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Lipid and Aqueous Metabolomic Responses of Lettuce (*Lactuca sativa*) from Organic Farming Practices

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Abstract

Organic farming and crop quality are important concerns in the food marketplace. This study employed an untargeted, semi-quantitative metabolomics and lipidomics workflow to characterise differences in aqueous metabolite and lipid profiles between organically and conventionally cultivated lettuce (*Lactuca sativa*). Using UPLC-Orbitrap-MS, we identified 38 aqueous metabolites at Metabolomics Standards Initiative (MSI) levels 1 and 2 and 37 lipids at MSI level 2 that showed significant differences between cultivation systems. Organic lettuce consistently exhibited elevated relative levels of phenylalanine, tryptophan, and the phytohormone indole-3-acetic acid (IAA), alongside increased relative concentrations of eleven secondary phenolic metabolites. Network analysis of these metabolites revealed that protocatechuic acid in organic lettuce was strongly associated with triacylglycerols (TAGs) and diacylglycerols (DAGs). In contrast, conventional samples exhibited a higher degree of lipid unsaturation. These findings demonstrate that lipids show important cultivation-dependent differentiation. The integrated untargeted workflow provides a useful analytical framework for profiling cultivation-associated biochemical variation in lettuce and supports future development of authentication approaches after validation in balanced, multi-farm and multi-season studies.

Highlights:

- First study applied integrated untargeted metabolomics and lipidomic to provide the comprehensive metabolic map of lettuce under different cultivations.
- Organic lettuce showed higher relative abundances of phenolic metabolites and aromatic amino acids.
- Lipidomics revealed cultivation-associated differences in selected glycerolipid, glycerophospholipid, glycosylglycerolipid and sphingolipid species.
- Association network analysis reveals novel interrelationships between lipid and aqueous metabolites influenced by organic and conventional farming practices.
- Untargeted lipidomics demonstrated exceptional discriminatory power for authentication of organic farming.

Keywords:

Organic farming; Lactuca sativa; Lipidomics; Metabolomics; Mass spectrometry

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1. Introduction

Lettuce (*Lactuca sativa*) is the most widely consumed leafy vegetable in the United States and globally (Packer, 2023; Sharma et al., 2014). Reflecting its massive agricultural footprint, the global lettuce market is valued at approximately \$4.3 to \$4.5 billion, with steady expansion projected toward roughly \$5.6 to \$6.5 billion (6Wresearch, 2025). Driven by modern supply chains and surging demand for fresh-cut produce, nearly 80% of consumers routinely use lettuce as a primary ingredient in fresh salads, and over 45% explicitly identify it as a mandatory household grocery staple (IFPA, 2026). Its popularity can be attributed to several factors, including its mild flavour and crisp texture (Derossi et al., 2016). In addition to being a common ingredient in various dishes, lettuce provides several health benefits. It serves as a valuable source of essential minerals and dietary fibre, including iron, zinc, phosphorus, and magnesium (Kim et al., 2016). Lettuce also contains various bioactive secondary metabolites with health-promoting activities, such as lutein, phenolic compounds and folate (Kim et al., 2016).

In recent years, organic lettuce has gained increasing popularity among health-conscious consumers. Globally, organic lettuce production exceeds 350,000 metric tons annually, reflecting a robust expansion in certified organic farmland across North America, Europe, and parts of Asia-Pacific (MarketGrowthReports, 2026). Lettuce ranks among the top organic vegetables in the United States by harvested acreage and production value (Skorbiansky, 2025), with an average retail price nearly twice that of conventionally grown lettuce (PMG, 2025). This price premium reflects consumer perception of organic produce as being healthier and more environmentally sustainable. Indeed, organic farming is often regarded as a more sustainable agricultural practice (Oliveira et al., 2010) and is known to improve soil quality and nutrient retention. Studies have shown that lettuce cultivated with organic fertilization results in higher soil organic carbon (Leogrande et al., 2013), as well as increased levels of nitrogen, phosphorus, and potassium compared to conventional methods (Pavlou et al., 2007). However, organic farming is not without challenges. The use of animal manure as fertilizer raises concerns about potential microbial contamination, particularly since lettuce is often consumed raw (Maffei et al., 2019). There are many studies on organic lettuce, most of them focused on environmental impact and food safety. Surprisingly, little is known about how different cultivation practices affect its nutritional and chemical composition. Understanding these differences at the molecular level could provide new insights into the quality, functionality, and authenticity of organically grown produce.

Metabolomics, a branch of omics sciences that focuses on the comprehensive study of metabolites (<2000 Da) in samples, is particularly well suited for examining the chemical profiles of lettuce because it enables the simultaneous detection and quantification of a wide range of primary and secondary metabolites, including sugars, amino acids, organic acids, phenolic compounds, and vitamins. These metabolites directly reflect the physiological and biochemical state of the plant and are highly responsive to environmental conditions, cultivation practices, postharvest handling, and stress factors. Consequently, metabolomics provides a comprehensive snapshot of lettuce biochemical composition and allows the identification of metabolic pathways and biomarkers associated with quality traits, stress responses, and food safety. It has therefore been widely

applied to study the metabolic changes in lettuce in response to a range of factors, including environmental stresses (Cheng et al., 2023; Corrado et al., 2021; Hameed et al., 2022; Najafi et al., 2022; Savarese et al., 2022; Wei et al., 2021; Zhang et al., 2021a; Zhou et al., 2021a), post-harvesting browning (García et al., 2017; Liu et al., 2021), biotic stress (Liu et al., 2023), and contaminants (Li et al., 2021; Najafi et al., 2022; Tang et al., 2023; Zhang et al., 2024; Zhao et al., 2022). However, despite extensive research, no comprehensive metabolomics study has yet compared the metabolic profiles of lettuce grown under organic and conventional cultivation systems.

Moreover, most metabolomics studies in crops and vegetables were focused primarily on polar metabolites, with lipids remaining an underexplored class. This gap is largely due to the limited metabolite coverage of conventional reversed-phase liquid chromatography–mass spectrometry (RP-LC-MS), which is better suited for semi-polar compounds (Wang et al., 2020). However, emerging research highlights the critical roles of lipids in plant physiology, including photosynthesis, membrane integrity, environmental adaptation, and cell signaling (Lamers et al., 2020; Liu et al., 2019; Lu et al., 2020; Mamode Cassim et al., 2019). Lipid composition is highly responsive to environmental conditions (Henschel et al., 2024), making it a promising target for agricultural and geographical authentication. A recent targeted metabolomics study on the geographical origin of white asparagus (Creydt et al., 2022) demonstrated a 76% prediction accuracy using 20 lipids, even surpassing the performance of isotope ratio mass spectrometry which is the most popular analytical technique for geographic origin authentication in regulatory and commercial applications. Given the dynamic responses of lipid metabolism to different environmental stresses or factors, it is reasonable to expect that different farming practices would significantly impact lipid composition of lettuce. Therefore, integrating lipidomics into an untargeted metabolomics workflow offers a more comprehensive view of how cultivation practices influence metabolic regulation.

The present study employed an integrated untargeted lipidomics and metabolomics approach utilizing an ultra-high-performance liquid chromatography–Orbitrap-mass spectrometry (UPLC–Orbitrap-MS) analytical platform to provide a comprehensive metabolic profile of conventional and organic lettuce. The aim was to evaluate the impact of different farming practices and assess the feasibility of differentiating lettuce based on cultivation methods. To broaden the metabolite coverage, a liquid-liquid extraction (LLE) protocol was implemented to separate the polar metabolites and lipids. The polar fraction was examined using a hydrophilic interaction liquid chromatography (HILIC) column to enhance the detection of polar metabolites. For lipid identification, a detailed mass fragmentation analysis was conducted and validated against commercial lipid standards to ensure structural accuracy. Furthermore, a correlation-based metabolite network analysis was performed to further examine the relationships among metabolites. This systems-level analysis provided insights into the physiological status of lettuce and revealed interconnections among primary, secondary, and lipid metabolites in relation to cultivation practices. Overall, this integrative analytical strategy offers a more comprehensive view of metabolic alterations induced by farming systems and enables better identification of potential biomarkers associated with organic vegetables.

2. Materials and methods

2.1 Reagents

Deuterated cholic acid (2,2,4,4-d₄) and L-tryptophan (indole-d₅) were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The complex lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA), including 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (PA (16:0/18:1)), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (PG (16:0/18:1)), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol (PI (16:0/18:1)), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (PS (16:0/18:1)), N-16:0 phytosphingosine (Cer(t18:0/16:0)), N-24:0(2R-OH) phytosphingosine (Cer(t18:0/24:0(2OH))), glucosylceramide (GluCer), digalactosyl diacylglycerols (DGDGs), monogalactosyl diacylglycerols (MGDGs) and sulfoquinovosyl diacylglycerols (SQDGs). Ammonium formate, mono-, di-, & triglyceride mixture, tryptophan, phenylalanine and indole-3-acetic acid (IAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetonitrile, chloroform, isopropanol, and methanol were purchased from Duksan Pure Chemicals (Gyeonggi-do, South Korea). Optima LC/MS grade formic acid was obtained from Fisher Chemical (Radnor, PA, USA). Double-deionised water was freshly prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Alpha-ketoglutarate was acquired from Honeywell International (Phoenix, AZ, USA). Quinic acid and 7-hydroxycoumarin were acquired from LGC Labor GmbH (Augsburg, Germany). Shikimic acid was obtained from Acros Organics (Morris Plains, NJ, USA). Ferulic acid and vanillin were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan).

