博 士 論 文 の 要 約

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学 位 論 文 題 名

Application of untargeted lipidomics for the identification and characterization of novel lipids in plant and yeast samples
(植物および酵母サンプルにおける新規脂質の同定と特性評価のための非標的脂質オミクスの応用)

Introduction

Lipids constitute a diverse group of hydrophobic biomolecules that play indispensable roles in maintaining cellular structural integrity, serving as energy reservoirs, and mediating intricate signalling pathways. Beyond their physiological functions, food-derived bioactive lipids, particularly those enriched in polyunsaturated fatty acids (PUFAs), exert profound health-promoting effects, including anti-inflammatory, cardioprotective, and neuroprotective activities. In plants, lipids are compartmentalized within cellular organelles such as the plasma membrane, chloroplasts, and endoplasmic reticulum, where they participate in maintaining membrane fluidity, permeability, and protein function. Microbial lipids, particularly in yeasts, also fulfil essential structural and metabolic roles, facilitating stress tolerance and energy homeostasis. Bioactive lipids derived from plant tissues, including leaves, stems, flowers, fruits, and seeds, exhibit significant nutritional and therapeutic potential. These lipid species have been increasingly recognized for their involvement in the prevention and management of chronic diseases, such as cancer, diabetes, cardiovascular disorders, inflammatory bowel diseases, and neurodegenerative conditions. The biochemical mechanisms underlying such bioactivities are primarily attributed to polyunsaturated fatty acids (PUFAs), sphingolipids, sterols, and specialised signalling molecules that modulate oxidative stress, inflammatory responses, and cellular homeostasis. Lipidomic investigations in plants, therefore, provide valuable insights into lipid metabolism, post-harvest physiology, adaptive responses to environmental stimuli, and the compositional diversity that underlies nutritional quality and functional food development.

Oilseeds constitute one of the richest reservoirs of plant lipids, with certain species such as

chia (*Salvia hispanica*), flax (*Linum usitatissimum*), sunflower (*Helianthus annuus*), pumpkin (*Cucurbita pepo*), and hemp (*Cannabis sativa*) recognized for their high concentrations of essential fatty acids and bioactive lipid species. Lipidomic profiling in oilseeds enables the evaluation of compositional diversity, oxidative stability, and cultivar-specific quality parameters, ultimately underpinning breeding, metabolic engineering, and industrial applications. Quantitative and structural characterization of triacylglycerols, phospholipids, and minor lipid constituents through advanced LC/MS platforms provides critical insight into small-molecule interactions, such as lipid-based interactions that determine oil quality and nutritional value.

In yeast systems such as *Saccharomyces cerevisiae* (SC), lipids contribute significantly to membrane integrity, energy storage, and environmental stress adaptation. Yeast membranes are primarily composed of phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), accompanied by sphingolipids, sterols (predominantly ergosterol), and neutral lipids such as triacylglycerols (TAGs) stored within lipid droplets. Lipidomic analysis in *S. cerevisiae* has provided comprehensive insight into lipid droplet biogenesis, membrane remodelling under stress, and the molecular basis of fermentation efficiency. Moreover, yeast-derived lipids play key roles in the fermentation process of alcoholic beverages by modulating yeast metabolism, influencing flavour development, cell viability, and product stability during winemaking.

The continuous advancement of high-resolution liquid chromatography–mass spectrometry (LC/MS) techniques has revolutionized the field of lipidomics. Modern analytical platforms enable the sensitive, accurate, and high-throughput identification of hundreds of lipid classes and molecular species within complex biological matrices. In plant, oilseed, and yeast lipidomes, five principal lipid categories, such as fatty acyls (FAs), glycerophospholipids (GPs), sphingolipids (SPs), sterols (STs), and glycerolipids (GLs), predominate and exhibit critical variations such as ω-3/ω-6 ratios, triacylglycerol species distribution, and phospholipid subclass composition, all of which have direct implications for nutritional quality and functional food applications. Untargeted lipidomics, in particular, allows the discovery of novel lipid species, the mapping of lipidome dynamics under stress or developmental cues, and the elucidation of metabolic plasticity across biological systems. Comprehensive lipidomic analyses in plants and yeasts thus provide essential insights into metabolic diversity, facilitating the improvement of food quality, crop breeding, and industrial biotechnology. The integration of LC/MS-based lipidomics with complementary omics

disciplines, such as metabolomics, transcriptomics, and proteomics, will further accelerate the identification of novel bioactive lipids, metabolic biomarkers, and regulatory networks. Such integrative approaches promise to advance our understanding of lipid biology, guiding the development of sustainable lipid resources and innovative applications in food science, biotechnology, and biomedical research.

