions and cell wall modification as a stress protector (Li, Cui, Dai, Liu, Cheng, & Luo, 2021). Higher levels of T6P inhibited hexokinase activity and regulated glycolytic pathways (Wang, Wang, Pan, Sun, Chen, Chen et al., 2019). The results of our study revealed that in the starch and sucrose metabolic pathway, sucrose and T6P were up-regulated and trehalose was down-regulated in NS vs. CS, and there were no significant differences in sucrose, T6P and trehalose in RS vs. CS. This may be due to that NS (hypoxic) impeded the synthesis of trehalose, leading to the accumulation of sucrose and T6P. It implied that the starch and sucrose metabolism was down-regulated in NS vs. CS. While in RS vs. CS, peanut consumed sucrose and T6P to enhance tolerance to oxidative stress.

Amino acid metabolism

Proline was the product of the glutamate synthesis pathway and the ornithine synthesis pathway, which was commonly used as an osmotic regulator and a scavenger of ROS in plant (Chen, Xie, Wei, Guo, Zhang, Lu et al., 2022). Yang et al. reported that plants up-regulated the glutamate synthesis pathway to produce proline when faced with abiotic stresses (Yang, Zhao, & Liu, 2020). In our study, proline was significantly down-regulated in NS vs. CS (the ratio was 1.00:1.72) and RS vs. CS (the ratio was 1.00:1.51), and proline was less in NS than in RS (the ratio was 1.00:1.14). We hypothesized that peanuts underwent oxidation under CS condition and the oxidative stress induced up-regulation of proline to resist environmental stress, the same trend was exhibited by proline under RS conditions.

L-isoleucine was shown significantly down-regulated in NS vs. CS and no significant difference in RS vs. CS. Llanes et al. elaborated that isoleucine could improve salt resistance, and maintain metabolic and osmotic homeostasis in plants under stress (Llanes, Arbona, Gómez-Cadenas, & Luna, 2016). The protein oxidation index (free sulfhydryl content, carbonyl content, etc.) indicated that RS and CS possessed the same level of oxidation at the storage endpoint, which was consistent with our findings that peanut up-regulated L-isoleucine to maintain normal internal metabolism under oxidative stress.

In aromatic amino acid metabolism, serine was the precursor substance of D-serine and tryptophan. Tryptophan could then synthesize 5-hydroxy-L-tryptophan (Fig. 6). In our research, D-serine accumulation was found in peanuts under NS. And 5-hydroxy-L-tryptophan was significantly down-regulated in NS vs. CS. This suggested that NS allowed for more conversion of serine to D-serine, resulting in less 5-hydroxy-L-tryptophan being produced. In contrast, there was no significant difference between D-serine and 5-hydroxy-L-tryptophan in RS vs. CS, which may indicate that the oxygen levels in the environment affected the conversion between aromatic amino acids.

Fatty acids metabolism

Unsaturated fatty acids played a protective role in seed longevity and viability by means of oxidation (Ma, Zhu, Li, Zhang, Li, Dong et al., 2015). Notably, significant upregulation of unsaturated fatty acids such as arachidonic acid (in the arachidonic acid metabolism pathway) and gamma-linolenic acid (in the linoleic acid metabolism pathway) was detected in the present study in the RS vs. CS, and no significant difference of the two substances was observed in NS vs. CS. This could imply that re-exposure to the air environment was associated with more intense oxidative reactions. And RS stimulated the peanuts to increase their unsaturated fatty acid contents so as to counteract the detrimental effects of increased oxygen levels.