2.2 Sample collection

A total of twenty-six lettuce samples (*Lactuca sativa* L. cv. Seven Wonders) were collected from local farms in Hong Kong, China. To capture a comprehensive representation of the regional agricultural landscape, the sampling strategy aimed to encompass all active lettuce-producing farms in the territory. Specifically, organic lettuce samples (LO, n = 16) were sourced from local farms certified by the Hong Kong Organic Resource Centre (HKORC) and supplied by the Vegetable Marketing Organization (VMO). Conventional lettuce samples (LF, n = 10) were cultivated on local farms operating under the Hong Kong government's Accredited Farms Scheme and were obtained directly from individual local farmers. Detailed sample collection information is provided in the supplementary material (Table S1).

2.3 Sample preparation for extended metabolomics analysis

A quality control (QC) sample was prepared following the methodology described in our previous study on water spinach (Man et al., 2024). To extract the lipids and small molecules from the lettuce samples, a modified liquid-liquid extraction method was used, employing a chloroform/methanol/water solvent system, as outlined in the earlier investigation. This approach enabled the separation of lipids into the organic fraction and small molecules into the aqueous fraction. Both fractions were immediately stored at -80 °C after nitrogen stream drying until analysis by UPLC-MS.

For the aqueous profile analysis, the dried aqueous layer was re-dissolved in 150 μ L of pre-chilled HILIC solvent (85% v/v acetonitrile: 15% v/v water with 10 mM ammonium acetate, pH 3) spiked with internal standards (1 ppm cholic acid- d_4 and tryptophan- d_5). After centrifugation at 14,000 rpm for 15 min at 4°C, the supernatant was transferred into an HPLC vial for HILIC-UPLC-Orbitrap-MS analysis. For the lipid profile analysis, the dried organic layer was re-dissolved in 150 μ L of pre-chilled lipid solvent (65% v/v acetonitrile: 30% v/v isopropanol: 5% v/v water) spiked with an internal standard (1 ppm cholic acid- d_4). After centrifugation at 14,000 rpm for 15 min at 4°C, the supernatant was transferred into an HPLC vial for RP-UPLC-Orbitrap-MS analysis.

2.4 UPLC-Orbitrap-MS based extended metabolomics analysis

A Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was connected to a Waters ACQUITY UPLC System (Waters Corp., Milford, MA, USA) via a heated electrospray ionisation (H-ESI) interface.

The untargeted metabolomics analysis of the aqueous fraction was conducted using our previously established HILIC-UPLC-MS method (Man et al., 2024). Chromatographic separation was achieved using a Merck SeQuant ZIC-cHILIC column (100 mm $\times$ 2.1 mm, 3 μ m) with a corresponding Merck SeQuant ZIC-cHILIC guard column (2.1 mm $\times$ 20 mm) (Merck, Darmstadt, Germany) at a column temperature of 60°C. The mobile phase consisted of 10 mM ammonium acetate (pH 3) in water (A) and 85% v/v acetonitrile (B). The gradient elution program was as follows: 0–0.5 min, 98% B; 0.5–7.5 min, 98–70% B; 7.5–8 min, 70–50% B; 8–9.5 min, 50% B; 9.5–11 min, 50–98% B; 11–15 min, 98% B for re-equilibration.

For lipid profiling of the organic fraction, a modified reverse-phase (RP)-UPLC-MS method was applied, incorporating a shortened elution program based on our validated untargeted lipidomics workflow (Man et al., 2021). Chromatographic separation was carried out using a Waters ACQUITY UPLC HSS T3 column (2.1 mm $\times$ 100 mm, 1.8 μ m) with an HSS T3 pre-column (2.1 mm $\times$ 5 mm, 1.8 μ m) at a column temperature of 40°C. A gradient elution was applied using solvent A (60% v/v water: 40% v/v acetonitrile containing 10 mM ammonium formate and 0.1% formic acid) and solvent B (90% v/v isopropanol: 10% v/v acetonitrile containing 10 mM ammonium formate and 0.1% formic acid). The RP gradient for lipid separation was as follows: 0–1 min, 30% B; 1–3 min, 30–65% B; 3–4 min, 65–70% B; 4–6 min, 70–75% B; 6–7 min, 75–80% B; 7–8 min, 80–85% B; 8–10 min, 85–90% B; 10–11.5 min, 90–97% B; 11.5–13 min, 97% B; 13–14 min, 97–30% B; 14–16 min, 30% B for re-equilibration.

For both methods, a 3- μ L sample was injected and eluted at a flow rate of 0.3 mL/min, with the sample chamber temperature maintained at 4°C. Mass spectrometry settings were also consistent across both analyses. The H-ESI source parameters were as follows: a spray voltage of 3500 V for (+)ESI and 2300 V for (–)ESI; nebulizer auxiliary gas flow set to 10 arbitrary units; sheath gas flow at 35 arbitrary units; and sweep gas flow at 0 arbitrary units. The ion transfer tube temperature and vaporizer temperature were set at 350°C and 300°C, respectively. MS1 spectra were acquired over m/z 70–1200 at Orbitrap resolution 120,000, with AGC target of 2.0×10^5 and maximum injection time 100 ms. Data-dependent

MS/MS (DDA) acquisition was performed using higher-energy collisional dissociation (HCD) at stepped normalized collision energies of 15, 30, and 45 eV. Precursors were isolated in the quadrupole using a 1.6 m/z isolation window and analyzed at an MS/MS resolution of 15,000. Other DDA parameters included a dynamic exclusion duration of 6 s with a mass tolerance of ± 10 ppm, a charge state selection of 1, and a minimum precursor intensity threshold of 1.0×10^4 . The automatic gain control (AGC) target was set to standard, with an automated maximum injection time.

2.5 UPLC-Orbitrap-MS data preprocessing

The preprocessing of the UPLC-MS data was performed in Progenesis QI (version 2.0; Nonlinear Dynamics) according to the established methodology outlined in our previous study on water spinach (Man et al., 2024). This process included essential steps such as peak picking, alignment, deconvolution, and peak area fitting and filtering. Peak detection, chromatographic deconvolution, retention-time alignment, isotope grouping, adduct annotation, and peak-area integration were performed separately for each chromatographic platform and ionization mode. Peak picking was performed using the automated co-detection algorithm, with sensitivity set to automatic (default level 3) and a minimum peak width constraint of 0.1 min. Compound identification was subsequently conducted using the MetaScope search engine with a precursor mass tolerance of 5 ppm and a fragment mass tolerance of 10 ppm. Features detected in procedural blanks were removed when the blank/sample intensity ratio exceeded 20% or when the feature was not at least 3-fold higher in biological samples than in blanks. Features were further filtered based on QC reproducibility, and only features with a coefficient of variation (CV) below 20% across pooled QC injections and present in at least 80% of samples within one group were retained for statistical analysis. Missing values were imputed using half of the minimum detected value for that feature. The scaling mode applied in the multivariate statistical analysis was unit variance. The potential marker selection was based on the variable importance in the projection (VIP, threshold ≥ 1.0) and jackknife confidence.

In the metabolomics platform, isotopes and common adducts, including $[M+H]^+$, $[M+Na]^+$ and $[M-H_2O+H]^+$ for (+)ESI, $[M-H]^-$ and $[M+FA-H]^-$ for (-)ESI, were grouped based on accurate mass and retention-time coelution. Conversely, in the lipidomics analysis, software limitations regarding certain predominant adducts required all features to initially be processed as $[M+H]^+$ and $[M-H]^-$. Final adduct grouping for the lipid data was subsequently refined using subclass-specific ionization behaviors and empirical MS/MS fragmentation patterns established through our rigorous in-house analysis.

2.6 Statistics analysis

The data matrix was subjected to principal component analysis (PCA) after unit variance scaling (autoscaling). The orthogonal projections to latent structures discriminant analysis (OPLS-DA) were applied using the Python package pypls (<https://github.com/Omicometrics/pypls>, version 1.0.0) with leave-one-out cross-validation and Pareto scaling. To evaluate the statistical robustness of the OPLS-DA model, a permutation test with

10,000 iterations was conducted. Potential marker differences between sample groups were assessed using a two-tailed, unpaired Student's t-test, with statistical significance defined as $p \leq 0.05$. The jackknife confidence interval method was further applied to validate the identified discriminative markers, which have variable importance in projection (VIP) score > 1 and $p \leq 0.05$.

