

Metabolic profiling of the liquid core and solidified outer layer of preserved duck egg yolk using widely targeted metabolomics

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Abstract: Preserved duck egg yolk (PEY) gradually formed a distinctive liquid inner core (L-PEY) and solidified outer layer (S-PEY) during alkaline pickling. However, the distinctions in small molecule metabolites between these two regions have remained unclear. In this study, PEY, S-PEY, and L-PEY were extensively compared using widely focused metabolomics. The majority of the 596 metabolites found were fatty acyls (FAs) (14.4%), organic acids and their derivatives (12.9%), and amino acids and their metabolites (22.9%). The major classes, which combined accounted for 56.4%–79.1% of the total abundance, were glycerophospholipids (GPs), amino acids and their metabolites, and organic acids and their derivatives, according to relative quantitative abundance analysis. A total of 117, 47, and 169 differential abundance metabolites were identified in the comparisons of L-PEY vs. PEY, S-PEY vs. PEY, and L-PEY vs. S-PEY, respectively, mainly belonging to amino acids and their metabolites and FAs. GPs and amino acids were more abundant in L-PEY than in S-PEY, but FAs were significantly more abundant in S-PEY. Specifically, lysophospholipid (20:4/0:0), pyroglutamic acid, *L*-valine, lysine, and histidine were significantly more abundant in L-PEY, whereas free fatty acid (FFA) (22:5), FFA (20:2), and 12,13-epoxyoctadecenoic acid were significantly more abundant in S-PEY. These findings provide new insights into the metabolic basis of regional differentiation and quality formation in PEY.

Keywords: preserved duck egg; metabolomics; alkali pickled; amino acid

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1 Introduction

Preserved duck egg yolk (PEY) is traditional egg products originating from China. During processing, complex component migration and structural reconstruction occur due to the synergistic influence of strong alkali and metal ions, ultimately resulting in their distinctive color, flavor, and texture^[1]. Prior research has demonstrated that protein denaturation, aggregation, and gelation are essential to the development of preserved eggs^[2–4]. Compared with egg white, egg yolk contains a higher content of lipids and proteins and exhibits a more intricate structural organization. Egg yolk is identified as a natural supramolecular assembly composed of lipids and proteins, which can be further separated into a yolk plasma fraction, predominantly containing low-density lipoproteins (LDL), and a yolk granule fraction, primarily consisting of high-density lipoproteins (HDL) and phosvitin (Pv)^[5].

In the middle ground and later phases of alkaline pickling, duck egg yolk progressively formed a distinct inner-outer partition, distinguished by a solidified outer layer (S-PEY) and a liquid inner core (L-PEY)^[6]. Xu et al.^[6] reported that during the gelation of PEY, the outer yolk gradually developed a dense fibrous gel network, whereas the inner yolk underwent granule disruption followed by reaggregation. The two regions exhibited significant differences in physical state, microstructure, and intermolecular interactions. Specifically, the outer gel was mostly maintained by ionic bonds, hydrophobic interactions, and disulfide bonds, while the inner region was dominated by ionic bonds and hydrogen bonds. In

addition, lower pickling temperatures accelerated outer-layer solidification and promoted the formation of a denser and more ordered gel network^[6]. It has been reported that alkali treatment induces the release of constituent components from yolk plasma and granules, followed by their reaggregation. In this process, the alkali-induced unfolding and subsequent aggregation of plasma proteins were considered important factors driving yolk gel formation, whereas granules mainly contributed to greater gel hardness and structural stability through disulfide bonding^[7]. These findings suggested that the formation of the characteristic S-PEY and L-PEY in preserved egg yolk was controlled by both alkali diffusion and the breakdown, migration, and reassembly of different lipoprotein fractions. In our previous study, the nutritional composition and lipidomic characteristics of PEY, S-PEY, and L-PEY were further analyzed. Nutritional analysis showed that the L-PEY contained higher contents of total protein, total lipids, and phosphorus than the solidified outer layer. Lipidomic research revealed that the total lipid abundance in the L-PEY was 1.7 times more than that in the S-PEY. The abundances of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in the L-PEY were approximately 19.6 and 13.6 times, respectively, more than those in the S-PEY^[8]. These results indicated that significant nutrient migration and lipid modification occurred across various parts of the yolk during alkaline pickling, further suggesting that the formation of the L-PEY may be associated with a unique small-molecule foundation. Studies have shown that free amino acids and small peptides in PEY experience significant

alterations during alkaline pickling, which are intricately linked to umami development^[9–10]. In addition, metabolomics has been employed to elucidate changes in precursors, oxidation products, and Maillard reaction-associated metabolites involved in the formation of advanced glycation end products (AGEs) in preserved egg yolk during pickling^[11]. Small molecule metabolites generally include amino acids, organic acids, nucleosides, and their derivatives, many of which are nutritionally relevant and play important roles in flavor precursor accumulation and chemical reactions during food processing^[12–13]. However, the systematic differences in small molecule metabolites between the S-PEY and L-PEY remain unclear.

In this study, a widely targeted metabolomics approach was employed to systematically compare the small molecule metabolite profiles of PEY, S-PEY, and L-PEY. This study aims to enhance the limited knowledge of individuals regarding the redistribution of metabolites between the S-PEY and the L-PEY during the alkaline pickling process, and to elucidate the potential correlation between these component changes and the formation of their unique structural and quality characteristics. The findings of this study provide new insight into the chemical basis underlying regional heterogeneity in PEY and offer useful support for quality regulation and product development of preserved eggs.

2 Materials and methods

2.1 Materials

PEY were purchased from Sichuan Chuangu Lao Food Co., Ltd. (Chengdu, China). Methanol, acetonitrile, and formic acid of chromatographic grade were purchased from Merck KgaA (Darmstadt, Germany). All standards were of chromatographic grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sample preparation

The S-PEY and L-PEY of PEY were prepared according to our previously reported method with slight modifications^[8,14]. Briefly, seven preserved eggs from the same batch were randomly selected, shelled, and carefully separated from the egg white while preserving the yolks intact. The S-PEY and L-PEY of the yolks were then carefully separated and collected using a spatula. Samples obtained from the seven preserved eggs were pooled and thoroughly homogenized to generate one composite sample for each group. The resulting pooled sample for each group was then divided into four portions and analyzed as four technical replicates. All collected samples were stored at -80°C until further analysis.

2.3 Metabolomics analysis

2.3.1 Solid sample extraction

Samples were removed from the -80°C freezer and thawed on ice, and all subsequent pretreatment procedures were performed on ice. Approximately (20 ± 1) mg of each sample was weighed into the corresponding centrifuge tube, followed by the addition of 400 μL of precooled 70% methanol aqueous extraction solution containing internal standards. The mixture was vortexed for 3 min, and if the sample was not fully dispersed, a steel bead was added and vortexing was continued for an additional 3 min. The samples were then sonicated in an ice-water bath for 10 min, vortexed again for 1 min, and incubated at -20°C for 30 min to facilitate protein

precipitation. After centrifugation at 12 000 r/min for 10 min at 4°C , 300 μL of the supernatant was transferred into another corresponding centrifuge tube. The supernatant was then centrifuged again at 12 000 r/min for 3 min at 4°C , and 200 μL of the resulting supernatant was transferred into the insert of an autosampler vial for metabolomics analysis^[12].

