博 士 論 文 の 要 約

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Development of a quantitative analytical method for unsaturated short-chain fatty acid esters of hydroxy fatty acids and their biological applications
（ヒドロキシ脂肪酸の不飽和短鎖脂肪酸エステルの定量分析法の開発とその生物学的応用）

Lipids represent one of the most structurally diverse and functionally significant classes of biomolecules in living systems. They serve as fundamental building blocks of cellular membranes, act as highly efficient energy reservoirs, and participate in numerous signaling processes that regulate metabolism, inflammation, immunity, and homeostasis. Alterations in lipid composition and metabolism have been widely implicated in a broad spectrum of diseases, including obesity, diabetes, neurodegeneration, and chronic inflammatory disorders. With the advancement of analytical technologies, particularly liquid chromatography coupled with mass spectrometry (LC/MS), the field of lipidomics has expanded rapidly, enabling the qualitative and quantitative profiling of thousands of lipid species across biological matrices. LC/MS-based lipidomics has become indispensable for understanding the complexity of lipid metabolism, uncovering novel lipid classes, and identifying potential biomarkers for disease progression and therapeutic response.

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a newly recognized class of endogenous lipids that have rapidly gained attention due to their regulatory roles in metabolic and inflammatory pathways. Structurally, FAHFAs consist of a hydroxy fatty acid esterified to another fatty acid, generating substantial chemical diversity through differences in chain length, unsaturation, and the position and orientation of the hydroxyl and ester linkages. This structural complexity underpins the broad biological functions attributed to FAHFAs. They were initially identified in adipose tissue of insulin-sensitive mice, where their levels were significantly higher than in insulin-resistant models. Subsequent studies demonstrated that certain FAHFA families, such as PAHSAs, enhance insulin secretion, promote glucose uptake, activate lipid-sensing receptors (GPR120 and GPR40), and suppress inflammatory cytokines, highlighting their potential as protective metabolic lipids. FAHFAs are also stored within triacylglycerols (FAHFA-TGs), which serve as a dynamic reservoir whose mobilization appears impaired in insulin resistance. Their widespread presence in liver, intestinal mucosa, circulation, and muscle suggests important systemic functions that remain incompletely understood.

Short-chain fatty acid esters of hydroxy fatty acids (SFAHFAs) have recently been identified as a subclass of FAHFAs that are linked to gut microbial metabolism. SFAHFAs are typically composed of short-chain fatty acids such as acetate, propionate, or butyrate esterified to long-chain 2-hydroxy fatty acids. Their abundance in cecal and colonic contents suggests that their biosynthesis depends on microbial

fermentation products and host–microbiome interactions. Emerging evidence shows that SFAHFAs are dynamically regulated by diet, microbial composition, infection, and physiological state. Reductions in SFAHFAs have been observed in high-fat–diet–fed mice, whereas increases occur during influenza infection or in later stages of bacterial infection, indicating roles in metabolic adaptation and immune response. Antibiotic depletion of the microbiota causes a marked reduction in propionate- and butyrate-derived SFAHFAs, confirming that microbial populations are essential for their production. Human studies also show strong inter-individual variability linked to specific microbial taxa, and declines in SFAHFAs with ageing have been associated with neurocognitive impairment. Collectively, these findings suggest that SFAHFAs function as gut-derived lipid mediators involved in intestinal homeostasis, immune regulation, and microbiota–host communication.

Despite their growing biological relevance, the analytical characterization of SFAHFAs remains challenging. Their endogenous levels are extremely low in the biological matrix, causing significant ion suppression during mass spectrometric detection. Additionally, SFAHFAs include a wide variety of positional and stereoisomers formed from different combinations of short-chain fatty acids and 2-hydroxy fatty acids, leading to subtle structural differences that are hard to separate chromatographically. Tandem mass spectrometry frequently yields overlapping or non-specific fragment ions, making confident identification difficult without authentic standards. Complex biological matrices contain high levels of interfering compounds, further complicating detection. Furthermore, commercially available standards for SFAHFAs are lacking, preventing accurate quantification, calibration, and cross-study comparison. As a result, most existing studies rely on semi-quantitative measurements, limiting the mechanistic understanding of these lipids.

These challenges form the rationale for the present study, which aims to develop a comprehensive analytical framework for the investigation of SFAHFAs. Although FAHFAs have been reported to possess metabolic and anti-inflammatory activities, research has predominantly focused on long-chain species, leaving gut-associated SFAHFAs largely unexplored. Their low abundance, structural diversity, and dependence on microbial metabolism make them important yet poorly understood mediators of gut–host interactions. Addressing the analytical gaps is therefore essential for elucidating their biosynthesis, physiological regulation, and potential roles in metabolic and neurocognitive health. The objectives of this research were to synthesize a structurally defined library of unsaturated SFAHFAs, develop and validate a targeted LC–MS/MS method for their sensitive and accurate quantification, enhance detection sensitivity through derivatization method, and apply these analytical tools to murine and human biological samples to investigate how SFAHFAs respond to dietary stress and early neurocognitive decline.