2.7 Potential markers identification and validation

Following multivariate statistical analysis, discriminant features exhibiting a VIP score > 1 , $p \leq 0.05$ and robust jackknife confidence intervals were selected for downstream structural identification. The annotation of potential markers in the aqueous fraction was achieved by combining literature review, online database matching, and direct experimental alignment with the retention times and MS/MS fragmentation patterns of authentic commercial standards. Specifically, metabolite annotation was conducted using the METLIN database (<https://metlin.scripps.edu/>) and the RefMetaPlant database (<https://www.biosino.org/RefMetaDB/>). For lipid marker identification, our independently developed in-house lipid fragmentation database was utilized in conjunction with the LIPID MAPS database (<http://www.lipidmaps.org/>).

2.8 Correlation network analysis

Metabolite–metabolite correlation networks were constructed separately for conventional and organic lettuce samples using the probabilistic context likelihood of relatedness (PCLRC) algorithm (Saccenti et al., 2015). Pearson correlation coefficients (PCCs) were calculated to assess pairwise associations between metabolites. Due to the limited sample size, a bootstrap subsampling approach was employed: in each iteration, 75% of the samples were randomly selected to construct the correlation network, and the top 30% of PCCs were retained as valid associations. This procedure was repeated 10,000 times. Metabolite pairs that were consistently identified as valid associations in over 90% of the iterations were used to calculate metabolite connectivity values, defined as the sum of absolute PCCs between a metabolite and all others (Jahagirdar & Saccenti, 2020).

Differences in metabolite–metabolite correlation networks between conventional and organic lettuce were assessed by comparing metabolite connectivity values. To determine statistical significance, permutation tests were performed by independently randomizing the concentrations of each metabolite and recalculating connectivity differences using the PCLRC algorithm over 10,000 iterations. The resulting p-values were adjusted using the Benjamini–Hochberg (BH) method to control the false discovery rate (FDR) at 0.05. The Python package used for differential network analysis is available at: <https://github.com/Omicometrics/pclrc>.

3. Results and Discussion

3.1 Aqueous and lipid profiles of lettuce

Representative MS chromatograms of the aqueous and organic layers of lettuce samples, acquired under both positive and negative ionization modes using UPLC-Orbitrap-MS, are provided in the Supplementary Material (Fig. S1). The aqueous metabolite profile of lettuce was comprehensively characterized using the HILIC-UPLC-Orbitrap-MS platform. Using the HILIC-UPLC-Orbitrap-MS metabolomics platform, we identified 69 aqueous metabolites in lettuce with confidence levels of MSI 1 and 2. Consistent with findings from water spinach (Man et al., 2024), lettuce exhibited a diverse range of water-soluble primary metabolites, including amino acids, organic acids, purines, tricarboxylic acids, sugars, and their derivatives. In addition, polar to semi-polar secondary metabolites such as vitamins, flavonoids, phenolic compounds, and phenylpropanoids were also detected. The lipid profile of the organic layer was investigated using the RP-UPLC-Orbitrap-MS platform. A wide variety of lipid subclasses were identified in the lipidomic profiles of the organic fraction (Fig. 1a). Glycerophospholipids (GPLs) constituted the most abundant subclass (30%), followed by glycosylglycerolipids (glycosyl-GLs, 23%) and glycerolipids (GLs, 20%). Other lipids, including sphingolipids (SPs, 15%), fatty acids (FAs, 6%), and plant pigments and benzoquinones (6%), were also detected. In accordance with the literature (Mongrand et al., 1998), which reports the absence of DGDG (18:3/16:3) in lettuce, our mass fragmentation analysis similarly confirmed the lack of this lipid species. This result supports the classification of lettuce as an 18:3 plant, indicating the absence of a plastidial prokaryotic pathway in its lipid biosynthesis.

3.2 Reliability of MS results for subsequent multivariate analysis

The selection of metabolite characteristic of lettuce grown in different cultivation practices requires a comprehensive comparison of MS results obtained from the metabolomics and lipidomic analysis. To evaluate the stability and reliability of the MS instrumental response throughout the entire analysis, aliquots of a pooled quality control (QC) sample were injected regularly within the sample sequence. The QC samples from both the aqueous metabolite profiles obtained using HILIC-UPLC-Orbitrap-MS (HILIC-MS) (Fig. 1b and c) and the lipid profiles from HSS T3-UPLC-Orbitrap-MS (HSS T3-MS) (Fig. 1d and e) formed tight clusters in the corresponding PCA score plots. This indicates that a robust instrumental performance has been maintained, and the observed sample group differences are primarily due to differences in farming practices.

A feature-selection workflow was applied independently to each platform and ionization mode. Raw features were first filtered by blank contribution, QC reproducibility and missing-value frequency. The retained feature matrices were then used for PCA and OPLS-DA. To characterize cultivation-specific metabolic alterations, an OPLS-DA model was constructed using cross-validation (Fig. 2). The cross-validated OPLS-DA score plot (Fig. 2a) demonstrates the separation between organic and conventional lettuce samples based on their metabolomic/lipidomic profiles. Most platforms showed a separation between organic and conventional lettuce, except positive-mode metabolomics. S-plots (Fig. 2b) for individual platforms were used to discover the variables with the greatest contribution and reliability for class discrimination and potential biomarker selection. To evaluate the risk of model overfitting, permutation tests were performed. The resulting

permutation plots (Fig. 2c), generated from 10,000 random permutations, consistently showed negative Q^2 intercepts, confirming that the observed group separations were statistically robust and not due to overfitting. Features contributing to group separation were prioritized using $VIP > 1$ and further evaluated using jackknife confidence intervals (Fig. 2d). The number of detected features, retained features after QC filtering, VIP-selected features, jackknife-supported features, and MSI Level annotated features is summarized in Table S2. The untargeted data-processing workflow successfully reduced the immense chemical complexity of the raw datasets to a highly refined cohort of robust biomarkers. Overall, the multivariate analyses demonstrate that cultivation practice is a major driver of metabolic variation in lettuce, with lipidomics providing stronger discrimination than metabolomics.

3.3 Aqueous metabolite signature of organic lettuce

This study also showed a remarkable separation between organic and conventional lettuce in the OPLS-DA plots of HILIC-based metabolomics platforms (Fig. 2a). In the metabolomics platforms, a total of 334 and 375 features exhibited VIP scores greater than 1 in the positive and negative ionization modes, respectively. Of these, 135 features in the positive mode and 243 in the negative mode passed the jackknife resampling analysis. Database searches enabled the tentative annotation of 38 features, among which 16 were confirmed with reference standards and assigned Metabolomics Standards Initiative (MSI) Level 1 identification (Table S3). Additional features assigned to MSI Levels 3 and 4 also contributed to the OPLS-DA separation but were excluded from biological interpretation because insufficient MS/MS information, unavailable diagnostic ions, or mismatches with commercial standards precluded reliable structural assignment. Thus, the annotated metabolites represent a chemically validated subset of the broader discriminatory metabolomic profile. Previous studies have shown that environmental stresses primarily impact lettuce by altering primary metabolites such as amino acids, TCAs, and sugars (Chung et al., 2022; Gao et al., 2022; Najafi et al., 2022; Savarese et al., 2022; Zhao et al., 2016; Zhou et al., 2021b) with comparatively limited effects on secondary metabolites. In this study, primary metabolites, particularly simple sugars, tricarboxylic acids, and most amino acids, did not show significant variation between organic and conventional lettuce samples. Because relative abundance measurements provide a static representation of metabolite levels, these findings do not directly indicate changes in metabolic flux or pathway activity. Therefore, the following discussion focuses strictly on differences in metabolite abundance rather than inferring pathway rates.

Organically farmed lettuce consistently exhibited higher relative abundances of thirteen metabolites compared to conventionally farmed samples in the aqueous fractions. These included two aromatic amino acids, phenylalanine and tryptophan, one plant hormone, indole-3-acetic acid (IAA), and ten secondary metabolites: chicoric acid, chlorogenic acid, coumarin, dihydroferulic acid 4-O-glucuronide, ferulic acid, neochlorogenic acid, protocatechuic acid, quercetin-4'-glucuronide, quinic acid, and vanillin. A simplified metabolic pathway linking these compounds is illustrated in

Fig. 3 and the corresponding heatmap illustrating their abundance changes is provided in Fig S12. Collectively, these metabolites identified not only reflected stress resilience in organic systems but also potentially serve as promising biomarkers to distinguish organically cultivated lettuce from conventional counterparts. Although shikimic acid did not differ significantly between cultivation systems, the overall metabolite profile suggests that organic cultivation is associated with the enrichment of phenolic compounds, aromatic amino acids, and IAA. As this study employed a semi-quantitative metabolomics approach, the observed differences reflect relative abundances rather than absolute concentrations, further validation with targeted quantification would significantly strengthen future investigations.