2.3.2 Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis

Metabolomics analysis was carried out by Wuhan Metabot Biotechnology Co., Ltd. (Wuhan, China). Metabolites were analyzed using an ExionLC AD UPLC system coupled with a QTRAP[®] tandem mass spectrometer (SCIEX; Framingham, MA, USA). Chromatographic separation was performed on a ACQUITY UPLC HSS T3 C_{18} column (2.1 mm $\times$ 100 mm, 1.8 μm , Waters, Milford, MA, USA). The mobile phase consisted of 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile (B). The mobile phase was delivered using the following gradient: 95:5 (A:B, V/V) at 0 min, 80:20 at 2.0 min, 40:60 at 5.0 min, 1:99 from 6.0 to 7.5 min, and 95:5 from 7.6 to 10.0 min^[13,15]. The flow rate was 0.4 mL/min, the column temperature was maintained at 40°C , and the injection volume was 2 μL . MS detection was conducted using an electrospray ionization (ESI) source in both positive and negative ion modes. The ion source temperature was set at 500°C , and the ion spray voltage was 5 500 V in positive mode and $-4\,500$ V in negative mode. The ion source gases I and II were set at 55 (379 kPa) and 60 psi (414 kPa), respectively, and the curtain gas was set at 25 psi (172 kPa)^[16]. The collision-activated dissociation (CAD) gas was set to high. Data were acquired in multiple reaction monitoring mode, and each ion pair was optimized using the corresponding declustering potential and collision energy.

2.3.3 Qualitative and quantitative analysis of metabolites

Metabolites were quantified using a mass spectrometer (TripleTOF 6600, SCIEX, Framingham, MA, USA). Peak area integration was performed for all detected metabolites, and the peaks corresponding to the same metabolite in different samples were corrected for comparative analysis. Metabolite identification was conducted based on retention time (RT), precursor/product ion pairs, and MS/MS spectra by comparison with the in-house MWDB database (Wuhan MetWare Biotechnology Co., Ltd., Wuhan, China).

2.4 Statistical analysis

Statistical differences among groups were evaluated by one-way analysis of variance, followed by post hoc multiple comparison tests when appropriate. $P < 0.05$ was considered statistically significant. For metabolomics data, false discovery rate (FDR) correction was performed using the Benjamini-Hochberg method, and adjusted P values were used for multiple testing control. Data visualization was performed using OriginPro 2025 software (OriginLab Corp., Northampton, MA, USA).

3 Results

3.1 Metabolomics profiling of PEY, S-PEY, and L-PEY

A total of 596 metabolites were identified, including 137 (22.9%) amino acids and their metabolites, 86 (14.4%) fatty acyls (FAs),

77 (12.9%) organic acids and their metabolites, 64 (10.7%) glycerophospholipids (GPs), 50 (8.4%) benzene and substituted derivatives, 37 (6.2%) heterocyclic compounds, 34 (5.7%) nucleotides and their metabolites, 32 (5.4%) alcohols and amines, 21 (3.5%) carbohydrates and their metabolites, 19 (3.2%) aldehydes, ketones, esters, 13 (2.2%) coenzymes and vitamins, 11 (1.9%) hormones and hormone-related compounds, 6 (1.0%) bile acids, 3 (0.5%) tryptamines, cholines, pigments, 1 (0.2%) sphingolipid (SL), 1 (0.2%) glyceride, 4 (0.7%) others (Figure 1A). A quantitative comparison based on metabolite abundance revealed that the

cumulative metabolite abundance in the L-PEY group was approximately 1.37-fold of that in the S-PEY group (Figure 1B). However, this difference did not reach statistical significance ($P = 0.851$). Overall, no significant differences in total metabolite abundance were observed among the groups. GPs were the predominant class of metabolites, followed by amino acids and their metabolites, as well as organic acids and their derivatives, collectively representing 56.4%–79.1% of the total relative abundance across PEY, S-PEY, and L-PEY. Principal component analysis (PCA) was employed to evaluate the overall differences in