Despite the recognized significance of lipids of plant and microbial origin, there remains a paucity of comprehensive lipidomic studies focusing on the discovery, identification, and functional characterization of novel bioactive lipids within dietary plants and yeasts. This research was conducted to bridge these knowledge gaps through an untargeted lipidomic approach encompassing extensive lipid profiling, molecular characterization, and comparative analyses. By employing a state-of-the-art untargeted high-resolution LC/MS workflow, this study systematically investigated the lipidomes of various plant-based matrices, such as edible rose petals, herbal teas, grapes, and oilseeds, as well as yeast (*Saccharomyces cerevisiae*). Through this approach, several novel lipid classes, including acyl steryl glycosides (ASGs), fatty acid esters of hydroxy fatty acids (FAHFAs), N-acyl-lysophosphatidylethanolamines (LNAPEs), and acylhexosylceramides (AHexCers), were identified and structurally characterized for the first time. These findings expand the current lipidomic catalogue and highlight the nutritional, functional, and biotechnological potential of plant and yeast-derived lipids.

Materials and Methods

Chemicals

LC/MS-grade solvents, including methanol, isopropanol (IPA), chloroform, and 1 M ammonium acetate solution, were procured from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Internal standards, namely oleic acid-d9 and the EquiSPLASH LIPIDOMIX quantitative standard mixture, were supplied by Avanti Polar Lipids (Alabaster, AL, USA). Zirconium ceramic oxide beads (1.4 mm, Fisher brand) used for sample homogenization were obtained from Thermo Fisher Scientific (Tokyo, Japan).

Sample Information

The biological samples analyzed in this study encompassed a diverse collection of plant- and yeast-derived materials obtained from various sources in Japan to ensure species variance and sample integrity. Five grape varieties harvested in Hokkaido, Japan, were freshly collected for lipidomic profiling: Kerner (*Vitis vinifera*), commonly referred to as White Herald, a white vinifera variety used in winemaking; Kiyomai, Kiyomi, and Yamasachi (*Vitis coignetiae*),

which are Japanese hybrid grape cultivars; and Zweigelt (*Vitis vinifera* 'Zweigelt'), an Austrian red hybrid grape variety widely used in red wine production. Grapes were separated into fruit (comprising pulp and skin) and seed fractions. The seeds were air-dried at 25 °C for 48–72 h, mechanically ground into a fine powder, and stored at −80 °C to inhibit lipid oxidation before analysis.

Five nutritionally relevant oilseed samples, such as chia (*Salvia hispanica*), flax (*Linum usitatissimum*), hemp (*Cannabis sativa*), pumpkin (*Cucurbita pepo*), and sunflower (*Helianthus annuus*), were obtained from local commercial markets in Japan. Seeds were air-dried under ambient laboratory conditions, finely ground using a mortar, and hermetically sealed before storage at −80 °C to preserve lipid stability for LC/MS-based lipidomic analysis.

Additionally, forty-eight *Saccharomyces cerevisiae* (SC) yeast strains were collected from multiple wineries across Hokkaido, Japan. The yeast cultures were maintained under cryogenic conditions (−80 °C) until lipid extraction and analysis were conducted.

Collectively, these diverse biological samples represent a comprehensive spectrum of plant and microbial lipid sources, enabling comparative lipidomic investigations across taxonomic groups and supporting the evaluation of lipid diversity, nutritional potential, and bioactive compound discovery.

Total lipid extraction

For grape samples, approximately 200 mg of finely ground seed powder from each sample type was accurately weighed for analysis. Each sample was combined with 5–6 zirconium ceramic oxide beads (1.4 mm) and 1 mL of methanol, followed by mechanical homogenization for 30 s with two successive cycles. The homogenized mixtures were centrifuged at 15,000 rpm for 10 min at 4 °C, and the resulting supernatants from extractions were pooled into a single tube to prepare the stock solution for the fruit and seed samples. Total lipid extraction was subsequently performed using a modified Bligh and Dyer method previously standardized in our laboratory. Briefly, 100 µL (n = 5) of the homogenate (equivalent to approximately 10 mg/100 µL) was transferred into a 1.5 mL microcentrifuge tube. To this, 100 µL of internal standard solution (a methanolic mixture containing 10 µg/mL oleic acid-d9 and 1 µg/mL EquiSPLASH LIPIDOMIX), 100 µL of chloroform, and 20 µL of Milli-Q water were added. The samples were vortexed at 3,500 rpm for 5 min to achieve efficient phase mixing and subsequently incubated at room temperature (~25 °C) for 2 h. The resultant monophasic extracts were concentrated using a centrifugal evaporator at 4 °C overnight. The dried lipid residues were reconstituted in 100 µL of methanol, centrifuged at