Phenolic compounds are key antioxidants in plants, playing critical roles in regulating growth and providing defense against biotic and abiotic stresses (Kumar et al., 2020; Sambangi, 2022). Environmental stresses can induce oxidative stress, which activates oxidative signaling pathways and leads to the biosynthesis and accumulation of secondary metabolites, particularly phenolic compounds (Oliveira et al., 2013). In this study, organically cultivated lettuce exhibited higher relative abundances of ten phenolic compounds compared to conventionally grown counterparts. The relative increase of phenolic compounds aligns with previous findings in the Asteraceae family. For instance, an NMR-based metabolomics study showed increased chicoric acid in lettuce treated with compost tea derived from aerobically fermented plant materials (Lanzotti et al., 2022). Similarly, globe artichoke, another edible plant from the Asteraceae family, demonstrated higher levels of five phenolic compounds, including apigenin, caffeic acid, chlorogenic acid, ferulic acid, and luteolin, under organic versus conventional management (Salata et al., 2022). These consistent trends suggest that the phenylpropanoid pathway and its downstream metabolites are particularly responsive to organic cultivation practices in Asteraceae species. Beyond plant stress response, phenolics are widely recognized for their roles in plant defense, and stress responses, as well as for their well-documented anti-inflammatory and antidiabetic properties (Cheng et al., 2014; Zekrumah et al., 2023).

Aromatic amino acids such as tryptophan and phenylalanine play crucial roles in plant physiology, serving not only as essential components for protein synthesis but also as key precursors for various secondary metabolites, including flavonoids, phenolic compounds, and phenylpropanoids. These metabolic derivatives are crucial for plant defense and overall stress response. Moreover, tryptophan is a well-established precursor in the biosynthetic pathway of indole-3-acetic acid (IAA), a fundamental auxin that regulates diverse aspects of plant growth and development, including cell division, elongation, and fruit formation (Zhao, 2012). In the present study, organically cultivated lettuce exhibited consistent upregulation of tryptophan, phenylalanine, and IAA compared to conventionally farmed lettuce.

An elevated relative abundance of specific amino acids is frequently highlighted as a notable metabolic feature of organically cultivated plant products (Rembiałkowska, 2007). Under limited nitrogen-supply conditions, lettuce has been shown to accumulate certain metabolites such as chlorogenic acid, phenylacetic acid, and tryptophan (Zhou et al., 2021b). In addition to nitrogen availability, phosphorus deficiency has been implicated in the regulation of aromatic amino acid biosynthesis, particularly phenylalanine and tryptophan. This response has been well

documented in the roots of several plant species, including peanut roots (Wu et al., 2022), rice roots (Tawaraya et al., 2013) and tea roots (Kc et al., 2022). However, the response of above-ground tissues, such as leaves, to phosphorus limitation, especially in terms of aromatic amino acid metabolism, has barely been studied. Therefore, the elevated levels of aromatic amino acids observed in organically grown lettuce leaves cannot be readily attributed to nutrient limitation alone.

Recent studies have highlighted that indole-3-acetic acid (IAA), a key phytohormone involved in regulating plant growth and development, is synthesized not only by plants but also by certain beneficial rhizosphere bacteria. In the rhizosphere, IAA functions as a signaling molecule that significantly influences plant-microbe interactions (Etesami & Glick, 2024). For example, using ToF-SIMS imaging, Zhang et al. provided molecular evidence of IAA biosynthesis by the Gram-negative plant growth-promoting rhizobacterium *Pseudomonas fluorescens* in *Brachypodium* seeds (Zhang et al., 2021b). In parallel, Feng et. al reported that phenylalanine, a common metabolite in compost, alters soil bacterial community composition, increasing the abundance of plant growth-promoting bacteria that are positively associated with plant biomass, including *Burkholderia-Caballeronia-Paraburkholderia*, *Azospirillum*, *Cupriavidus*, and *Achromobacter* (Feng et al., 2023). These findings suggest that organic farming practices, particularly the use of amino acid-rich composts, may indirectly promote plant health by modulating the rhizospheric microbiome. This microbial shift may enhance biological nitrogen fixation and stimulate microbial phytohormone production, especially IAA, thus improving the plant's capacity to cope with various environmental stresses associated with cultivation.

3.4 Mass fragmentation analysis for complex lipid identification

Although lipidomics is valuable for authenticating agricultural practices, untargeted lipid annotation remains constrained by the scarcity of reference standards, making MSI Level 1 annotation difficult. Structural validation therefore relies heavily on rigorous MS/MS interpretation. Five major challenges persist: mobile-phase- and subclass-dependent multi-adduct formation; database omission of uncommon adducts and in-source fragments; adduct-specific fragmentation patterns; co-elution and isobaric overlap of structural isomers; and the absence of stereospecific stereospecific (*sn*) notation in reference databases, which limits acyl-chain positional assignment using diagnostic fragment intensity ratios (Higashi & Saito, 2019). Therefore, lipid identification in this study was not based solely on accurate mass or database matching. Instead, lipid annotation was performed using a multi-criteria workflow centred on an in-house lipid fragmentation library. The library was generated from authentic commercial standards analysed under identical RP-LC-Orbitrap-MS conditions and covers major adduct species and diagnostic fragments in both ionisation modes for 29 lipid subclasses relevant to plant lipid metabolism, including fatty acyls, glycerolipids, glycerophospholipids, glycolipids, and sphingolipids. Annotation confidence was strengthened by integrating library-based MS/MS matching with precursor mass accuracy, retention behaviour, adduct form, subclass-specific headgroup ions, fatty acyl fragments, neutral-loss ions, and, where

applicable, diagnostic fragment intensity patterns for acyl-chain positional assignment. Representative fragmentation spectra for the significantly altered lipid subclasses are provided in Figs. S2–S11.

Glycosylglycerolipids (glycosyl-GLs), including MGDG, DGDG and SQDG, were detected in both positive and negative ionisation modes. Although their structural elucidation has predominantly relied on positive-mode MS/MS of adduct ions (Jouhet et al., 2017; Tatituri et al., 2012), particularly predominant ammonium adduct ions $[M+NH_4]^+$ (Fig. S2, S4a), we found that negative ionisation provided more direct structural information for MGDG and DGDG. Specifically, the carboxylate ions required for fatty acyl identification were absent from the $[M+NH_4]^+$ spectra. In negative mode, MGDG and DGDG produced both $[M+HCOO]^-$ and $[M-H]^-$ ions (Fig. S3), whereas SQDG was detected predominantly as $[M-H]^-$ (Fig. S4b). For MGDG and DGDG, negative-mode spectra provided galactose-related headgroup ions and fatty acyl carboxylate ions, which enabled subclass assignment and acyl-chain identification. The shared galactose-related fragment at m/z 235.0816, the MGDG-specific ion at m/z 253.0935, and DGDG-related ions at m/z 379.1238, 397.1354 and 415.1448 were used as diagnostic evidence. SQDG species were assigned using sulfoquinovosyl headgroup fragments at m/z 80.9651, 125.0243, 164.9865 and 225.0080, together with fatty acyl carboxylate ions and neutral-loss fragments. Fatty acyl composition was confirmed by diagnostic carboxylate ions, and *sn*-positions were inferred from their relative intensities when supported by authentic standard spectra, with the *sn*-2-derived carboxylate ion exhibiting greater intensity than the *sn*-1-derived ion. For example, the QC ion at m/z 815.4989 was annotated as SQDG (16:0/18:3), while the ion at m/z 937.5918 was assigned as DGDG (18:2/18:3).

Glycerophospholipids (GPLs), including PA, PC, PE, PG, and PI, were detected in lettuce in both ionisation modes, but fatty acyl chain lengths were resolved only in negative mode. Except for PC, which predominantly formed the formate adduct $[M+HCOO]^-$, PA, PE, PG and PI were mainly detected as deprotonated ions $[M-H]^-$ (Fig. S5-7). Common GPL headgroup fragments at m/z 152.9958 and 78.9589 supported subclass assignment, while PG showed an additional diagnostic ion at m/z 227.0325. PI was further characterised by inositol-related fragments at m/z 223.0013, 241.0120, 297.0380 and 315.0488. Fatty acyl chains were identified from carboxylate ions and neutral losses, with the stronger fragment indicating the *sn*-2 position. Based on these criteria, representative QC ions were annotated as PG (18:1/16:0), PA (18:2/18:3) and PI (16:0/18:3). The fragmentation behaviour of PC and PE was consistent with that described in our previous study (Man et al., 2021).

