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Development and validation of chromatography and mass spectrometry-based lipidomic methods for pharmaceutical lipid emulsion components in total parenteral nutrition

MARK DENNIS CHICO RETRATO

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Abstract
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Total parenteral nutrition (TPN) is a life-sustaining therapy that delivers essential nutrients intravenously to patients unable to meet their dietary requirements through oral intake. TPN formulations typically contain a mixture of carbohydrates, proteins, lipids, vitamins, and minerals, with pharmaceutical lipid emulsions (PLEs) serving as a key component. Ensuring the stability and quality of TPN lipids is critical as compositional changes—particularly in PLEs, can impact formulation efficacy and patient safety.

This thesis explores the lipidomic analysis of PLEs by investigating lipid stability and degradation over time. This research develops and applies chromatography coupled with mass spectrometry (MS): gas chromatography (GC-MS) for **Paper I**, supercritical fluid chromatography (SFC-MS) for **Papers II-III** and liquid chromatography (LC-MS) for **Paper IV** methods to investigate important lipid groups, including free fatty acids (FFAs), cholesterol and cholesterol oxidation products (COPs), phospholipids (PLs), and triacylglycerols (TAGs). These tailored lipidomic techniques provided critical insights into compositional changes that may indicate PLE degradation and potential TPN instability.

To ensure analytical robustness, all methods were validated according to ICH Q2(R2) guidelines meeting pharmaceutical quality standards. Present study also addresses matrix effects and emphasizes the importance of using appropriate internal standards for accurate lipid quantification. The developed strategies were applied to pharmaceutical-grade egg yolk powders, a key raw material for PLE formulations. These findings contribute to improving lipidomic methodologies for quality control, enabling high-throughput, and reproducible analysis of TPN formulations, supporting safer and more effective patient care.

Keywords: Chromatography, mass spectrometry, targeted lipidomics, method development, method validation, pharmaceutical lipid emulsion, total parenteral nutrition

Mark Dennis Chico Retrato, Department of Chemistry - BMC, Analytical Chemistry, Box 599, Uppsala University, SE-75124 Uppsala, Sweden.

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To everyone who has my back through thick and thin.

For those who persevere amidst adversities,
Padayon.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Retrato, MDC.**, Qiu, S., Lundquist, A., Muratovic AZ., Rad, FM., Ubhayasekera SKJA., Bergquist, J. (2023) Simultaneous determination of 22 fatty acids in total parenteral nutrition (TPN) components by gas chromatography-mass spectrometry (GC-MS). *Analytical Methods,* 15, 2480-2489

II. **Retrato, MDC.**, Anh, VN., Ubhayasekera SKJA., Bergquist, J. (2025) Comprehensive quantification of C4 to C26 free fatty acids using a supercritical fluid chromatography-mass spectrometry method in pharmaceutical-grade egg yolk powders intended for total parenteral nutrition use. *Analytical and Bioanalytical Chemistry,* DOI: 10.1007/s00216-025-05732-3

III. **Retrato, MDC.**, Ubhayasekera SKJA., Bergquist, J. (2025) Quantitative analysis of cholesterol and 14 cholesterol oxidation products using supercritical fluid chromatography tandem mass spectrometry method applied in pharmaceutical-grade egg yolk powders. *Submitted*

IV. **Retrato, MDC.**, Ubhayasekera SKJA., Bergquist, J. (2025) Targeted analysis of 30 key lipid species in pharmaceutical-grade egg yolk powders for total parenteral nutrition products using ultra-high performance liquid chromatography tandem mass spectrometry. *Submitted*

Contribution Report

The author would like to clarify his contributions to the research presented in **Papers I-IV**.

I. Conceptualization, performed experiments, method development, sample analysis, data processing, wrote the original draft of the manuscript, helped in finalizing the manuscript

II. Conceptualization, performed experiments, method development, sample analysis, data processing, wrote the original draft of the manuscript, helped in finalizing the manuscript

III. Conceptualization, performed experiments, method development, sample analysis, data processing, wrote the original draft of the manuscript, helped in finalizing the manuscript

IV. Conceptualization, performed experiments, method development, sample analysis, data processing, wrote the original draft of the manuscript, helped in finalizing the manuscript

This thesis was written by the author alone and without content generation using generative artificial intelligence (AI) tools.