The second chapter of this thesis details the development of a comprehensive synthetic strategy to create a library of unsaturated short-chain fatty acid esters of hydroxy fatty acids (SFAHFAs), addressing a major limitation in lipidomics: the lack of authentic standards necessary for precise quantification and biological studies. Building on our laboratory's previous success in synthesizing saturated SFAHFAs, which allowed the first targeted LC–MS/MS quantification of this lipid class, the current work expands synthetic access to structurally diverse unsaturated SFAHFAs that represent the biochemical complexity of

gut-derived lipid profiles. Unsaturated fatty acids are vital for membrane dynamics, lipid signaling, and inflammatory regulation, making their inclusion in SFAHFA structures biologically significant; however, their synthesis is technically difficult due to the vulnerability of double bonds to isomerization and overoxidation during α-hydroxylation. To address these challenges, a reliable two-step synthetic route was developed, starting with the α-hydroxylation of six commercially available unsaturated fatty acids (C16:1, C18:1, C18:2, C20:1, C22:1, and C24:1), producing the corresponding 2-hydroxy fatty acids, followed by acylation with short-chain fatty acid chlorides (C2–C6) to form the target unsaturated SFAHFAs. Reaction conditions were carefully optimized to maintain double-bond configuration and ensure high structural accuracy. All 30 synthesized compounds were thoroughly characterized through proton nuclear magnetic resonance (1H NMR) spectroscopy and high-resolution mass spectrometry (HRMS). These analyses confirmed their expected molecular structures and demonstrated high chemical purity. This chapter demonstrates, for the first time, the successful synthesis of a structurally validated library of unsaturated SFAHFAs, establishing a vital analytical foundation that enables precise quantification, broadens biological research possibilities, and supports future mechanistic studies into the metabolic and physiological roles of this emerging class of gut-derived lipids.

Chapter 3 describes the development and validation of a targeted LC–MS/MS method for the quantitative analysis of unsaturated SFAHFAs using the synthetic standards established in Chapter 2. The analytical workflow was constructed through an iterative process of mass spectrometric and chromatographic optimization, recognizing the need for high sensitivity, selectivity, and structural discrimination due to the presence of numerous isomeric species and the extremely low endogenous abundance of these lipids in biological matrices. Initial optimization of mass spectrometric parameters on a triple quadrupole platform enabled the identification of highly specific product ions in the negative electrospray ionization mode of unsaturated SFAHFAs and their deuterated internal standards. Chromatographic conditions were subsequently refined using a reversed-phase C18 column to achieve efficient separation of unsaturated SFAHFA species within a 22-minute gradient, ensuring adequate resolution of structurally similar analytes. Addressing the inherent chemical instability of unsaturated lipids, antioxidants such as BHT were incorporated to mitigate oxidation and preserve analyte integrity during sample preparation and analysis. Comprehensive validation demonstrating excellent linearity with high correlation coefficients ($R^2 > 0.99$), limits of detection between 0.5 and 5 fmol and quantification between 5 and 50 fmol, and consistent retention times across all analytes. Accuracy and precision assessments across multiple concentration levels yielded acceptable ranges (typically 95–110% accuracy and <20% RSD), supporting the method's reliability for biological applications. Collectively, this chapter establishes the first rigorously validated targeted LC–MS/MS method for the quantification of unsaturated SFAHFAs, providing a robust analytical platform capable of detecting trace-level species and enabling precise quantitative investigation of their roles in metabolic and gut-associated physiological processes.

Chapter 4 applies the validated targeted LC–MS/MS method to investigate the quantitative profiles and biological regulation of SFAHFAs in fecal samples from murine models subjected to obesogenic diet and exercise, as well as human subjects with mild cognitive impairment (MCI). Using chemically defined

standards, the method enabled absolute quantification of both saturated and unsaturated SFAHFAs across diverse physiological conditions, providing the first detailed assessment of how diet, physical activity, and early neurocognitive decline influence gut-derived lipid mediators. All samples were extracted using a modified Folch extraction method, which uses a two-solvent system (chloroform–methanol) to effectively separate lipids into the organic phase. This approach minimizes matrix interference and maintains lipid integrity. This robust extraction workflow ensured reproducible recovery of low-abundance SFAHFAs before LC–MS/MS analysis. In mice, OBD displayed a pronounced reduction in saturated SFAHFA across long-chain and very-long-chain species, consistent with disrupted intestinal lipid metabolism, altered microbial composition, and potential suppression of enzymes implicated in SFAHFA biosynthesis, such as Pla2g2a. In contrast, unsaturated SFAHFAs were significantly elevated under OBD conditions, particularly acetate- and propanate-derived forms and very-long-chain monounsaturated species, suggesting a diet-induced shift toward desaturated lipid intermediates potentially linked to increased intestinal SCD1 and ELOVL activities. Although exercise enhanced SFAHFA abundance in control-diet animals, it did not reverse obesogenic diet–induced reductions, indicating that dietary stress mitigates the beneficial metabolic effects of physical activity on gut lipid homeostasis. Application of the method to human fecal samples revealed no significant differences in SFAHFA concentrations between MCI patients and age-matched healthy controls, despite greater inter-individual variability in the MCI group. These findings align with prior observations that SFAHFA reductions occur in more advanced neurocognitive impairment, suggesting that early-stage MCI may not yet exhibit detectable alterations in these gut-derived lipid mediators, or that such changes are masked by dietary and microbiota-related variability. Collectively, this chapter demonstrates the application of the developed analytical method for biological applications and provides novel insights into the divergent metabolic regulation of saturated and unsaturated SFAHFAs in response to diet, exercise, and early cognitive decline.