Plant sphingolipids were annotated using complementary information from both ionisation modes. Phytoceramides (Cert) were mainly interpreted in positive ionisation mode (Fig. S8), where the protonated ion $[M+H]^+$ and sequential dehydration ions $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and

$[M+H-3H_2O]^+$ reflected the hydroxylated long-chain base structure (Hsu, 2021). In the standard Cer (t18:0/16:0), the phytosphingosine ion at m/z 318.2983 and its dehydrated fragments confirmed the long-chain base and hydroxylation pattern, while the amide-linked fatty acid fragment at m/z 256.2620 supported acyl-chain assignment. Similar fragmentation was observed for α -hydroxylated fatty acid-containing phytoceramides (Cert- α OHFA), supporting the annotation of Cert and Cert- α OHFA species in the QC samples. Glucosylceramides (GluCer) were mainly confirmed in negative ionisation mode (Fig. S9), which provided useful information for distinguishing GluCer from related glucosylceramide isomers (Han & Cheng, 2005). The standard GluCer (d18:2/16:0(2OH)) produced $[M-H]^-$ and $[M+HCOO]^-$ ions, together with neutral losses of the glucose moiety and low-mass sugar-derived fragments, which is more complex than Han and Cheng study due to the higher resolution of Orbitrap-MS. The agreement between standard and QC spectra supported the confident annotation of GluCer species.

Glycerolipids, including DAG and TAG, were characterised in positive ionisation mode because negative ionisation generated limited structural fragments. Both subclasses were predominantly observed as ammonium adducts $[M+NH_4]^+$. DAG species were assigned using the corresponding protonated ion, dehydrated ion, monoacylglycerol-type fragments and fatty acid ketene ions (Fig. S10). TAG species were annotated from diagnostic diacylglycerol fragments, dehydrated monoacylglycerol fragments and fatty acid ketene ions generated by the neutral loss of constituent fatty acids (Fig. S11). Using this approach, representative sample ions were assigned as DAG (18:3/18:3) and TAG (18:0/18:1/18:2), consistent with the fragmentation behaviour of the corresponding standards.

Although this workflow cannot fully resolve all structural features, such as double-bond position, cis/trans geometry, or every possible co-eluting isomer, it provides a high-confidence annotation strategy for untargeted plant lipidomics. Importantly, the reported lipid species were assigned only when accurate mass, chromatographic behaviour, adduct type, subclass-specific fragments and fatty acyl-related ions were mutually consistent with standard-derived fragmentation rules. Within the current practical limitations imposed by the limited availability of authentic lipid standards, this RP-LC-Orbitrap-MS/MS scheme provides a robust and conservative basis for lipid species annotation across the major lipid subclasses detected in lettuce, providing the structural confidence required to interpret the cultivation-associated lipid differences described in Section 3.5.

3.5 Lipid signature of organic lettuce

Changes in lettuce lipid composition in response to environmental and cultivation conditions remain poorly characterized because of the limited lipid coverage of conventional analytical platforms. Previous studies have reported only a small number of responsive fatty acids, particularly palmitic acid (FA 16:0) (Gao et al., 2022; Najafi et al., 2022; Savarese et al., 2022) and myristic acid (FA 14:0) (Gao et al., 2022; Savarese et al., 2022) under various environmental changes. In the present study, a systematic complex-lipid identification workflow was used to characterize differences in lipid profiles between organic and conventional lettuce. OPLS-DA of the untargeted RP-UPLC-Orbitrap-MS lipidomics data clearly

distinguished the two cultivation systems (Fig. 2a). A total of 644 and 583 features with $VIP > 1$ were identified in the positive and negative ionization modes, respectively. Subsequent jackknife resampling analysis retained 166 features in the positive mode and 295 in the negative mode. Based on matches with established lipid databases and our in-house spectral library, 37 features were successfully annotated with MSI Level 2 confidence (Table S2), with all targeted annotations successfully resolved beyond the basic sum-composition level. Although a substantial portion of features assigned to MSI Levels 3 and 4 also contributed to the OPLS-DA separation, they were excluded from biological discussion interpretation because of insufficient MS/MS spectra and the absence of diagnostic ions for lipid headgroups and fatty acyl chains, hereby avoiding speculative structural and biochemical interpretations. Consequently, the identified lipids represent a chemically validated subset of the broader discriminatory lipidomic profile. The validated discriminatory lipids are summarized in Fig. 4, and their abundance patterns are displayed in Fig. S13. Organic lettuce generally exhibited lower levels of TAG, DAG, and SQDG species, whereas PE, PG, and PC showed species-dependent changes. Fatty acids and phytoceramides also exhibited variable responses. Collectively, these findings indicate that cultivation system is associated with remodeling across multiple lipid classes in lettuce.

Changes in SQDG and PG subclasses are noteworthy because these anionic lipids are important components of photosynthetic membranes. In this study, conventional lettuce showed higher relative abundances of SQDG, TAG and DAG, while showing lower levels of PG compared to organically grown samples. The potential causes of these changes may relate to variations in phosphorus availability and salt stress. It is well-documented that SQDGs substitute PGs in the plastidial membranes under phosphate-starvation conditions across a broad range of photosynthetic organisms, including cyanobacteria (Abida et al., 2015; Sato et al., 2000; Van Mooy et al., 2009), phytoplankton (Maat et al., 2016), and model plant *Arabidopsis thaliana* (Essigmann et al., 1998; Maat et al., 2016), while previous studies have linked the accumulation of GLs, including TAGs and DAGs, to phosphorus deprivation in various plant tissues such as in *Arabidopsis* (Gaude et al., 2008; Nakamura et al., 2005; Pant et al., 2015), tomato (Pfaff et al., 2020), and soybean (Okazaki et al., 2017). Beyond phosphorus dynamics, the observed lipid profiles in different could hypothetically relate to differing levels of salt or osmotic pressures between the cultivation systems. Under salt stress, SQDG levels often rise to support membrane fluidity (He & Ding, 2020). Concurrently, salinity-induced osmotic stress can trigger the accumulation of TAGs in lipid droplets, helping cells adjust to altered osmotic potentials (Karimi et al., 2021; Shetty et al., 2019). In plant leave tissues, these dynamic lipid reservoirs can undergo hydrolysis to generate ATP (Matkowski & Daszkowska-Golec, 2023), providing the necessary metabolic energy to fuel proton pumps involved in stomatal regulation (McLachlan et al., 2016; Pautov et al., 2022). While the observed lipidomic profiles closely align with documented stress responses to phosphorus limitation and salinity, confirming these specific physiological mechanisms remains hypothetical without direct tissue-nutrient analysis. Such physiological validation falls beyond the scope of the present study.

A notable feature of the lipidomic profile of organically grown lettuce was the altered partitioning of FA16:0 and FA18:3 among lipid subclasses. Reduced abundances were observed in DAG, TAG, SQDG, and lyso-GPLs, whereas selected PC and PE species accumulated under organic cultivation (Fig 4). Given the central role of PC in plant glycerolipid metabolism (Botella et al., 2017), the observed accumulation of PC (18:3/18:3), a terminal product of the eukaryotic glycerolipid biosynthesis pathway, may indicate enhanced desaturation of FA18:2 to FA18:3 through increased fatty acid desaturase 3 (FAD3) activity, the enzyme responsible for introducing the third double bond into linoleic acid (FA 18:2) to form linolenic acid (FA 18:3) (He et al., 2020). Similarly, the elevated abundances of PE (16:0/18:1) and PE (16:0/18:2) may reflect increased conversion of DAG to PE via aminoalcohol phosphotransferase (AAPT), the enzyme responsible for PE biosynthesis in non-plastidial membranes (Nakamura, 2017). While these observations suggest altered glycerolipid metabolism in organically cultivated lettuce, confirmation of changes in metabolic fluxes and enzyme activities will require targeted biochemical analyses beyond the scope of the present untargeted metabolomics study.

3.6 Authentication power of lipidomics platform and metabolomics platform

Our previous study on water spinach (Man et al., 2024) demonstrated the remarkable discriminatory power of a HILIC-based metabolomics platform in differentiating between various farming practices, with classification accuracies ranging from 80% to 100%. Similarly, the present study achieved comparable discrimination rates (80% to 100%) between organic and conventional cultivation systems in lettuce using an untargeted metabolomics approach (Fig. 2a). Comparing to the polar metabolites, lipidome responses to cultivation and environmental factors remain relatively underexplored in foodomics research. Using an untargeted lipidomics platform, we identified significant alterations across multiple lipid subclasses in response to organic cultivation, as discussed in Section 3.4. Notably, lipidomics achieved 100% classification accuracy for organically grown lettuce (Figure 2a), highlighting its considerable potential for farming practice verification and origin authentication. Moreover, the pronounced changes observed in lipid composition indicate that lipid metabolism is highly responsive to cultivation conditions. While most previous metabolomics studies have focused on aqueous-phase metabolites such as amino acids, sugars, and secondary metabolites, our findings demonstrate that lipids provide complementary information and represent an important component of plant responses to agricultural management practices. These results show that integrating metabolomics and lipidomics enables a more comprehensive characterization of biochemical variation between cultivation systems. Although less frequently investigated, lipids exhibit distinct and reproducible responses to environmental and agronomic factors, underscoring the value of lipidomics as both a tool for crop authentication and a means of understanding plant metabolic adaptation.