List of Papers not included in this thesis

V. Amortegui, JCE., Pekar, H.; **Retrato, MDC.,** Persson, M., Karlson, B., Bergquist, J., Zuberovic-Muratovic, A. (2023) LC-MS/MS Analysis of Cyanotoxins in Bivalve Mollusks-Method Development, Validation and First Evidence of Occurrence of Nodularin in Mussels (*Mytilus edulis*) and Oysters (*Magallana gigas*) from the West Coast of Sweden. *Toxins*, 15 (5).

VI. Isgren, AR., Carlhall, S., **Retrato, MDC**., Kodikara, C., Ubhayasekera, SJKA., Kjolhede, P., Bergquist, J., Blomberg, M. (2023) The association between maternal body mass index and serial plasma oxytocin levels during labor. *PLOS One*, 18 (8)

Contents

Abbreviations

APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
API	Active pharmaceutical ingredient
ATP	Adenosine triphosphate
BEH	Ethylene bridged hybrid
CAD	Charged aerosol detector
CRM	Charged residue model
CSH	Charged surface hybrid
CE	Epoxycholesterol
CE (eV)	Collision energy
CI	Chemical ionization
COP	Cholesterol oxidation product
CT	Cholestanetriol
CV	Cone voltage
%CV	Percent coefficient of variation
DC	Direct current
EFA	Essential fatty acid
EI	Electron ionization
ELSD	Evaporative light scattering detector
EMA	European Medicines Agency
ESI	Electrospray ionization
ESI^+	Electrospray ionization in positive mode
ESI^-	Electrospray ionization in negative mode
FA	Fatty acid
FAME	Fatty acid methyl ester
FDA	U.S. Food and Drug Administration
FFA	Free fatty acid
FID	Flame ionization detector
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GI	Gastrointestinal
GPC	Glycerophosphatidylcholine
HC	Hydroxycholesterol
HILIC	Hydrophilic interaction liquid chromatography
HSS	High strength silica

ICH	International Council for Harmonization
IDM	Ion desorption model
IS	Internal standard
IV	Intravenous
KC	Ketocholesterol
LC	Liquid chromatography
LCFFA	Long-chain free fatty acid
LOD	Limit of detection
LLOQ	Lower limit of quantification
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPL	Lysophosholipid
MCFFA	Medium-chain free fatty acid
MCT	Medium chain triglyceride
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
PLE	Pharmaceutical lipid emulsion
Q2(R2)	Guidelines for validation of analytical procedures
QA	Quality assurance
QC	Quality control
RF	Radio frequency
R^2	Correlation coefficient
SCFFA	Short-chain free fatty acid
SF	Supercritical fluid
SFC	Supercritical fluid chromatography
SM	Sphingomyelin
SIR	Selected ion recording
S/N	Signal-to-noise ratio
TAG	Triacylglycerol
TPN	Total parenteral nutrition
UPLC	Ultra-performance liquid chromatography
UPC^2	Ultra-performance convergence chromatography
UV	Ultraviolet

1. Introduction

Providing the daily nutritional requirements of severely ill patients is necessary for their total rehabilitation. Oral food intake is not feasible for comatose individuals, premature babies in intensive care units, or treatment recipients with a dysfunctional gastrointestinal (GI) tract[1]. Therefore, alternative methods to enteral feeding (the process of food administration through the mouth to the digestive system) for supplying nourishment are explored to revitalize clinical subjects.

The parenteral pathway, which bypasses the GI system, is widely utilized for delivering dietary needs of these subjects in critical condition. This is achieved through intravenous administration, facilitating direct delivery of nutrition and calories into the bloodstream. The components of total parenteral nutrition (TPN) are categorized into macronutrients, micronutrients, and other additives. TPN products incorporate carbohydrates, lipids, proteins, vitamins, minerals, electrolytes, trace elements, and water[1, 2], aiming to provide therapeutic outcomes in diseased individuals and long-term patients. Since TPNs are injected directly into circulation, these products are classified as pharmaceuticals and are subject to stringent regulations and rigid manufacturing checkpoint practices. As such, ensuring the TPN product quality is crucial for optimizing critical patient care.