15,000 rpm for 10 min at 4 °C, and the clear supernatants were transferred to LC/MS vials. A 10 μL aliquot from each vial was injected into the LC/MS system for lipidomic analysis.

For the five oilseed samples and 48 *Saccharomyces cerevisiae* (SC) strains, total lipid extraction was conducted using a modified Folch method that had been optimized and validated in our laboratory. In brief, 200 μL (n = 5) of homogenized sample solution for oilseed samples and 100 μL of yeast suspension in Milli-Q water mixed with 100 μL of methanol was transferred into a 2 mL Eppendorf tube for homogenization. Subsequently, 100 μL of internal standard (IS) solution in methanol, comprising oleic acid-d9 (100 μg/mL) and EquiSPLASH (10 μg/mL), was added to each sample. To induce biphasic separation, 600 μL of chloroform and 150 μL of Milli-Q water for oilseeds and 400 μL of chloroform and 100 μL of Milli-Q water were introduced, and the mixture was vortexed for 5 min followed by centrifugation at 15,000 rpm for 10 min. The lower chloroform layer containing the extracted lipids from each tube was carefully collected into a new 2 mL microcentrifuge tube. A second extraction step was performed by adding an additional 600 μL of chloroform to the remaining upper layer for seeds (400 μL of chloroform for yeast samples), followed by vortexing and centrifugation under identical conditions. The two chloroform layers were combined and evaporated to dryness under vacuum overnight at 4 °C. The dried lipid residues were reconstituted in 100 μL of methanol, centrifuged for 10 min at 15,000 rpm, and the clarified supernatants were transferred into LC/MS vials. A 10 μL volume from each sample was subsequently injected into the LC/MS system for comprehensive lipid profiling. The total protein content of the 48 *S. cerevisiae* strains was quantified following the protocol described in our previous study to facilitate normalization of lipid abundance data.

Nontargeted lipidomic analysis using an LC/MS

Comprehensive lipidomic analysis was conducted using a high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) coupled to a high-resolution mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA). Chromatographic separation was achieved on a reverse-phase Atlantis T3 C18 column (2.1 × 150 mm, 3 μm; Waters, Milford, MA) maintained oven temperature of 40 °C, with a constant flow rate of 0.2 mL/min. The mobile phase system consisted of three solvents: (A) 10 mM ammonium acetate (CH_3COONH_4) in Milli-Q water, (B) isopropyl alcohol (IPA), and (C) methanol. A linear gradient elution program was applied as follows: 0–1 min, 30% B / 35% C / 35% A; 1–14 min, 80% B / 10% C / 10% A; 14–27 min, 85% B / 10% C / 5% A; followed by a 3 min re-equilibration to the initial conditions before the

next injection. Mass spectrometric detection was performed under data-dependent acquisition (DDA) mode in both positive and negative electrospray ionization (ESI) modes under conditions previously optimized in our laboratory. For the negative ionization mode, the ESI voltage was set at 3.0 kV. Nitrogen was used as the sheath and auxiliary gases at flow rates of 50 and 5 arbitrary units, respectively. Full-scan MS spectra were acquired in Fourier transform mode across an *m/z* range of 160–1900 with a resolving power of 60,000. High-resolution MS^2 spectra were obtained by collision-induced dissociation (CID) at a normalized collision energy of 40 eV and an isolation window of *m/z* 3. Parameters for the positive ionization mode were identical to those validated in prior studies from our laboratory. Raw MS data were processed and interpreted using MS-DIAL (version 4.9) and Xcalibur 2.2 software for accurate peak detection, alignment, annotation, and identification of lipid molecular species. Semi-quantitative analyses were performed by normalizing the detected peak areas to sample weight and calculating relative abundances based on the ratio of analyte peak area to that of the internal standards.