3.7 Primary-secondary metabolites association networks

Pathway analysis is widely used to study metabolic interconnections and understand how environmental factors regulate biological processes in model animals and plants. However, lipid metabolism is often underrepresented in most pathway analysis tools. Technical challenges in

constructing comprehensive lipidomics maps, together with the oversimplification of lipid diversity in many most open-source databases and commercial pathway analysis software, for example, assigning identical identifiers to different lipids within the same subclass. Although lipid-specific databases exist, they are primarily based on human and mouse data, and therefore lack many plant-specific lipids, including SQDGs and MGDGs. This limitation is significant because growing evidence indicates that lipids play central roles in plant growth, development, and stress adaptation. For instance, phytoceramides containing hydroxylated and saturated very-long-chain fatty acids are essential for fiber cell elongation in cotton (Chen et al., 2021), while sphingolipid biosynthesis dynamics in Arabidopsis are tightly regulated and central to metabolic balance (Osinuga et al., 2024). Furthermore, membrane lipid remodeling, involving changes in lipid head groups, acyl-chain lengths, and degrees of unsaturation, is a key mechanism by which plants adapt to environmental stress (Yu et al., 2021). Consequently, expanding plant lipid databases is essential for improving our understanding of plant physiology and responses to environmental and climate-related challenges.

Given these limitations in current pathway analysis platforms, we integrated the lipidomics and metabolomics results to construct a overall metabolic pathway map illustrating the relationships among discriminant metabolites identified in lettuce grown under different cultivation methods (Fig. 5). Notably, no common nodes linking lipid and aqueous metabolites were identified. While this pattern could reflect variations in how these metabolic domains adapt to farming conditions, the absence of shared nodes does not necessarily imply independent regulatory mechanisms.

To better understand the global correlations among the biomarkers identified in lettuce, we constructed an association network integrating aqueous and lipid metabolites (Fig. 6) using the Probabilistic Context Likelihood of Relatedness on Correlation (PCLRC) algorithm (Saccenti et al., 2015). In this network, metabolites are represented as nodes and significant correlations as edges, providing a systems-level view of metabolic coordination. Differences in network topology reflects cultivation-dependent changes in metabolic regulation, stress responses, and nutrient utilization, and could potentially serve as distinctive signatures for the authentication of organically grown lettuce. The metabolite association networks for organic (Fig. 6a) and conventional farming (Fig. 6b) were constructed using a total of 75 metabolites identified through HILIC-based metabolomics and lipidomics analysis. Comparison of the two networks (Fig. 6c) revealed that 16 metabolites exhibited significantly different connectivity patterns ($P < 0.05$), with corresponding Pearson correlation coefficients (PCC) provided in Tables S4 and S5. These differential connectivity patterns highlight metabolites that may be particularly responsive to cultivation practices and therefore useful for biomarker selection. Distinct network topologies were observed between the two cultivation systems, with the organic network displaying a greater degree of

connectivity among aqueous and lipid metabolites than the conventional network. This broader metabolic coordination suggests that organic cultivation may induce more extensive interactions among metabolic pathways.

A shift in the accumulation of phenolic compounds in lettuce was observed between conventional and organic farming methods. In conventional lettuce, dihydroferulic acid 4-O-glucuronide showed predominantly negative correlations with various unsaturated lipid metabolites, including lyso PC(18:3), lyso PE(18:3), lyso PC(18:2), FA 18:3, SQDG(16:0/18:3), SQDG(18:3/18:0), and SQDG(18:3/18:3). In organic farming lettuce, L-tyrosine, phenylalanine, and protocatechuic acid were negatively correlated with several lipid species, although each of these compounds showed fewer associations than dihydroferulic acid 4-O-glucuronide in the conventional network. Protocatechuic acid, which demonstrated more associations with TAG and DAG in the organic network, is an antioxidant compound and was found at higher levels in organically grown lettuce. These correlations may reflect coordinated responses between phenolic metabolism and lipid remodeling under oxidative stress from reactive oxygen species.

Ceramides (Cer), including Cer (18:0/16:0(OH)), Cer (18:1/22:0(OH)), and Cer (18:0/22:0(2OH)), formed a distinct cluster associated with phenylalanine in the organic network. Although direct evidence linking these metabolites to organic farming practices is limited, both sphingolipid metabolism and phenylpropanoid biosynthesis are known to respond to biotic and abiotic stress. Increased phenylalanine availability may promote the accumulation of defense-related phenolics, such as protocatechuic acid and ferulic acid (Sharma et al., 2019), while elevated ceramide levels have been implicated in membrane stabilization and stress-signaling processes (Liang et al., 2003). Therefore, the observed association between ceramides and phenylalanine suggests coordinated activation of stress-response pathways in organically cultivated lettuce.

In both cultivation systems, DAG and TAG species formed highly interconnected subnetworks, consistent with their close biosynthetic relationship. However, a separate cluster comprising FA 18:3, lysophospholipids, and SQDG species was observed only in the conventional network, suggesting differences in lipid coordination and membrane lipid metabolism between cultivation practices.

Associations among aqueous metabolites were comparatively sparse in both networks. Interestingly, phenylalanine was positively associated with oxoglutaric acid and IAA in the organic network. This association suggests coordinated interactions between primary metabolic processes related to energy and nitrogen cycling and secondary metabolic pathways involved in defense and growth (Rojas et al., 2014). The elevated relative abundances of these metabolites under organic farming may reflect metabolic adjustments to more variable environmental conditions associated with the absence of synthetic fertilizers and pesticides. While correlation networks cannot directly infer pathway flux or definitive metabolic reprogramming, this observed association might reflect an adaptive shift in metabolism under organic farming conditions. Network analysis

indicates that organic cultivation is associated with a more interconnected metabolic network and altered relationships between phenolic compounds and lipids.

Although this study demonstrates the potential of untargeted profiling for lettuce authentication, several limitations must be acknowledged. The experimental design was unbalanced ($n = 16$ organic vs. $n = 10$ conventional) and utilized market-representative samples rather than a controlled field trial, leaving potential geographic, agronomic, and microclimatic confounding factors unresolved. Nevertheless, these effects may have been partially minimized because Hong Kong is geographically compact, vegetable farms occupy less than 0.7% of the land area and are concentrated primarily in the northern region, and all sampled vegetables were obtained from accredited farms. Furthermore, because soil characteristics and plant physiological stress markers were not directly measured, the proposed mechanisms involving rhizosphere interactions and metabolite network crosstalk remain speculative. Additionally, the intentional focus on a single cultivar to minimize baseline genetic noise limits the immediate generalizability of the model to other cultivars and production systems. Finally, the untargeted LC-MS workflow is inherently semi-quantitative, and several discriminant features remaining unannotated below MSI Level 2. Future investigations employing balanced, multi-farm, and multi-season sampling paired with targeted absolute quantification will be required to fully validate the broader robustness and generalizability of this authentication framework.

4. Conclusion

This study demonstrates that an integrated untargeted metabolomics and lipidomics approach, utilizing the UPLC-MS analytical platform, offers valuable insights into the distinct aqueous and lipid metabolic profiles associated with organic farming practices in lettuce. By applying this comprehensive strategy, we successfully characterized the impact of organic cultivation on a diverse set of 60 aqueous and lipid metabolites. Furthermore, we constructed the interrelationships between primary and secondary metabolites in lettuce grown under both organic and conventional systems. These findings offer new insights into how organic farming practices influence the overall metabolic regulation in lettuce and provide a deeper understanding of the biochemical mechanisms underlying the benefits of organic farming. Importantly, the analytical workflow developed in this study demonstrated exceptional discriminatory power for the authentication of organically produced vegetables and other agricultural products, with the lipidomics platform showing particularly strong performance. These results underscore the potential of this integrated approach as a robust and effective tool for food authenticity testing, traceability, and quality assurance in the context of agricultural and food chemistry. Besides, the overall analytical workflow presented in this study holds significant potential for the authentication of organically produced vegetables and other agricultural products. Notably, the lipidomics approach exhibited exceptional discriminatory power for origin authentication, highlighting its value as a robust tool in food traceability and quality assurance.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT 4.0 to improve readability and language. After using this service, the authors reviewed and edited the content and take full responsibility for the content of the publication.