Recent developments in TPN formulations have explored the incorporation of active pharmaceutical ingredients (APIs), as TPNs are regarded as potential mediums for targeted drug delivery systems[1-3]. Among the components of TPN, variations in lipid content are strongly associated with the stability and effectiveness of pharmaceutical lipid emulsions (PLEs), which is considered the backbone and primary raw material for manufacturing TPNs[1, 4, 5]. This emphasizes the need for precise, reliable, and robust techniques to identify, distinguish, and quantify major lipid groups in PLEs, ensuring the integrity of TPN products and compliance with manufacturing specifications and quality standards. Here, the skills of analytical chemists and their developed methodologies can make important contributions to the improvements and this thesis is dedicated to push the analytical strategies and technologies forward into robust and validated tools.

1.1 Total parenteral nutrition

Parenteral nutrition (PN) is the intravenous administration of essential calories and nutrients directly into the bloodstream, bypassing the digestive system. PN is classified into two types—peripheral parenteral nutrition (PPN) and total parenteral nutrition (TPN)[5, 6]. PPN is typically used for short-term nutritional support and is delivered through a peripheral vein, often in the arm. On the other hand, TPN is administered through a central vein, usually accomplished by a central venous catheter (CVC) inserted into large veins such as the internal jugular or subclavian veins. TPN is most commonly used for individuals with chronic malabsorption, digestive tract dysfunction, or those in neonatal intensive care units who require long-term, specialized nutritional support that cannot be provided through the gastrointestinal tract[7].

TPN products are carefully formulated to provide essential macronutrients, including carbohydrates (glucose), proteins (amino acids), and lipids (lipid emulsion)[2, 3]. These macronutrients form the bulk of TPN formulations and are designed to meet energy needs. Glucose, typically in the form of dextrose, serves as the primary energy source, making up 5% to 70% of the formulation depending on the patient's caloric requirements. Essential and non-essential amino acids are included to support protein synthesis and maintain nitrogen balance. For critically ill subjects with kidney or liver complications, specialized TPN formulations with modifications in amino acid content may be used[4, 8]. Pharmaceutical lipid emulsion (PLE) is incorporated to provide essential fatty acids and serve as a calorie-dense energy source, which will be further discussed in section 1.2.

Micronutrients are also included in TPN to satisfy daily vitamin and mineral requirements[1]. Electrolytes such as sodium, potassium, calcium, magnesium, phosphate, and chloride are essential for maintaining electrolyte balance and supporting cellular functions. Trace elements like zinc, copper, chromium, manganese, and selenium are included for their role in enzymatic processes and physiological pathways[8]. Fat-soluble vitamins (A, D, E, and K) and water-soluble vitamins (C and B-complex) are also supplied due to their importance in promoting recovery and supporting overall health[9].

Tailored TPN formulations can be customized based on the patient's specific therapeutic needs by incorporating additional components such as insulin, heparin, and carnitine[10, 11]. Insulin helps manage hyperglycemia induced by dextrose infusion, while heparin prevents catheter-related thrombosis. The addition of carnitine in TPN formulations aids in fatty acid metabolism. All of these ingredients are incorporated in sterile water, which ensures proper hydration and maintains the solution volume for patients receiving TPN.

1.2 Pharmaceutical lipid emulsion and its components

PLE serves as the fundamental component of TPNs, which acts as a calorie reservoir supplying sufficient energy for clinical subjects through IV administration[12]. It supplies essential fatty acids[13] (EFAs) such as omega-3 and omega-6 FAs necessary for several physiological process, such as maintaining the structure of cell membranes[13, 14], production of hormones[6] and facilitates inflammatory responses[6, 15]. Lipids aid in the uptake of fat-soluble vitamins crucial in numerous biological pathways. These benefits contribute to the overall therapeutic outcome associated in consuming TPN products. Emulsions, characterized by two phases existing as one upon admixture (*e.g.* oil-in-water) are only stabilized by surfactants for a definite period. At some point, TPN product formulations start degrading due to compositional changes, as a consequence of short-lived stability of PLEs incorporated requiring immediate consumption. This decline in PLE stability is linked to lipid content variations, which may lead to adverse effects instead of improved clinical progression. Previous studies associated these negative clinical impacts to some stereospecific placement of FAs in triacylglycerols (TAGs) present in PLEs[16, 17].