Respiratory metabolism

The major pathways of respiratory metabolism included the tricarboxylic acid cycle (TCA cycle), the pentose phosphate pathway (PPP), and the glycolysis/gluconeogenesis (Cai, Yang, Liao, Song, & Zhang, 2021). Aerobic respiratory metabolism was frequently accompanied by the production of ROS, which were used to regulate intracellular redox homeostasis. Sun et al. noted that excessive oxidation destroyed the structure of proteins and promoted protein carbonylation and degradation of protein sulfhydryl groups, thereby altering their functional properties such as reducing protein solubility and emulsification properties (Sun, Wu, & Mao, 2022). In the present experiment, we found that the TCA cycle and PPP of peanuts were inhibited in NS vs. CS, whereas the TCA cycle and glycolysis/gluconeogenesis of peanuts were significantly up-regulated in RS vs. CS (Fig. 5c and d). This suggested that aerobic respiratory metabolism of peanut was suppressed during NS, thus reducing the accumulation of ROS and avoiding the alteration of spatial structure and functional properties of peanut proteins. After re-aeration, aerobic respiration was active again due to the sudden increase in oxygen levels, resulting in rapid oxidation, aggregation and alteration of the functional properties of peanut proteins. In summary, under different storage conditions (oxygen level), TCA and PPP regulated the degree of peanut oxidation, thus affecting the spatial structure and functional properties of peanut proteins.

Proteomics analysis

Lipoxygenase

Lipoxygenase (EC:1.13.11.12) is an enzyme closely related to biotic and abiotic stress resistance in plants. It was reported that lipoxygenase played an important role in lipid metabolism to help plants resist osmotic stress (Zhao, Xu, Liu, Li, Zhao, Liu et al., 2020). Chang et al. found that pumpkin up-regulated the gene encoding cryo-protective

compounds, such as lipoxygenase, during cold stress (Li, Chang, Zheng, Dong, Liu, Yang et al., 2017). These articles indicated that plants synthesize more lipoxygenase when faced with the environmental stresses. Our results showed that lipoxygenase was significantly down-regulated in peanuts in NS vs. CS, while no significant difference in RS vs. CS. The results suggested that peanuts in NS confronted less oxidative stress than CS, leading to the down-regulation of lipoxygenase. However, the intense oxidative reaction occurred after re-aeration, resulting in up-regulation of the synthesis pathway of lipoxygenase. Ultimately, there was no significant difference in lipoxygenase in RS vs. CS.

Aldehyde dehydrogenase

Acetaldehyde dehydrogenase (EC:1.2.1.3, NAD^+) was expressed when plants were under stress and it helped the plants to overcome the environmental stress and survive (Zeng, Peng, Zhao, Wu, Chen, Ren et al., 2019). It could catalyze the oxidative dehydrogenation of aldehydes to reduce the accumulation of aldehydes, which occurred in membrane peroxidation, helping to reduce toxicity in plant cells. Up-regulation of acetaldehyde dehydrogenase in citrus fruit in response to cold stress had been reported (Yun, Jin, Ding, Wang, Gao, Pan et al., 2012).

In our study, acetaldehyde dehydrogenase in peanuts for NS vs. CS and RS vs. CS showed low expression and no significant difference, respectively. Therefore, acetaldehyde dehydrogenase was up-regulated in the oxygen-enriched environments. It also indicated that peanut cells increased the expression of acetaldehyde dehydrogenase in response to oxidative damage. This result suggested that at the end of storage, peanut proteins were oxidized to a higher extent in RS and CS than in NS, thereby affecting the functional properties of peanuts and stimulating an increase in acetaldehyde dehydrogenase levels in peanuts. Metabolomics results showed that peanut respiratory metabolism was inhibited in NS vs. CS and reactivated in RS vs. CS, and the combined results of proteomics and metabolomics suggested that respiration was inhibited rather than disrupted in NS peanuts.

Heat shock proteins are a group of emergency proteins associated with stress response and signaling in plants. Overexpressed heat shock proteins could enhance plant resistance to adverse environmental stresses (Mishra, Shteinberg, Shkolnik, Anfoka, Czosnek, & Gorovits, 2022). Furthermore, the production of heat shock proteins also was accompanied by some biological processes, such as embryonic development, germination and plant growth (Aldubai, Alsadon, Migdadi, Alghamdi, Al-Faifi, & Afzal, 2022).