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List of figure and table

Figure 1: a) Relative abundance of lipid subclasses in lettuce samples. PCA score plots derived from b) HILIC-ESI(+)-MS, c) HILIC-ESI(-)-MS, d) HSS T3-ESI(+)-MS, and e) HSS T3-ESI(-)-MS platforms;

Figure 2. OPLS-DA model evaluation and marker selection for distinguishing organic and conventional lettuce based on positive-mode metabolomics (HILIC (+)), negative-mode metabolomics (HILIC (-)), positive-mode lipidomics (RP(+)), and negative-mode lipidomics datasets (RP(-)). (a) Cross-validated OPLS-DA score plots showing the separation between organic and conventional lettuce samples. (b) S-plots highlighting variables with the greatest contribution and reliability for class discrimination and potential biomarker selection. (c) Permutation test results demonstrating model validity and indicating that the observed discrimination is not due to overfitting. (d) Loading plots with jackknife confidence intervals showing the contribution and statistical reliability of individual variables;

Figure 3: Cultivation-dependent differences in aqueous metabolites detected by metabolomics and associated with the shikimate and phenylpropanoid pathways in lettuce;

Figure 4: Cultivation-dependent alterations in free fatty acids, glycerolipids, phospholipids, and sphingolipids detected by lipidomics in lettuce;

Figure 5: Integrated metabolic network illustrating cultivation-dependent alterations in lettuce metabolites and lipids;

Figure 6: Association networks of primary and secondary metabolites in (A) organic and (B) conventional lettuce. Metabolites with significant associations identified by PCLRC are highlighted in red and annotated; non-significant metabolites are shown in grey. Edges between nodes (metabolites) represent pairwise correlations, with line thickness proportional to the absolute Pearson correlation coefficients (PCCs). Blue solid: positive PCCs; pink dotted: negative PCCs. (C) Differential connectivity analysis comparing organic and conventional lettuce networks. p-values were obtained through permutation testing and adjusted using the Benjamini–Hochberg (BH) correction. Metabolites exhibiting statistically significant differences in connectivity (Level set at 0.05) are shown in red; non-significant metabolites are shown in grey

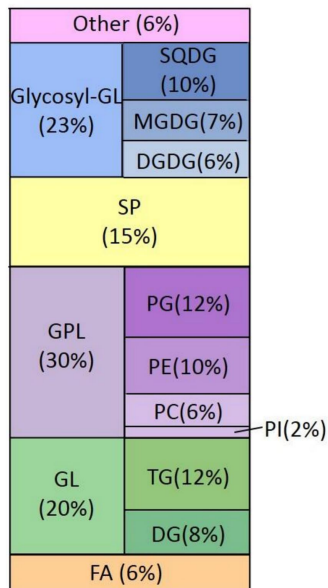
Declaration of interests

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

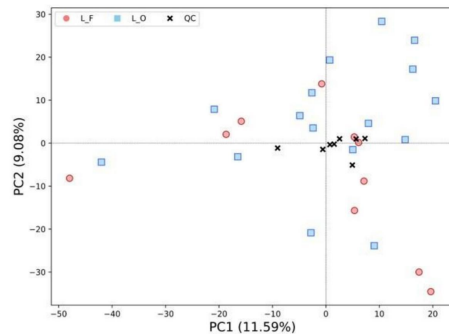
☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Daniel Kam-Wah MOK reports administrative support was provided by Vegetable Marketing Organization (VMO) , Hong Kong SAR, China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

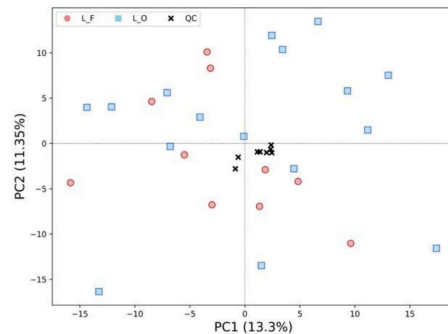
a)



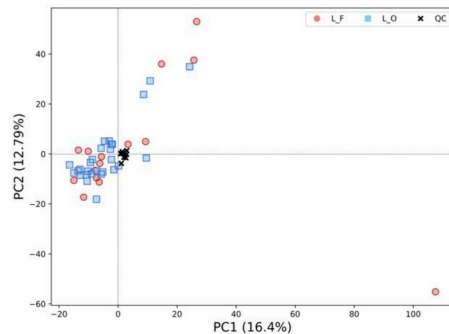
b) Metabolomics: HILIC(+)



c) Metabolomics: HILIC(-)



d) Lipidomics: RP(+)



e) Lipidomics: RP(-)