Rancidity and premature separation of lipid emulsions were also correlated to variations in total free fatty acid (FFA) content[8]. Soybean oil was the exclusive lipid source in earlier PLE formulations, which contains approximately 50% linoleic acid (C18:2) in total FFA content. Exceeding other FFAs in concentration, the abundance of C18:2 in TPNs was correlated to a higher oxidative stress burden in the diseased individuals, resulting in greater metabolic risks and detrimental clinical conditions[18]. Therefore, conscious efforts in reducing C18:2 in TPN product formulation is achieved by lowering the amount of soybean oil and mixing it with alternative oil sources[12, 14]. This provides an opportunity for improving TPN product formulation with better admixture stability, overcoming the potential health risks and side-effects such as immunomodulation[1, 3] and inflammation[3, 5]. With these pharmaceutical developments, alternative oil sources such as fish[6, 19], olive[6, 20], palm kernel[14], and coconut[11, 14] are explored and incorporated in PLE modifications aiming for increased product potency. Therefore, the presence of FAs in these admixtures must be carefully monitored and regulated considering the inevitable compositional changes in manufactured TPNs.

Phospholipids (PLs) function as emulsifying agents in PLEs, making them its critical component. They allow fat droplet dispersion in the aqueous phase, resulting in uniform distribution of two immiscible liquid phases to a single state. Egg yolk and soybean oil are the main sources of PLs in TPN formulations[11, 21, 22]. The incorporation of PLs into PLEs contributes to its stability by

preventing fat droplets from coalescing through an electrostatic barrier generated by the dissociation of the anionic charge of PL polar ends toward the aqueous phase[23, 24]. An excess of PL content in the admixture leads to the creation of vesicular liposomes forming a lipid bilayer in the aqueous phase of PLEs. Liposome accumulation results in hypercholesterolemia in human studies as found in clinical studies[2, 25]. Moreover, changes in PL ratios are linked to instability and decreased efficacy of TPN products. For example, the hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholines (LPC) presents a drawback in TPN stability since LPC accumulation interferes with the lipid bilayer dynamics by modulating the phase transition temperature[9]. In effect, thermosensitive liposomes that are tailored for temperature-controlled drug delivery systems are disrupted[9, 25]. Monitoring the inevitable hydrolysis of PLs through quantification methods can offer valuable insights into the compositional changes of PLEs and enable predictions regarding the long-term potency and shelf-life of TPN.

Cholesterol maintains the structural integrity and function of cellular membranes by modulating their fluidity. Its unique structure, which consists of a rigid steroid ring system and a flexible isooctyl side chain at carbon 17, enables it to disrupt the tight packing of phospholipids, thereby enhancing membrane fluidity[26]. Cholesterol is an essential precursor for the synthesis of bile acids, vitamin D, and steroid hormones[26-28]. PLEs typically contain egg yolk, which serves as the primary source of cholesterol[5, 12, 15, 29]. However, cholesterol is susceptible to autooxidation through free radical reactions, mediated through enzymatic and non-enzymatic pathways[28]. Cholesterol is sensitive in the presence of oxygen, light and high temperatures, leading to the formation of cholesterol oxidation products (COPs) or oxycholesterols[30, 31]. Increase in the production of COPs is related to adverse health effects, mainly neurodegenerative[32, 33] and cardiovascular ailments[28, 34]. Unregulated cholesterol oxidation impairs cholesterol transport and metabolism in the body and induces inflammation, as it contributes to the instability and degradation of PLE[3, 23].

Recent TPN formulations include phytosterols (plant-based sterols) to help reduce cholesterol levels by inhibiting cholesterol absorption and accumulation in circulation[35, 36]. This is particularly beneficial for patients with hypercholesterolemia or cardiovascular diseases, as phytosterols can support better lipid management while they receive long-term PN[37]. Therefore, the use of low-cholesterol lipid sources in product formulations like soybean, fish, olive and medium-chain triglyceride (MCT) oils aid to regulate cholesterol levels and producing less amounts of COPs[4, 14, 18].

1.3 Lipidomic methods

Lipidomics is a discipline in metabolomics that emphasizes on the comprehensive study of lipids within a biological system[38]. Analysis of lipids relies on advanced analytical techniques, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS). In a biological system containing a multitude of lipids, coupling MS with chromatographic-based methods provides more information toward lipid identity and its content. The capability of chromatography for analyte distinction through column separation in combination with the high specificity and speed of the MS makes these hyphenated methods ideal for performing lipidomic work.