Chapter 5 introduces a derivatization-based strategy to enhance the analytical sensitivity of SFAHFAs, addressing a major limitation encountered in their native LC–MS/MS analysis due to poor ionization efficiency and low endogenous abundance. Recognizing that derivatization can markedly improve chromatographic behavior and mass spectrometric response for carboxyl-containing lipids, this study establishes a robust and efficient method using 2-(2-pyridyl)ethylamine (PEA) to convert SFAHFAs into highly ionizable amide derivatives. The reaction, facilitated by triethylamine (TEA) and 2-chloro-1-methylpyridinium iodide (CMPI) under mild conditions, selectively modifies the carboxyl group, introducing a protonatable pyridyl moiety that substantially enhances electrospray ionization in positive mode. Comprehensive MS/MS optimization demonstrated that PEA derivatives produce abundant and reproducible fragment ions suitable for sensitive SRM quantification. Comparative analyses revealed an approximately 1000-fold increase in signal intensity for derivatized vs. underivatized SFAHFAs, enabling reliable detection of trace-level species previously near or below quantification limits. Application of the method to murine fecal extracts confirmed dramatically improved peak intensities across all SFAHFA subclasses, demonstrating compatibility with complex biological matrices. While other derivatizing reagents have been explored for fatty acids, the PEA-based approach offers a simpler, faster,

and more analytically stable alternative without harsh reaction conditions. This chapter therefore establishes the first PEA derivatization workflow for SFAHFAs, providing a powerful enhancement to targeted LC–MS/MS and enabling deeper exploration of their biological roles. Although certain limitations remain, such as the absence of isotope-labelled standards for all species and the focus on fecal matrix analysis, the derivatization method significantly strengthens quantitative lipidomics capabilities and lays the groundwork for future mechanistic and multi-matrix investigations into the metabolic and microbiota-linked functions of this emerging lipid class.

Chapter 6 investigates the systemic and tissue-specific effects of a chronic obesogenic diet (OBD) and a subsequent two-week voluntary wheel-running intervention on lipid metabolism in mice using an untargeted LC-MS-based lipidomics approach. Male C57BL/6J mice were assigned to control and OBD feeding, with sedentary and exercise subgroups, and lipids were extracted from liver, left ventricle, spleen, plasma, and feces using a modified Folch two-solvent extraction, followed by high-resolution HPLC/LTQ-Orbitrap MS analysis in both positive and negative ion modes. Multivariate analyses, including sPLS-DA, volcano plots, and heatmaps, revealed that OBD induced pronounced lipid remodelling across multiple compartments, whereas short-term exercise produced only modest and tissue-dependent effects. In the liver and plasma, OBD was associated with a consistent depletion of DHA (docosahexaenoic acid) containing glycerophospholipids, particularly PG and PE species, accompanied by an increase in arachidonic acid (AA; 20:4)-containing phospholipids and elevated ceramides, indicating a shift toward a pro-inflammatory and lipotoxic lipid profile. In the left ventricle and spleen, OBD similarly reduced EPA (eicosapentaenoic acid) and DHA-derived phospholipids, including PG (22:6/22:6), LPC 22:6, and LPS 22:6, suggesting impaired resolution of inflammation and loss of cardioprotective lipid mediators. Fecal lipidomics showed decreased glycerolipids and accumulation of long-chain saturated and monounsaturated fatty acids under OBD, consistent with altered intestinal lipid handling and potential changes in microbial metabolism. Exercise partially attenuated some adverse signatures, most notably by lowering plasma ceramides and increasing selected anti-inflammatory LPC and LPE 22:5 species, but did not fully restore DHA-rich phospholipids or reverse OBD-driven shifts in all tissues. Overall, this chapter demonstrates that long-term obesogenic diet induces a robust, organ-specific pro-inflammatory reprogramming of the lipidome, while short-duration voluntary exercise exerts only partial effects, underscoring the dominant impact of chronic dietary stress on lipid-driven inflammation and cardiometabolic risk.