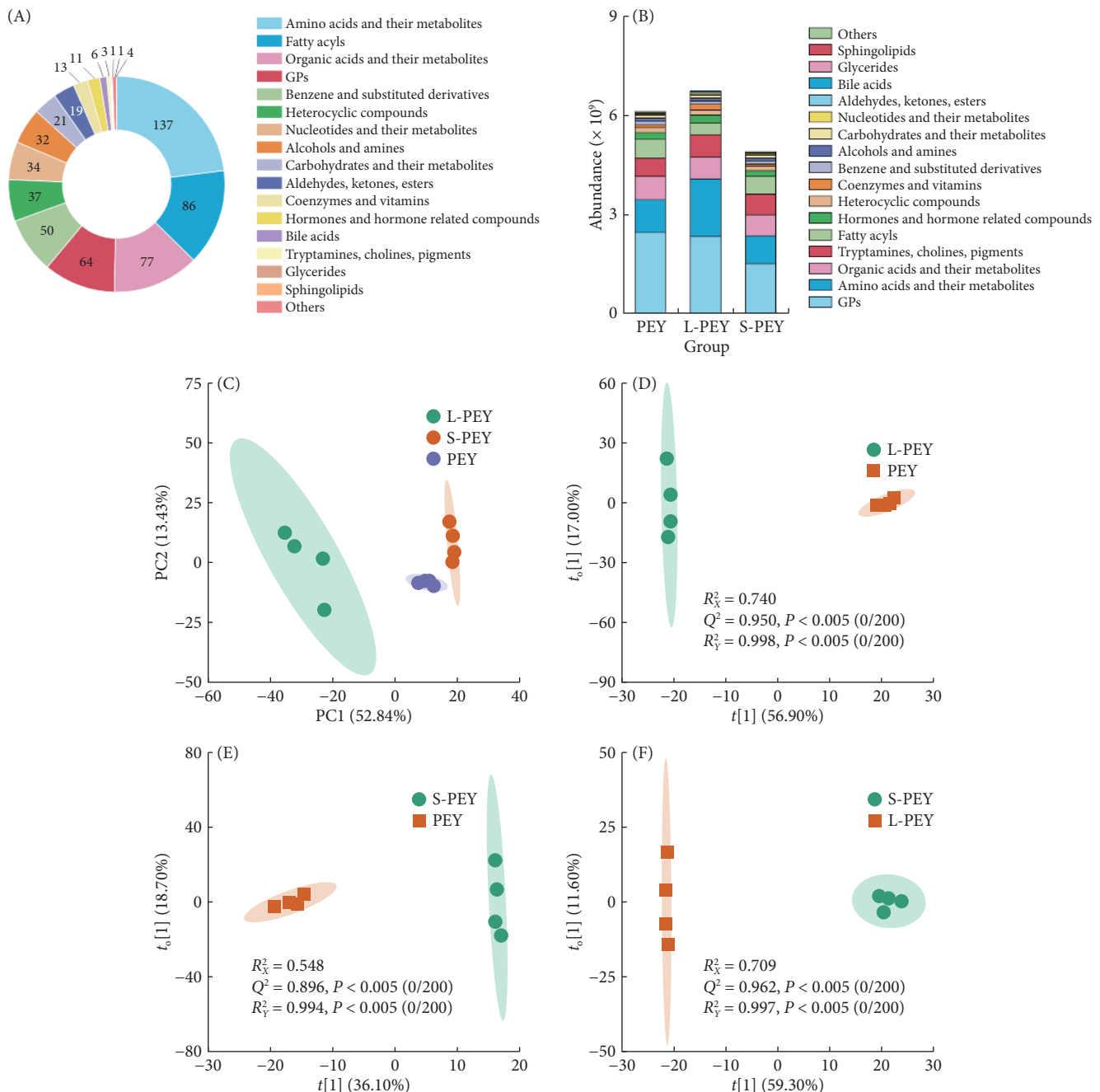


Figure 1 Overview of metabolite composition and multivariate analysis of PEY, S-PEY, and L-PEY. (A) Classification of the 596 identified metabolites into major categories; (B) total abundance of metabolite subclasses in PEY, S-PEY, and L-PEY groups; (C) PCA score plot showing the overall metabolic differences among the three sample groups; (D–F) OPLS-DA score plots for pairwise comparisons of PEY vs. L-PEY, PEY vs. S-PEY, and S-PEY vs. L-PEY, respectively, illustrating the distinct metabolic separation between groups.

metabolomics among the PEY, S-PEY, and L-PEY groups. The PCA plot indicated that the repeated samples within the same group clustered together, but a discernible separation trend emerged between different groups, especially a clear separation between L-PEY and S-PEY/PEY. The PC1 and PC2 accounted for 52.84% and 13.43% of the total variance, respectively (Figure 1C). Metabolomics data were examined using the orthogonal partial least squares discriminant analysis (OPLS-DA) model for supervised pattern recognition to optimize group differentiation. As shown in Figures 1D–F, the OPLS-DA score plots exhibited distinct group separation. The initial predictive component $t[1]$ accounted for 36.10%–59.30%. The model parameters R^2_X , R^2_Y , and Q^2 ranged from 0.548 to 0.740, 0.994 to 0.998, and 0.896 to 0.962, respectively.

3.2 Differential abundance metabolites (DAMs) of PEY, S-PEY, and L-PEY

Utilizing the OPLS-DA model, DAMs among PEY, S-PEY, and L-PEY were identified using variable importance in projection (VIP) values and further refined by integrating fold change (FC) and multiple testing correction. *P*-values were adjusted using the Benjamini-Hochberg procedure to regulate the FDR. Although a VIP value > 1.0 is commonly used in metabolomics studies, a more stringent threshold (VIP > 1.2) was adopted in this study to improve the robustness of metabolite selection and to reduce the

inclusion of marginally discriminative variables. Metabolites were considered significantly distinguished when they simultaneously satisfied the following criteria: VIP > 1.2, FDR < 0.05, and $|\log_2 FC| \geq 1$ (equivalent to FC ≥ 2 or FC ≤ 0.5). The identified DAMs are summarized in Table S1. In the comparison of L-PEY vs. PEY, 117 DAMs were identified, comprising 57 with decreased abundance and 60 with increased abundance metabolites, predominantly consisting of amino acids and their metabolites (36) and fatty acyls (29). As shown in Figure 2A, the five most significant DAMs with the highest VIP values were homovanillic acid sulfate, 13-hydroxyoctadeca trienoic acid (13-HOTrE), cyclo(His-Pro), ectoine, and ($\pm$)7,8-dihydroxydocosapentaenoic acid (($\pm$)7(8)-DiHDPE(A)). A total of 47 down-regulated DAMs were discovered in S-PEY compared to PEY. The majority of these DAMs were classified as FAs (26). The five DAMs exhibiting the greatest VIP values were Leu-Pro-Tyr, carnitine C_{18:3}, carnitine C_{16:2}, carnitine C_{22:5}, and carnitine C_{14:2} (Figure 2B). A total of 169 DAMs were identified in the comparison of L-PEY vs. S-PEY, comprising 105 with down-abundance and 64 with up-abundance. These DAMs were primarily composed of amino acids and their metabolites (57) and FAs (51). The five DAMs with the highest VIP values were ($\pm$)14,15-epoxyeicosatetraenoic acid (($\pm$)14(15)-EpETE), L-2-aminoadipic acid, Ser-Ala, carnitine C_{12:0}, and carnitine isoC_{4:0} (Figure 2C). Overall, the largest number of DAMs was observed in