The assessment of PLE stability in TPN products has mainly comprised the evaluation of physical properties in emulsions using droplet sizing techniques[19, 39, 40]. In recent past, lipidomic approaches have been developed and tailored for the analysis of FAs, TAGs, PLs, cholesterol and COPs in recent years using chromatography and mass spectrometry-based techniques. However, only a handful of these methods have evaluated lipid profiles from raw materials incorporated in TPN formulations[2, 15, 17]. Most of the available analytical methods are not capable for high-throughput analysis[17, 22, 39, 41, 42], mainly focused on a few lipid compounds and classes[43], untargeted and semi-quantitative[44], and requires extensive sample preparation[45-47], which reduces method applicability for quality control (QC) practices and routine performance checks. Therefore, the development of analytical methods that meet validation requirements is necessary to monitor the quality and stability of TPN.

Below are brief descriptions of the analytical methodologies employed in this thesis to investigate lipids. While these are all fairly well-established technologies, each new application typically requires tailored method development, refinement, and subsequent validation.

1.3.1 Gas chromatography

Chromatographic separations are based on the partitioning of analytes between a fixed stationary phase and a mobile phase, with the latter carrying the analytes through the system[48, 49]. Gas chromatography (GC) enables the separation of highly volatile compounds in a sample by introducing a small amount the sample, either in liquid or gaseous form into the sample injector. In this technique, an inert carrier gas acts as a mobile phase, carrying the injected multicomponent mixture through the column at a temperature suitable for vaporization. The analytes eluting from the chromatographic column can be identified based on their retention times before they reach the detector, as different components exhibit varying degrees of retention. As these analytes of

interest exit the GC column, they pass through a detector that measures their abundance and generates a chromatogram. Quantification of the analysed chemical species is possible through evaluation of their peak areas upon elution[50-52]. The two most widely used GC detectors are flame ionization detector (FID), which is best for detecting volatile organic compounds, and MS, which can support detailed compound identification based on their mass-to-charge (*m/z*) ratio[48, 49]. A block diagram showing GC-MS instrumentation is illustrated in Figure 1.

GC offers rapid analysis, excellent resolution for complex sample mixtures, and high sensitivity. However, a key limitation is that analytes must be volatile. Since lipids are generally non-volatile, they require derivatisation to be converted into GC-compatible compounds[46, 49, 53-55]. These principles are stressed in **Paper I**, where chemical derivatisation methods were performed and assessed for the conversion of FFAs to fatty acid methyl esters (FAMEs), as it is one of the most commonly used methods for FFA quantification. In this context, five different transesterification methods were tested, each based on various derivatisation approaches, to evaluate their effectiveness using the internal standard method. Additionally, the study aimed to incorporate short-chain FFAs (SCFFAs), since the smallest chain length detectable by the existing GC methods is C8:0[49, 56-58]. **Paper I** also sought to apply GC analysis to three TPN components: fish oil, olive oil, and an unidentified lipid sample as these three target components are the most sensitive for the changes in FFA levels over time.

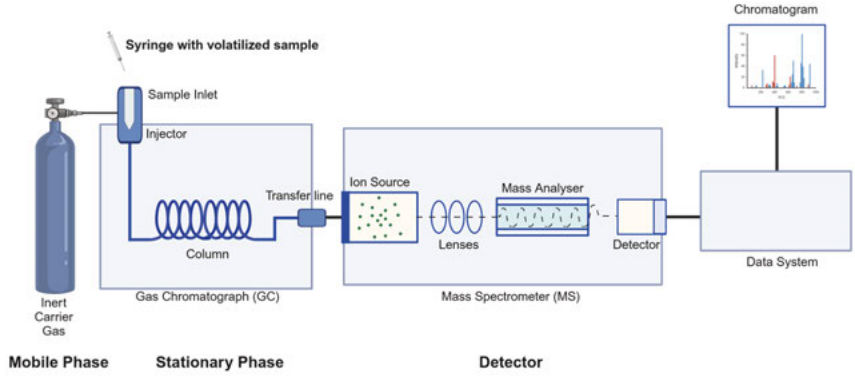


Figure 1. Schematic illustration of a typical gas chromatography coupled with mass spectrometry (GC-MS) instrumentation (Created in BioRender. Retrato, M. (2025))

1.3.2 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is an advanced analytical separation technique that uses a supercritical fluid (SF) as the mobile phase. Initially,

SFC was developed as an alternative to GC due to its unique combination of liquid-like solvation power and gas-like low viscocity[59-62]. The mobile phase in SFC plays an active role in analyte resolution, in contrast with GC where an inert carrier gas is used. In its early stages, SFC employed open tubular capillary columns and GC-compatible detectors[63]. The behaviour of SFs used is similar to a compressible fluid with high diffusivity, enabling rapid mass transfers in high linear velocities. This leads to efficient separations and shorter analysis times[60, 64].