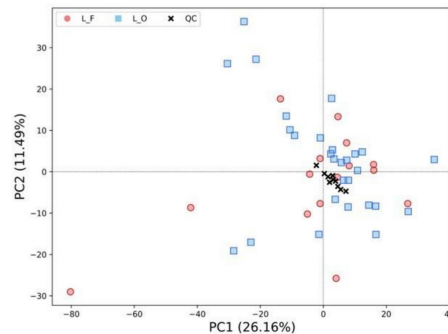


Figure 1

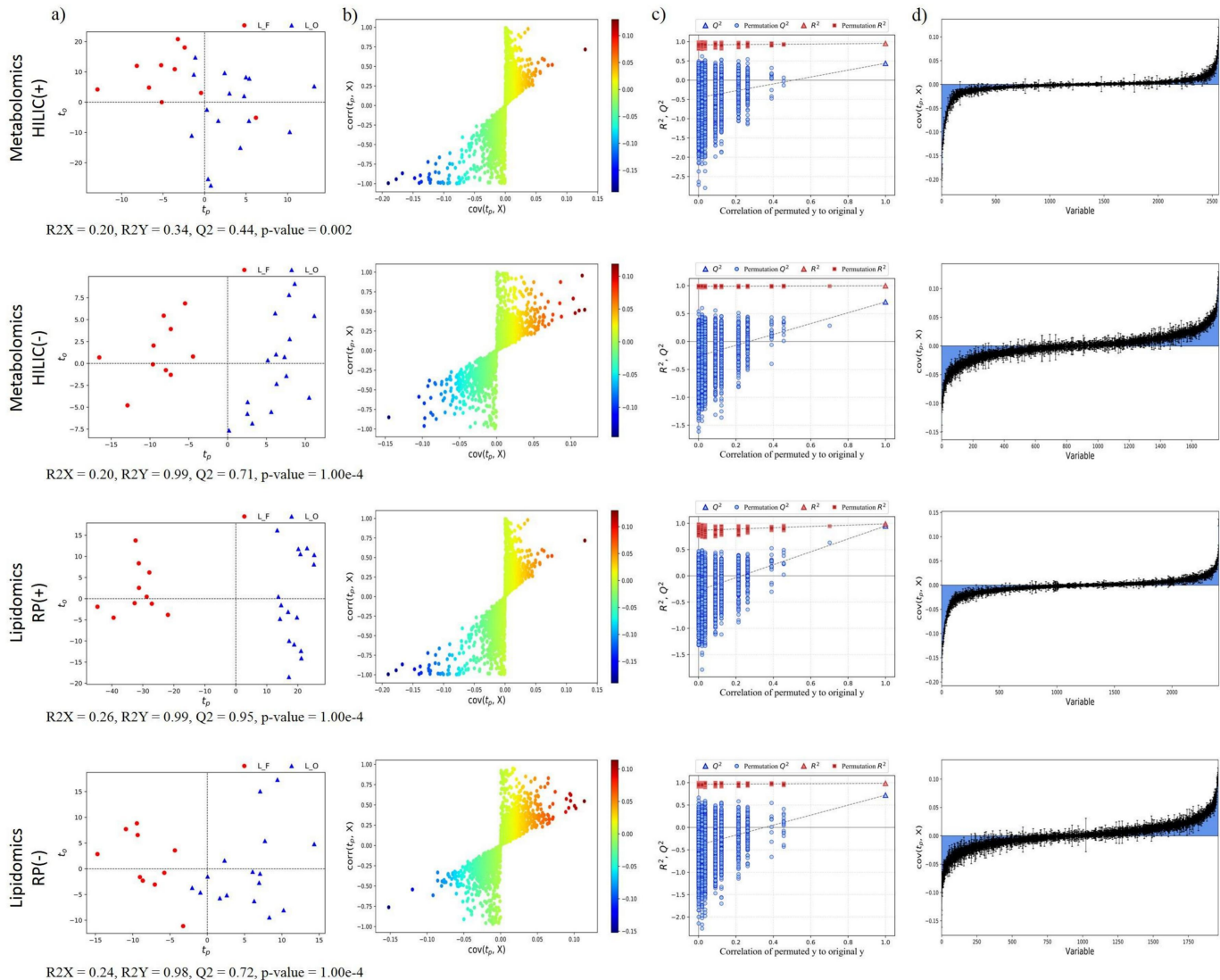


Figure 2

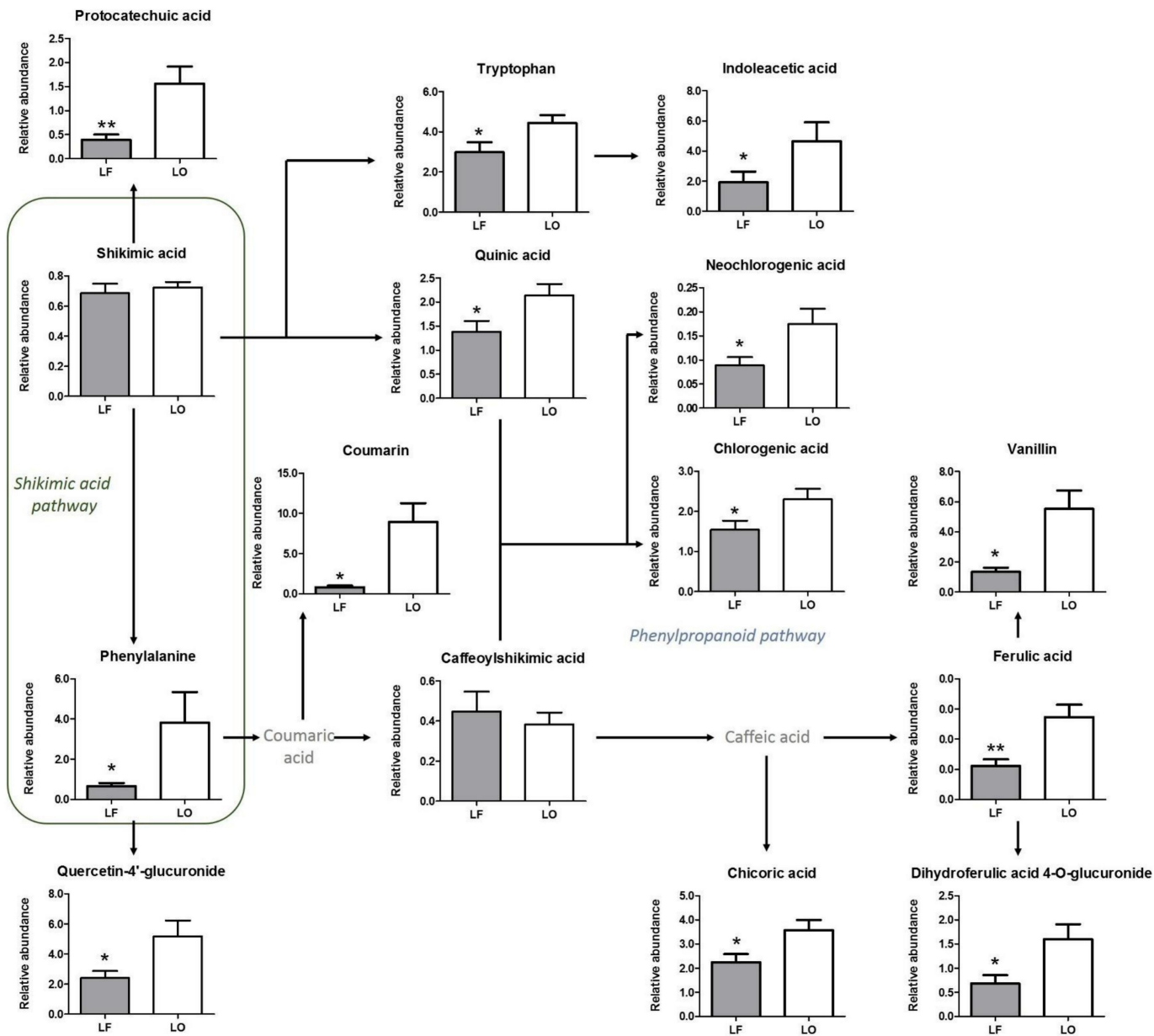


Figure 3

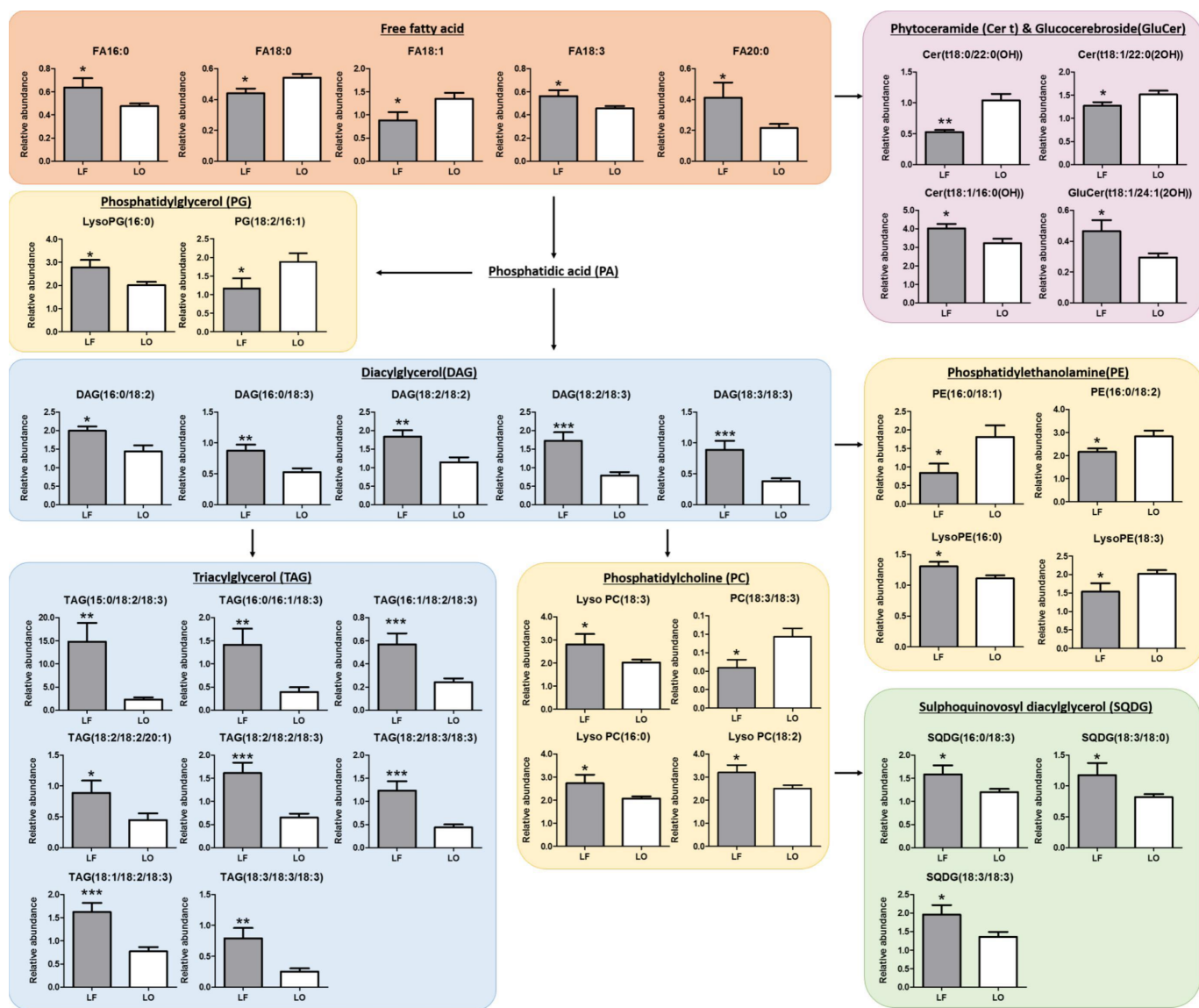


Figure 4

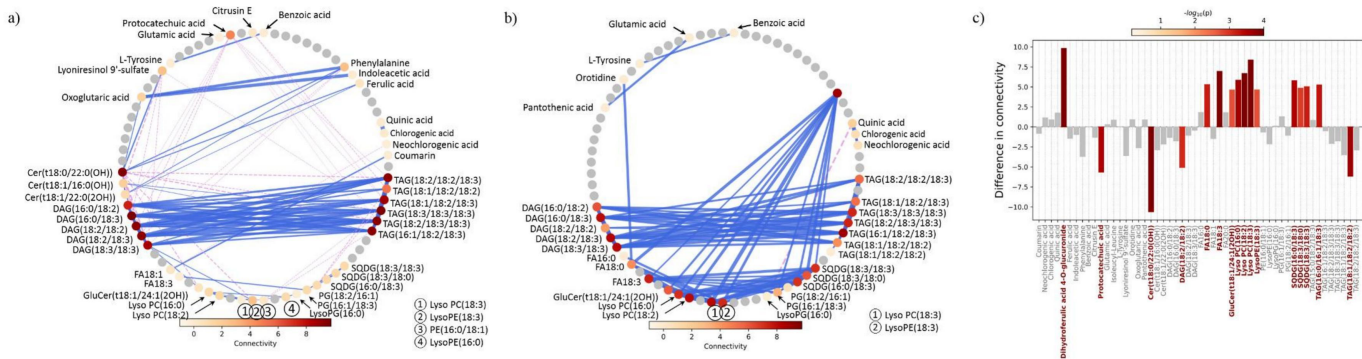


Figure 6