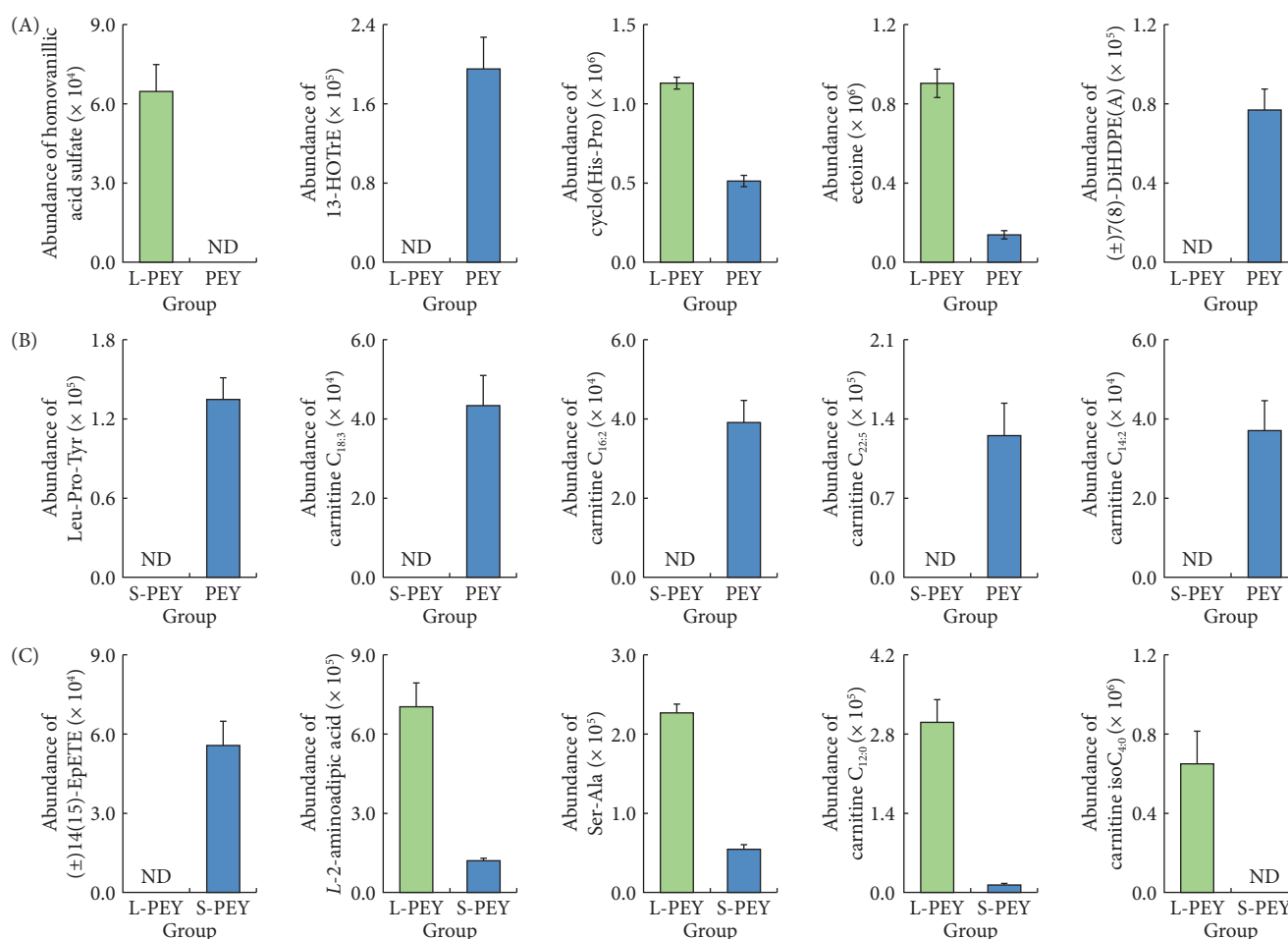


Figure 2 The top five metabolites of VIP scores from OPLS-DA for each pairwise comparison among PEY, L-PEY, and S-PEY. (A) L-PEY vs. PEY; (B) S-PEY vs. PEY; (C) L-PEY vs. S-PEY. ND indicates not detected.

the L-PEY vs. S-PEY comparison, indicating that the L-PEY and the S-PEY exhibited the most pronounced metabolic differences.

3.3 Changes in key metabolite classes of PEY, S-PEY, and L-PEY

Based on Figure 1B, metabolite categories with relatively high abundance or clear intergroup variation were selected for further analysis. GPs appeared as the predominant class, accounting for 30.4%–40.5% of the total abundance. The abundance of GPs in L-PEY group was significantly higher than that in S-PEY group ($P < 0.05$), indicating a 53.2% increase compared to S-PEY group. As shown in Figure 3, the ten most abundant GPs were lysophosphatidylethanolamine (LPE) (16:0/0:0), lysophosphatidylcholine (LPC) (0:0/16:1), LPE (18:1/0:0), LPE (0:0/20:4), LPE (18:2/0:0), LPC (20:4/0:0), LPC (0:0/14:0), LPC (0:0/18:3), LPC (0:0/20:3), and LPC (22:6/0:0). Among these, LPE (0:0/20:4), LPC (20:4/0:0), and LPC (22:6/0:0) exhibited

considerably greater abundance in L-PEY group compared to S-PEY group ($P < 0.05$).

Amino acids and their metabolites were 16.2%–25.8% of the total abundance, comprising 76 small peptides, 38 amino acid derivatives, and 23 amino acids. The abundance of amino acids and their metabolites in L-PEY group was significantly higher than that in S-PEY group ($P < 0.001$), reaching 2.21 times that of S-PEY group. This difference was mainly attributable to free amino acids rather than to small peptides or amino acid derivatives. Specifically, the abundances of small peptides and amino acid derivatives in L-PEY group did not differ significantly from those in S-PEY group ($P > 0.05$), whereas free amino acids were significantly more abundant in L-PEY, being 2.74 times that in S-PEY ($P < 0.001$). As shown in Figure 4, the ten most abundant amino acids and related derivatives were pyroglutamic acid, γ -aminobutyric acid, *L*-valine, *L*-phenylalanine, lysine, *L*-glutamic acid, aspartic acid, *N*-formylglycine, Val-Thr, and histidine. Among these,

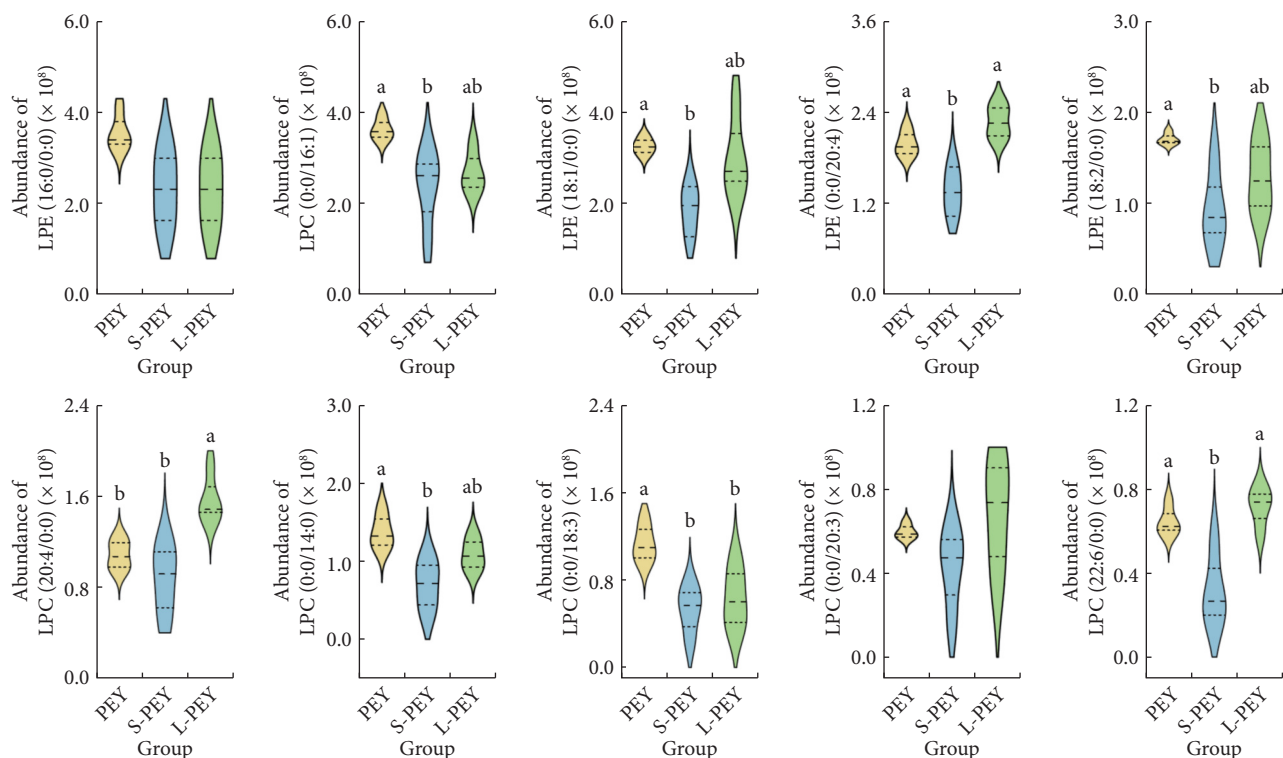


Figure 3 The top ten GPs by abundance in PEY, S-PEY, and L-PEY. The superscript letter (a–c) indicates a statistically significant difference ($P < 0.05$).