In our study, 17.5 kDa class I heat shock protein was detected in peanuts under all three different storage conditions. Compared to CS, 17.5 kDa class I heat shock proteins were lowly-expressed in NS and RS, suggesting that peanuts in CS were subjected to a more intense oxidative response, which in turn stimulated the synthesis of more heat shock proteins, while peanuts in RS underwent an intense oxidative response due to their re-exposure to the oxygen environment, stimulating the elevation of the level of 17.5 kDa class I heat shock proteins.

Glutathione S-transferase

Glutathione S-transferase (EC:2.5.1.18) was a crucial protein participating in ROS scavenging in plants. It was proven to deal with the adverse effects of oxidative stress (Choudhury, Rivero, Blumwald, & Mittler, 2017). Kruasuwan et al. reported that glutathione S-transferase was a signaling molecule for salt stress in rice plant (Kruasuwan, Lohmaneeratana, Munnoch, Vongsangnak, Jantrasuriyarat, Hoskisson et al., 2023). The expression of glutathione S-transferase was found to be significantly lowly expressed in peanuts for NS and RS compared with CS. The free sulfhydryl content, carbonyl content, etc. indicated that peanut in RS was less oxidized than in CS and when exposed to oxygen for a shorter period of time, it would have less accumulation of ROS. In contrast, long-term exposure of peanut in CS to oxidative stress caused a large accumulation of ROS and activation of the self-defense system to mitigate the deleterious effects of oxidation. This could indicate that the up-regulation of glutathione S-transferase accompanied with the oxidation of peanut proteins.

Succinate dehydrogenase

Succinate dehydrogenase (EC:1.3.5.1) was a vital protein for the regulation of redox levels in mitochondria (Sujoy, Kruger, Wicks, Simon, Ganesh Kumar, Johnson et al., 2016). It catalyzed the conversion of succinic acid to fumaric acid in TCA cycle. In our study, the succinate dehydrogenase was upregulated in both NS vs. CS, and RS vs. CS, which implied that hypoxic conditions protected it from oxidation. Before moving on to the RS stage, the oxygen-rich environment allowed it to continue catalyzing the above reaction in TCA cycle and enhance the respiratory metabolism.

Conclusion

In this study, we investigated the effect of NS and RS on peanut protein oxidation and peanut metabolism. In NS, oxidation was inhibited and the reduction of functional properties of peanut proteins was delayed. Compared with CS, peanuts down-regulated some of the enzymes associated with adversity stress and antioxidant, the TCA cycle and pentose phosphate pathway were inhibited, and sucrose and T6P levels were up-regulated to ensure signaling in response to hypoxia. After re-aeration, the increase of oxygen level stimulated the recovery of peanut respiratory metabolism and accelerated the oxidation of peanut proteins. In the face of oxidative stress, peanuts consumed sucrose and T6P, up-regulated the trehalose synthesis and proline synthesis to scavenge the accumulation of reactive oxygen species caused by oxidation. Peanuts up-regulated L-isoleucine to maintain normal internal metabolism, and up-regulated proteins related to environmental stress and antioxidants, such as lipoxygenase and acetaldehyde dehydrogenase, in response to the adverse effects of oxidation.

Overall, NS was an effective storage method to inhibit oxidation, but long-term hypoxic storage followed by re-aeration could lead to more intense oxidation of peanuts. This study provided new insights into peanut protein oxidation under hypoxic/re-aeration storage from metabolomics and proteomics perspectives.

CRediT authorship contribution statement

Wenhao Li: Writing – original draft, Methodology. **Yuhao Zhou:** Writing – review & editing. **Huayang Zhang:** Visualization, Data curation. **Mei Hu:** Project administration. **Peng Lu:** Project administration, Data curation. **Chenling Qu:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (No. 32372402), the earmarked fund for CARS (No. CARS-13), the Cultivation Programme for Young Backbone Teachers in Henan University of Technology, and the “Double First-Class” Project for Postgraduate Academic Innovation Enhancement Programme of Henan University of Technology (No. HAUTSYL2023TS04).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101173.

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