Carbon dioxide in its supercritical fluid (SF) state is created by applying heat and pressure above its critical temperature (31°C) and critical pressure (73.8 atm). Among SFs, supercritical CO_2 (sCO_2) is considered the most suitable mobile phase when compared to other SFs such as ethane, ammonia and nitrous oxide[59-62, 65]. This is due to its availability, relatively low critical point, inertness to most compounds, non-flammability, and miscibility with a wide range of organic solvents, such as methanol[59, 65]. While sCO_2 is an excellent solvent for non-polar compounds, its solvating power for polar analytes is enhanced by the addition of a modifier in SFC, enabling the separation of a broader range of analytes. Further modification of polar organic modifiers can be achieved by incorporating small volumes of water and additives, which can enhance chromatographic performance. The addition of water improves the solubility of polar analytes, while also enhancing selectivity and peak resolution, as it interacts with both the analyte and the stationary phase. Incorporating an acid or base adjusts the pH, aiding in analyte ionization and their interaction with the stationary phase, while also improving peak symmetry and reducing chromatographic tailing[35, 59].

Analyte separation in SFC is significantly influenced by the choice of stationary phase. SFC mimics normal-phase chromatography but can be tuned to accommodate a wider polarity range through the use of organic modifiers[35, 60]. The stationary phases used in SFC utilize small particle sizes to enhance interactions with the analyte, improve resolution, and enable higher flow rates, thereby reducing analyte retention time. However, these smaller particle sizes also lead to increased backpressure, necessitating instruments capable of operating at higher pressure[59, 65]. Similar to other chromatographic methods such as GC and LC, SFC can be coupled with MS (Figure 2), and is increasingly being applied for the analysis of a broad range of analytes, particularly semi-volatile, thermally labile, and structurally similar compounds[59, 65]. SFC-MS techniques are particularly effective at separating structurally similar compounds, such as isomers, within short analytical run times[35, 60, 66]. As a result, SFC-MS presents a promising alternative for the targeted analysis of endogenous compounds in various biological matrices[59-62]. Additionally, SFC-MS requires significantly less organic solvent than conventional LC, making it a

more environmentally friendly approach that aligns with the principles of green chemistry.

The application of SFC in method development was emphasized in **Papers II** and **III**, highlighting the technique's capability to analyse multicomponent matrices containing analytes with structurally-related lipids. The choice of SFC in **Paper II** for FFA quantification was a further development from the limitations encountered in **Paper I**, which requires chemical derivatisation of FFAs to FAMEs for GC analysis. **Paper II** establishes the direct analysis of FFAs, eliminating the need for transesterification. In addition, incorporation of 31 FFAs consisting from short to long-chain FFAs in a single SFC method is a first of its kind. In **Paper III**, the analysis of cholesterol and COPs without derivatisation advances the use of SFC for sterol analysis.

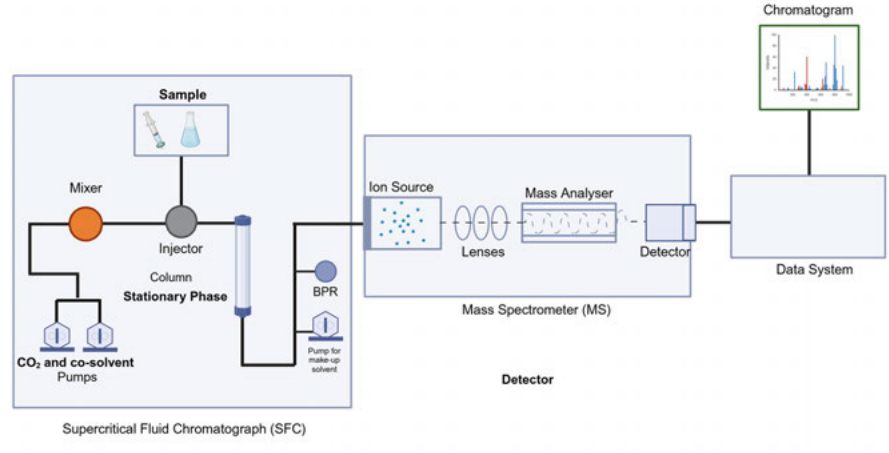


Figure 2. Schematic illustration of a typical supercritical fluid chromatography coupled with mass spectrometry (SFC-MS) instrumentation (Created in BioRender. Retrato, M. (2025))