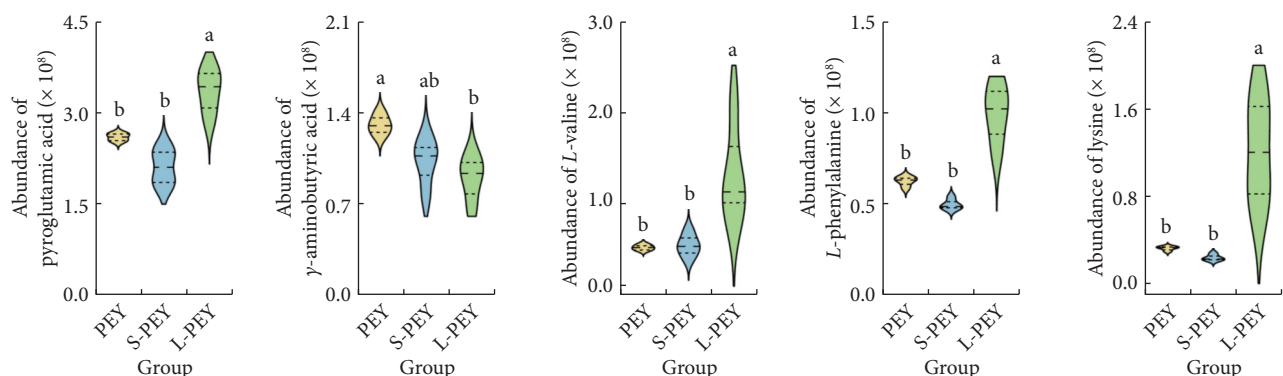


Figure 4 The top ten amino acids and their derivatives by abundance in PEY, S-PEY, and L-PEY. The superscript letter (a–c) indicates a statistically significant difference ($P < 0.05$).

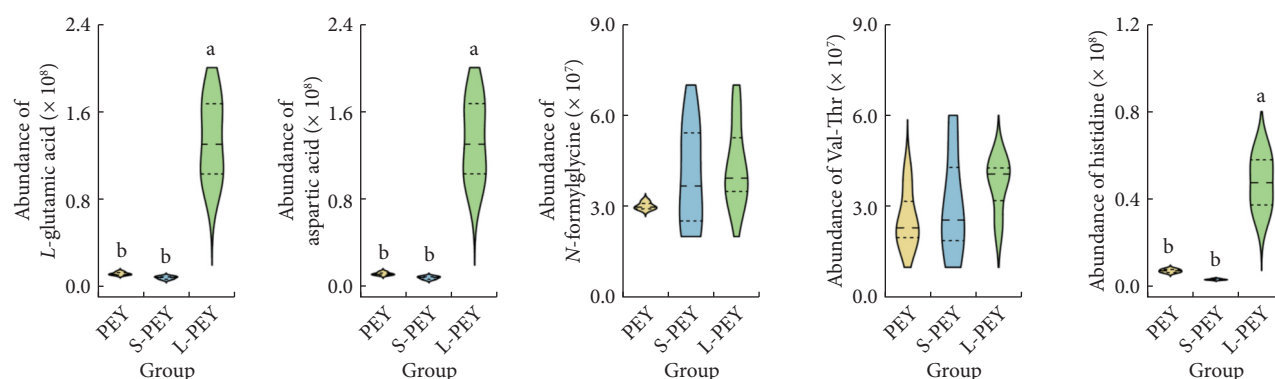


Figure 4 (Continued)

pyroglutamic acid, L-valine, L-phenylalanine, lysine, L-glutamic acid, aspartic acid, and histidine were significantly more abundant in L-PEY than in both S-PEY and PEY ($P < 0.05$).

Organic acid and its metabolites constituted 9.9%–12.8% of the total small molecule metabolites. No significant difference in the total abundance of this class was observed among L-PEY, S-PEY, and PEY. However, several individual compounds exhibited clear distribution-dependent variation. As shown in Figure 5, the top ten most abundant metabolite molecules included glycerol 3-phosphate, 2-dodecylbenzenesulfonic acid, 11-carboxyl- β -acetyl-boswellic acid, succinic anhydride, guanidineacetic acid, citric acid, iminodiacetic acid, isokaurenoic acid, quinic acid, and ethylsalicylate. Among these, succinic anhydride, guanidineacetic acid, and iminodiacetic acid exhibited considerably greater abundance in L-PEY compared to S-PEY, whereas citric acid, isokaurenoic acid, and quinic acid were significantly more abundant in S-PEY than in L-PEY ($P < 0.05$).

The discovered metabolites in the categories of tryptamine, choline, and pigment-related categories included choline, *sn*-glycero-3-phosphocholine, and tryptamine. Among these, choline accounted for 9.3%–10.1% of the total relative quantified abundance, whereas tryptamine represented 0.005%–0.012% of the total relative quantified abundance. As shown in Figure 6, the abundance of tryptamine was significantly higher in the L-PEY group compared to the S-PEY group ($P < 0.05$). FAs accounted for 5.3%–10.7% of the total abundance, including 38 acylcarnitines, 22 oxidized lipids, 20 free fatty acids (FFAs), and 6 other compounds. The total FFAs abundance in the S-PEY group was significantly higher than that in the L-PEY group ($P < 0.001$), representing a 49.2% increase relative to L-PEY. Specifically, the abundances of acylcarnitines and FFAs were significantly higher in S-PEY compared to L-PEY ($P < 0.05$), but no significant change was observed for oxidized lipids. The ten most abundant fatty acyls were tetranor-12(*R*)-hydroxy eicosatetraenoic acid (HETE), arachidonic