Data Processing and Statistical Analysis

All data were expressed as mean ± standard deviation (SD) and visualized using Microsoft Excel 2016 (Microsoft Corporation, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Comprehensive lipidomic data processing, normalization, and multivariate statistical analysis were performed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca) and ClustVis (https://biit.cs.ut.ee/clustvis/). These platforms were utilized to conduct principal component analysis (PCA), clustering analysis, and correlation mapping to identify patterns and relationships in the lipidomic profiles across different biological samples.

Results and discussions

This fourth chapter presents a comprehensive lipidomic investigation of five grape (*Vitis vinifera* and *Vitis coignetiae*) cultivars: Kerner, Kiyomai, Kiyomi, Yamasachi, and Zweigelt, representing both European and Asian species. The grape samples were fractionated into fruit (pulp and skin) and seed components prior to analysis. Lipid extraction was performed using a modified Bligh–Dyer method, which was optimized and validated within our laboratory to ensure maximal lipid recovery and structural preservation. Untargeted lipidomic profiling identified 245 distinct lipid molecular species distributed across five primary lipid classes: fatty acids (FAs), glycerophospholipids (GPs), glycerolipids (GLs), sphingolipids (SPs), and sterols (STs). Principal component analysis (PCA) revealed distinct and consistent segregation between fruit and seed lipidomes across all cultivars, emphasizing tissue-specific lipid

metabolic patterns. Seeds exhibited a substantially higher total lipid content relative to fruit tissues, reflecting their physiological role as energy reserves. Notably, Zweigelt seeds contained elevated levels of ω-3 and ω-6 polyunsaturated fatty acids (PUFAs), resulting in an increased PUFA-to-saturated fatty acid (SFA) ratio indicative of high nutritional value. The predominant FA chains observed C16:0, C18:0, C18:1, C18:2, and C18:3 were consistent with previously reported plant lipid profiles, reaffirming the prevalence of C16 and C18 acyl chains in plant lipid biosynthesis. Based on the observed lipid content and composition, Zweigelt seeds are suggested to play a significant role in modulating the lipid profile during winemaking and to provide potential value for functional food development. Kiyomi emerged as a promising source of bioactive ceramides, suggesting potential nutraceutical and cosmetic applications. Kerner seeds were enriched in acylhexosylceramides (AHexCers), a subclass of complex sphingolipids here reported for the first time in grape matrices. The identified glycerolipids, including monoacylglycerols (MGs), diacylglycerols (DGs), and digalactosyldiacylglycerols (DGDGs), were particularly abundant in seeds, underscoring their contribution to oil composition, cellular membrane structure, and germination metabolism. Complementary metabolomic profiling identified 59 metabolites, including isocitrate, palatinose, fructose, epicatechin, laurate, tryptophan, exifone, and digitoxin. These metabolites may contribute to the sensory characteristics of wine, influencing taste, flavor, texture, and color development. The identification and structural characterization of AHexCers in these grape varieties represent a novel finding, underscoring their potential role in grape-derived functional food innovation and quality enhancement in oenology.

The fifth chapter describes the comprehensive lipidomic profiling of five nutritionally significant oilseeds, namely, chia, flax, pumpkin, sunflower, and hemp. Lipids were extracted using a modified Folch procedure optimized in our laboratory for efficient recovery of both polar and non-polar lipid species. Untargeted LC/MS analysis identified 259 lipid molecular species categorized into five principal lipid groups. Distinct separation patterns among the oilseed species were observed based on multivariate analyses, with characteristic lipids such as FA 18:1, DGs, and triacylglycerols (TAGs) driving the variation. Saturated fatty acids dominated most oilseed lipidomes, with sunflower and pumpkin seeds showing higher proportions of saturated and monounsaturated fatty acids relative to polyunsaturated fractions. Hemp seeds exhibited the highest relative abundance of fatty acid esters of hydroxy fatty acids (FAHFAs), a recently recognized class of bioactive lipids possessing anti-inflammatory and antidiabetic properties and emerging as key modulators in metabolic and cardiovascular