1.3.3 Liquid chromatography

Analytical separation in liquid chromatography (LC) relies on the differential interactions between components of a complex mixture and the stationary phase packed within the column, while a mobile phase consisting of one or more solvents, transports the analytes[67, 68]. The separation of analytes in LC is primarily based on differences in compound polarity, molecular size, and specific interactions with the stationary phase. These differences result in differential migration, with each analyte having different retention times, enabling the separation of multiple components in LC[67, 68].

Ultra-performance liquid chromatography (UPLC) is an advanced form of LC that offers improved resolution and speed[38, 69]. UPLC utilizes columns with sub-2 µm stationary phase particles, enabling operation at higher pressures. This enables higher flow rates, faster analysis, and reduced sample volume requirements. Currently, UPLC is most commonly coupled with mass spectrometry (MS), and the combination of these techniques provides a comprehensive analysis of the analytes of interest, as illustrated in Figure 3. Together, UPLC-MS enables the analysis of diverse sample types, including those from environmental, food, clinical, and pharmaceutical sources[38, 70-76].

UPLC-MS is widely used in lipidomics due to its ability to analyse a broader range of lipid classes, particularly polar and intact lipids, compared to GC[38, 70-76]. Its adjustable parameters for developing analytical methods, such as the selection of various stationary phases, mobile phase combinations, gradient optimization, and flow rate adjustments, contribute to the versatility of UPLC[38, 70-76]. This makes it suitable for analysing a diverse array of analytes. **Paper IV** relies on the principles of LC as a suitable analytical technique for identification and quantification of key lipid species present in PLE raw materials. This work demonstrates a rapid and reproducible method for the detection and quantification of 30 lipid species. These species are highly monitored in pharmaceutical-grade egg yolk powders, an important component for PLE formulations and TPN products.

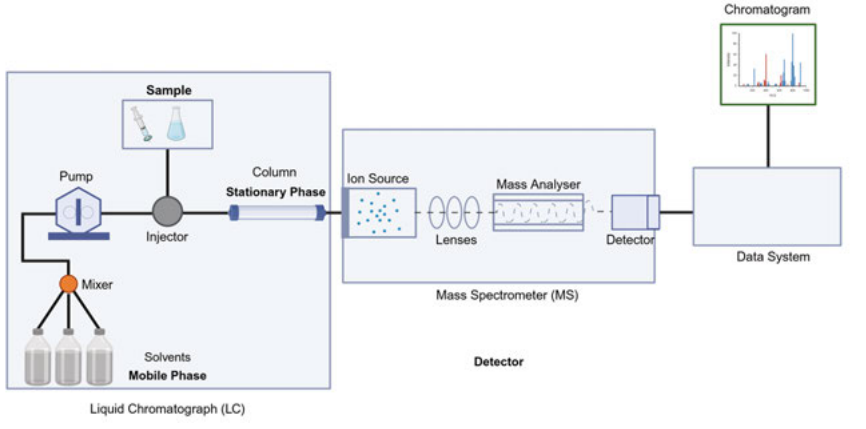


Figure 3. Schematic illustration of a typical liquid chromatography coupled with mass spectrometry (LC-MS) instrumentation (Created in BioRender. Retrato, M. (2025))

1.3.4 Mass spectrometry

Mass spectrometry (MS) is a powerful technique that ionizes chemical species into gas-phase ions and separates them based on their mass-to-charge ratio

(*m/z*). It stands out from other analytical methods by its several unique characteristics:

1. Exceptional molecular specificity, driven by its ability to precisely measure molecular mass,
2. High molecular specificity in determining the structures of diverse compound classes and individual elements,
3. High sensitivity, allowing for trace-level analysis,
4. Excellent compatibility with various chromatographic separation techniques, and
5. Broad applicability to a wide range of sample types, including volatile, non-volatile, polar, and non-polar compounds[77, 78].