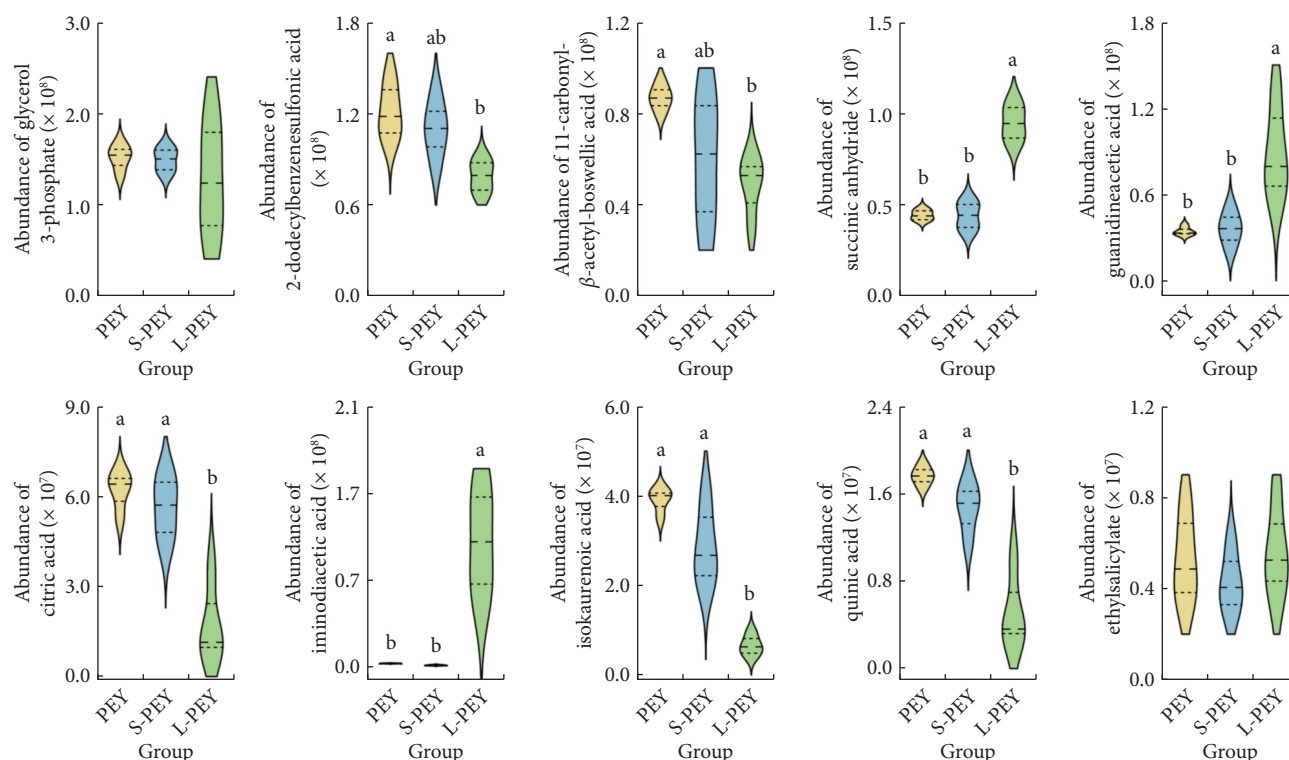


Figure 5 The top ten organic acids and their derivatives by abundance in PEY, S-PEY, and L-PEY. The superscript letter (a–c) indicates a statistically significant difference ($P < 0.05$).

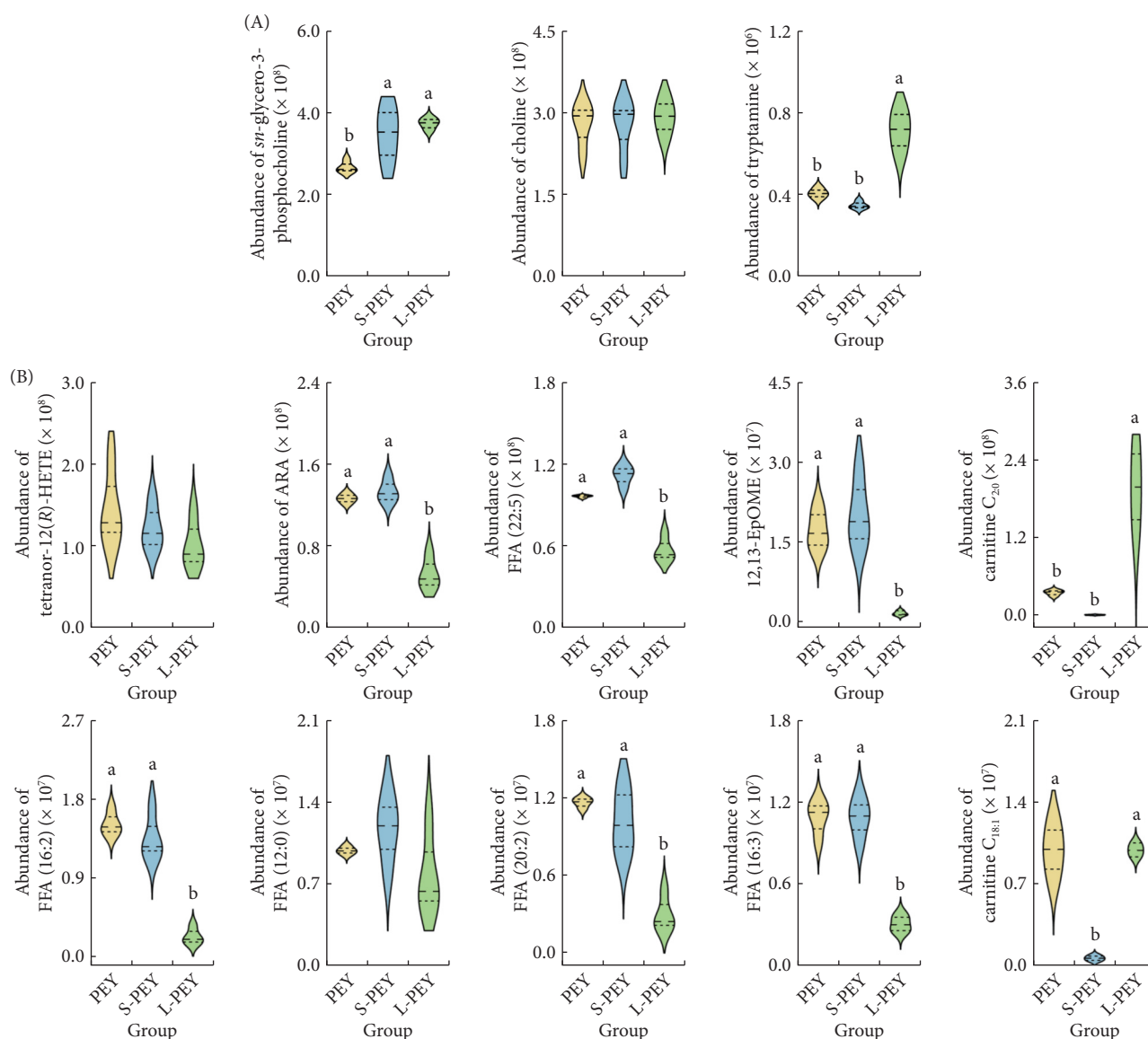


Figure 6 (A) Three identified tryptamine-, choline-, and pigment-related metabolites and (B) the top ten fatty acyl metabolites by abundance in PEY, S-PEY, and L-PEY. The superscript letter (a–c) indicates a statistically significant difference ($P < 0.05$).

acid (ARA), FFA (22:5), 12,13-epoxyoctadecenoic acid (12,13-EpOME), carnitine C₂₀, FFA (16:2), FFA (12:0), FFA (20:2), FFA (16:3), and carnitine C_{18:1}. The abundances of ARA, FFA (22:5), 12,13-EpOME, FFA (16:2), FFA (20:2), and FFA (16:3) were significantly higher in the S-PEY group than in the L-PEY group ($P < 0.05$), whereas carnitine C₂₀ and carnitine C_{18:1} were significantly more abundant in the L-PEY group than in the S-PEY group ($P < 0.05$). Overall, these results indicate that the major metabolite categories exhibited distinct distribution characteristics, with GPs and free amino acids being more abundant in L-PEY, while FAs were more abundant in S-PEY.