health. Glycerophospholipids (GPs) were most abundant in pumpkin seeds, followed by hemp, while flax displayed comparatively lower GP levels. The presence of cardiolipins (CLs), specialized mitochondrial phospholipids involved in electron transport and membrane organization, was confirmed across all species, highlighting their functional role in cellular respiration. Overall, flaxseeds exhibited lower relative abundances in glycerophospholipids (GPs), sphingolipids (SPs), and sterols (STs), yet these classes are critical for membrane integrity and cell signalling, forming part of the broader lipid regulatory network in plants. Pumpkin seeds demonstrated the most diverse and abundant lipidome, characterized by a rich composition of TGs and phospholipids, followed by flaxseed, whereas sunflower exhibited the lowest overall lipid abundance. Glycerolipids were notably enriched across all five oilseeds, reflecting their significance in membrane stabilization, signal transduction, and energy metabolism. Importantly, several novel FAHFA species were identified and structurally elucidated for the first time in multiple seed types, supporting their potential inclusion as bioactive ingredients in functional food and nutraceutical formulations. These findings provide valuable insights for comparative evaluation and selection of oilseed varieties suited for high-quality edible oil production and industrial applications.

The sixth and final chapter explores the lipidomic landscape of 48 *Saccharomyces cerevisiae* strains to investigate lipid diversity and its correlation with metabolic activity and fermentation performance. Untargeted LC/MS profiling quantified 135 lipid molecular species distributed among five major classes and fifteen subclasses. Fatty acid profiling revealed 22 key FAs, including palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3), which are known to modulate wine quality during fermentation. Strains SC19 and SC46 displayed particularly high levels of these PUFAs, suggesting strain-specific metabolic adaptations relevant to fermentation efficiency. Among the major lipid groups, glycerophospholipids and glycerolipids exhibited the most pronounced inter-strain variation, with triacylglycerols (TAGs) being markedly abundant in SC10, SC47, and SC48 strains. TAG accumulation likely supports stress tolerance during the fermentation process, reflecting intricate regulation of lipid biosynthesis by interconnected metabolic networks responsive to nutrient availability and cellular developmental stages. Correlation and multivariate analyses delineated strain-specific lipidomic patterns, linking metabolic heterogeneity to fermentation kinetics. Sterols (STs) and sphingolipids (SPs) were found to contribute significantly to both yeast lipidome functionality and oenological quality traits, influencing sensory attributes such as texture and mouthfeel. Ergosterol, synthesized under

aerobic conditions and phytosterols, generated under anaerobiosis, play essential roles in maintaining membrane fluidity and cell viability under ethanol stress, thereby preventing fermentation arrest. The detection of bioactive lysophosphatidylethanolamine (LPE-N) species within yeast lipidomes constitutes a novel finding, expanding our understanding of microbial lipid diversity and suggesting potential roles in signalling pathways and industrial fermentation optimization.

Conclusion

This doctoral research established an integrated lipidomic framework combining untargeted LC/MS with advanced bioinformatic and chemometric analyses to elucidate lipid structure, diversity, and functionality in plant and microbial systems. The lipidomic profiling of five grape cultivars identified 245 lipid molecular species with clear tissue-specific distributions. Zweigelt seeds exhibited the highest lipid content across multiple classes, whereas Kerner showed the lowest. Seeds contained significantly higher lipid concentrations than fruit, highlighting their metabolic role in energy storage. The discovery of acylhexosylceramides (AHexCers) represents a novel biochemical feature of grapes with potential applications in functional foods and winery bioproducts. The comparative analysis of five oilseed species identified 259 lipid species, revealing high interspecific lipid variability. Hemp seeds were rich in fatty acid esters of hydroxy fatty acids (FAHFAs), sunflower seeds showed the highest total fatty acid content, and pumpkin seeds were enriched in other lipid classes. Flax seeds contained the lowest sphingolipid and glycerophospholipid levels, reflecting species-specific lipid regulation. These results provide molecular insights into oilseed lipid complexity with implications for nutritional and industrial applications. Lipidomic profiling of 48 *Saccharomyces cerevisiae* strains uncovered 135 lipid species and 22 fatty acid subtypes. Strains SC19 and SC47 exhibited elevated FA levels, indicating strain-dependent lipid metabolism linked to fermentation performance. The identification of novel lipids, FAHFAs, AHexCers, acylated steryl glucosides (ASGs), and lysophosphatidylethanolamine-N (LPE-N), broadens understanding of lipid diversity in both plants and microbes. Overall, this work demonstrates the utility of untargeted lipidomics as a bridge between analytical chemistry, food science, and biotechnology. The discovery of novel bioactive lipids advances opportunities for developing functional foods, nutraceuticals, and optimized fermentation processes with enhanced sensory and metabolic outcomes.