4 Discussion

The presence of S-PEY and L-PEY in PEY indicates a unique structural heterogeneity resulting from alkaline pickling. This research thoroughly analyzed the spatial heterogeneity of small-molecule metabolites in PEY, L-PEY, and S-PEY using metabolomics. A total of 596 metabolites were identified, and

multivariate analyses revealed significant metabolic differentiation between L-PEY and S-PEY, indicating that the formation of the liquid inner core was associated with considerable metabolic reorganization. No significant difference was observed in the cumulative metabolite abundance among PEY, L-PEY, and S-PEY. However, significant distinctions in abundance were identified at the metabolite-class abundance. Significant differences were observed in several key metabolite classes, including GPs (53.2% higher in L-PEY compared to S-PEY), amino acids and their metabolites (2.2-fold higher in L-PEY than in S-PEY), free amino acids (2.7-fold higher in L-PEY than in S-PEY), and fatty acyls (49.2% higher in S-PEY than in L-PEY). L-PEY exhibited a greater tendency to enrich amphiphilic phospholipid-derived metabolites and free amino acids, whereas S-PEY revealed a stronger inclination to retain metabolites linked to FAs. These findings indicate the presence of distinct microenvironments within the pickled yolk, possibly influenced by gradients in salt-alkali diffusion, moisture content, and the restructuring of the protein-lipid matrix throughout the pickling process.

4.1 GPs and free amino acid enrichment in L-PEY

Metabolomics analysis revealed that GPs were the predominant metabolite class in L-PEY, accounting for 30.4%–40.5% of the total relative abundance across groups. Notably, the abundance of GPs in the L-PEY group (increased by 53.2%) was significantly higher than that in the S-PEY group, with lysophospholipids (LPC/LPE) being the dominant subclasses. In addition, some molecules, such as LPE (0:0/20:4), LPE (18:2/0:0), and LPC (22:6/0:0), exhibited considerable enrichment in the L-PEY group. The results indicated that the L-PEY may serve as a preferential enrichment region for phospholipid metabolites, particularly amphiphilic lysophospholipids. This discrepancy was likely associated with regional changes in physicochemical conditions and the structural evolution of lipoproteins during pickling, in conjunction with our prior findings. In our previous study, both pH and moisture content were significantly higher in S-PEY group than in L-PEY group. Under stronger alkaline conditions, PC, PE, phosphatidylinositol (PI), and phosphatidylglycerol (PG) in S-PEY may undergo hydrolysis, leading to the formation of LPC, LPE, lysophosphatidylinositol, and lysophosphatidylglycerol^[8]. However, the significant enrichment of lysophospholipids in L-PEY indicated that their spatial distribution was influenced not only by local hydrolytic release but also by subsequent migratory behavior. Previous transmission electron microscope observations further support this interpretation. In the L-PEY group, yolk granules remained a somewhat consistent shape, and the majority of LDL structures were preserved. Conversely, in the S-PEY group, several yolk granules exhibited significant structural damage and deformation, with typical LDL structures no longer observed. Rheological results indicated that both the storage modulus (G') and loss modulus (G'') of L-PEY group were markedly lower than those of PEY and S-PEY groups, indicating reduced viscoelasticity, higher fluidity, and weaker structural constraints in the L-PEY group^[6]. These results suggested that L-PEY group remains a relatively loose lipoprotein composite system, whereas S-PEY group has developed a denser gel-like network. Under strong alkaline conditions, the spherical LDL structure was disrupted, leading to the release of internal components, including triacylglycerols (TGs), cholesteryl esters, cholesterol, and proteins, which could subsequently reassemble into non-spherical LDL aggregates through hydrogen bonding and hydrophobic interactions^[2,17]. In contrast, HDL can establish a denser and more uniform network through using ionic bonds, hydrophobic interactions, and disulfide bonds. With the continued action of alkali and metal ions, lipids and proteins derived from both HDL and LDL gradually migrate and reorganize, resulting in a spatially heterogeneous yolk gel structure typical of L-PEY^[5]. The higher abundance of GPs, especially LPC and LPE, in L-PEY relative to S-PEY was unlikely to be explained solely by local hydrolysis, but was more likely related to the greater fluidity of the liquid core, weaker network confinement, and the inward migration of lipid- and phospholipid-derived metabolites. In addition to lysophospholipids, choline-containing metabolites were significant in the metabolome, with choline accounting for 9.3%–10.1% of the total relative abundance. The close association of choline and *sn*-glycero-3-phosphocholine with phosphatidylcholine-related metabolites^[18], along with their increased abundance, may further support the potential role of phospholipid-derived small molecules in the regional metabolic differentiation of PEY.

Amino acids and their metabolites accounted for 16.2%–25.8% of the total abundance across groups, and their abundance in L-PEY was 2.21 times that in S-PEY. Notably, this difference was predominantly attributed to free amino acids, whereas small peptides and amino acid derivatives exhibited no significant variation between L-PEY and S-PEY. The abundance of free amino acids in L-PEY was 2.74 times greater than that in S-PEY. This result indicated that the difference between L-PEY and S-PEY does not reflect a uniform increase in all nitrogen-containing metabolites, but rather a more pronounced accumulation of terminal hydrolysis products (free amino acids) in the L-PEY. This may indicate localized variations in microenvironmental conditions (e.g., pH, moisture content, fluidity, and structural organization), which can further influence protein-lipid interactions as well as the release and retention of hydrolysis products^[8]. Theoretically, the higher pH value in S-PEY will be expected to promote protein denaturation and alkali-induced hydrolysis. However, S-PEY has already developed a denser gel-like network structure, and the stronger structural confinement may restrict the release of protein hydrolysis products^[3,19]. In addition, in more pronounced alkaline conditions, the free amino acids may undergo additional chemical changes or interact with adjacent components^[20]. By contrast, the higher fluidity and weaker network confinement in L-PEY may promote the accumulation of free amino acids, resulting in a higher net accumulation level in the L-PEY. It was worth noting that, as the current analysis focuses on small molecule metabolites, the observed abundance differences may reflect not only regional formation and substance retention but also differences in matrix-dependent release and extraction accessibility.

4.2 FAs enrichment in S-PEY

In comparison to L-PEY, S-PEY exhibited a considerably greater abundance of total FAs (49.2% higher; $P < 0.001$), mainly caused by increased abundance of FFAs and acylcarnitines, whereas oxidized lipids showed no significant difference. This opposite trend indicated a distinct mode of lipid remodeling and/or enhanced retention of lipid-derived metabolites in the S-PEY. In the phase transition of L-PEY, a significant proportion of amphiphilic lipid species, particularly phospholipid and lysophospholipid molecules, tended to migrate inward, while the outer layer was the initial region to undergo structural solidification and develop a denser protein-lipid network^[8,21]. The early gelation changed the diffusion and partitioning behavior of hydrophobic/amphiphilic molecules and promoted local confinement of the remaining lipids in S-PEY^[5]. Previous studies have shown that granule gels were mainly maintained by ionic bonds, hydrophobic interactions, and disulfide bonds, which was consistent with the characteristics of the external yolk layer, whereas plasma gels were primarily stabilized by ionic bonds and hydrogen bonds, resembling the internal yolk layer^[6,22]. This supports the view that the S-PEY formed a more compact and strongly constrained matrix during alkaline pickling. In particular, such a denser matrix may favor the local accumulation of FAs-related metabolites by restricting their outward diffusion once released, thereby contributing to their higher abundance in S-PEY. These results were also consistent with our previous lipidomics findings, which demonstrated that during migration, the non-migrated TG molecules undergo hydrolysis under the strong alkaline conditions of S-PEY to form diacylglycerol (DG), monoacylglycerol (MG) and FFA, and DG could further decompose into MG and FFA^[8]. In addition to hydrolytic release,

the denser network in S-PEY may further reduce the outward diffusion of these lipid-derived end products, thereby increasing their abundance in the S-PEY. The higher abundance of ARA, FFA (22:5), 12,13-EpOME, FFA (16:2), FFA (20:2), and FFA (16:3) in S-PEY further indicated that lipid-derived metabolites were not uniformly distributed across the yolk. In addition to free fatty acids, acylcarnitines were also more abundant in S-PEY. Acylcarnitines are FA-related derivatives, and their higher abundance in S-PEY suggests region-selective accumulation in the outer layer, although the specific formation and retention mechanisms require further verification.

4.3 Flavor implications of differential metabolite distribution between L-PEY and S-PEY

The significant differences in metabolite abundance between L-PEY and S-PEY may have an impact on the flavor of the egg yolk in preserved eggs with a liquid core. In L-PEY, amino acids and their metabolites accounted for 16.2%–25.8% of the total relative abundance and were significantly higher than in S-PEY (2.21-fold; $P < 0.001$), with the difference primarily driven by free amino acids (2.74-fold higher in L-PEY than in S-PEY). Compared with S-PEY and PEY, the contents of several flavor-active amino acids and related compounds (including pyroglutamic acid, *L*-valine, *L*-phenylalanine, lysine, *L*-glutamic acid, aspartic acid, and histidine) in L-PEY were significantly increased ($P < 0.05$). Given that *L*-glutamic acid and aspartic acid are well-recognized umami contributors, their accumulation may be associated with a potentially higher umami intensity in L-PEY compared with S-PEY^[23–24]. In addition, the increased abundance of lysine, histidine, valine, and phenylalanine indicated that L-PEY may be involved in the broader modulation of taste complexity and balance. Free amino acids were not only direct taste-active compounds, but also important flavor precursors in preserved eggs. Previous studies have shown that amino acids might further participate in deamination, transamination, decarboxylation, Maillard-type reactions, Strecker degradation, and interactions with lipids under alkaline processing conditions, implying potential contributions to nuanced taste attributes^[25–26]. Organic acids and their metabolites constituted a substantial fraction of small molecule metabolites (9.9%–12.8%), and the overall abundance of this category did not differ significantly among L-PEY, S-PEY, and PEY. Nevertheless, several individual organic acids or derivatives showed region-dependent differences, implying potential contributions to nuanced taste attributes. Succinic anhydride, guanidineacetic acid, and iminodiacetic acid were significantly higher in L-PEY than in S-PEY, whereas citric acid, isokaurenoic acid, and quinic acid were significantly higher in S-PEY ($P < 0.05$). Previous studies have shown that organic acids are important taste-active substances in food systems and can influence sourness, overall taste intensity, and taste balance through interactions with other coexisting compounds^[3]. It was reported that organic acids such as malic acid and succinic acid can simultaneously enhance the perception of sourness and saltiness while suppressing sweetness^[27]. Besides, citric acid and succinic acid can provide a sour taste and act as flavor precursor molecules involved in the formation of the overall flavor^[28]. The contribution of organic acids to food taste was closely associated with their taste thresholds and taste activity values rather than concentration alone. Succinic acid, owing to its relatively low threshold, was reported to contribute to umami-related taste^[29]. Therefore, although no significant difference was observed in the

total abundance of organic acids and their derivatives between L-PEY and S-PEY, the differential distribution of specific organic acids between the two regions may still contribute to subtle regional differences in taste perception by modulating the balance among sour, salty, and umami-related sensations. However, these proposed associations require further validation through targeted quantitative analysis, volatile profiling, and sensory evaluation. In contrast, S-PEY exhibited significantly higher abundance of FAs than L-PEY (49.2% higher; $P < 0.001$), mainly attributable to higher abundances of free fatty acids and acylcarnitines. ARA, FFA (22:5), 12,13-EpOME, FFA (16:2), FFA (20:2), and FFA (16:3) were significantly higher in S-PEY than in L-PEY ($P < 0.05$). Free fatty acids and polyunsaturated fatty acid-derived species are important precursors of lipid-derived aroma compounds, and their higher abundance in S-PEY may suggest that the solidified region could be more strongly associated with lipid-related flavor cues and aftertaste characteristics^[30–32]. It should be noted that, since this study was based on the distribution of metabolites, it will be necessary in the future to conduct research that integrates targeted quantitative analysis of key taste compounds, volatile substance analysis, and sensory correlation to directly verify these proposed contributions.

5 Conclusion

This study systematically characterized the small molecule metabolite profiles of PEY, L-PEY, and S-PEY using a widely targeted metabolomics. A total of 596 metabolites were identified, among which amino acids and their metabolites, FAs, and organic acids and their derivatives were the major categories. In terms of cumulative relative abundance, GPs, amino acids and their metabolites, and organic acids and their derivatives emerged as the dominant classes. Clear metabolic differentiation was observed among PEY, S-PEY, and L-PEY, particularly between S-PEY and L-PEY, with 169 DAMs identified. L-PEY was characterized by higher abundance of amphiphilic GPs and free amino acids, including umami-related amino acids such as *L*-glutamic acid and aspartic acid, whereas S-PEY showed a significantly higher abundance of FAs, especially free fatty acids, which may serve as important precursors of lipid-derived volatile compounds. These findings provide new insight into the metabolic basis of regional differentiation and quality formation in PEY. Future studies integrating metabolomics with volatilomics, and microstructural analysis will help elucidate the mechanisms governing yolk heterogeneity during alkaline pickling.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Author contributions

Yunxiao Xie: Investigation, visualization, writing-original draft. Dingrui Jia: Investigation, methodology, data curation. Mohan Qiu: Conceptualization, writing-review & editing. Jinqiu Wang: Conceptualization, writing-review & editing. Fang Geng:

Conceptualization, supervision, validation, writing-review & editing, funding.

Appendix A. supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.26599/FSAP.2026.9240174>.

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