In MS, a small amount of sample is introduced into the ion source where the components of the sample are converted into gaseous ions by bombardment with electrons, photons, ions or molecules[65]. Ionization in the MS can also be achieved by applying thermal or electrical energy[65, 77, 78]. A stream of positive or negative gas-phase ions are the outputs of the ion source, in which are then accelerated into the mass analyzer[65, 77, 78]. The dispersion of the ions in the mass analyzer is characterized by their *m/z*. A vacuum system is required in MS to maintain a low pressure in all of the components except the signal processor and read out. The low pressure ensures infrequent collisions in the MS to produce and maintain free ions and electrons[65, 77, 78]. By optimizing the MS parameters, characteristic ions can be selectively generated, enhancing the technique's detection sensitivity[77, 78].

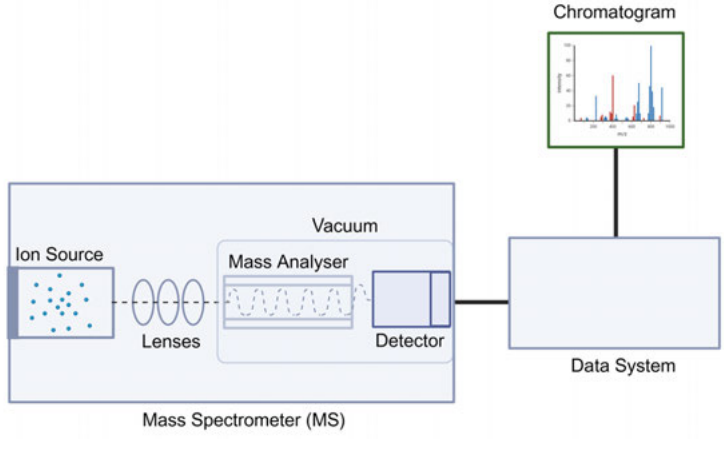


Figure 4. Block diagram of a mass spectrometer with an ionization source at atmospheric pressure. (Created in BioRender. Retrato, M. (2025))

Ionization techniques in GC

Electron ionization (EI) and chemical ionization (CI) are the two primary ionization techniques used in gas chromatography-mass spectrometry (GC-MS). EI is a hard ionization method, while CI is a softer alternative that reduces fragmentation. In CI, a reagent gas (*e.g.*, methane, ammonia, or isobutane) is introduced into the ionization chamber, where it undergoes ionization upon interaction with high-energy electrons, forming reagent ions that subsequently transfer charge to analyte molecules[48, 49]. A stream of electrons emitted from an electron gun ionizes the reagent gas, through electron-molecule collisions, generating primary reagent ions that participate in ion-molecule reactions with analyte molecules[48, 49]. These reagent ions transfer charge via protonation ($[M + H]^+$) or charge exchange, producing molecular ions with minimal fragmentation[48, 49]. CI is considered a gentler ionization method, as it produces less fragmentation and allows for the preservation of the intact molecular ion, making it particularly useful for the analysis of high molecular weight, thermally labile, or polar compounds[78].

In comparison, EI is achieved by a heated filament that emits electrons, creating an electron beam that passes through the ionization chamber, which is influenced by a magnetic field. As gas molecules eluting from the GC column collide with the electrons, they lose an electron and form positively charged molecular ions[48, 49]. While the ionization potential of organic compounds is typically around 10 eV, EI operates with an ionization energy of approximately 70 eV. This higher energy causes the molecular ions to fragment further, as the excess energy from the electron beam leads to the destabilization of the ionized species. Characteristic fragment ions are formed through specific cleavage patterns of the molecule, which include the loss of functional groups, the cleavage of carbon-carbon or carbon-heteroatom bonds, and molecular rearrangements. These principles were applied in **Paper I**, demonstrating the ability of EI to generate distinctive fragment ions of FAMEs that are essential for a selective and sensitive FFA quantification.

Ionization techniques in SFC and LC

SFC and LC are widely used analytical techniques for the separation and analysis of complex lipids, with SFC being particularly suited for non-polar lipids and LC offering flexibility for a broader polarity range. These techniques rely on efficient ionization methods when coupled with MS to detect and characterize a wide range of compounds.

Ionization techniques in SFC and LC employ electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). Each technique operates under different principles,

making them suitable for specific classes of compounds based on their polarity, volatility, and chemical structure[78, 79].

In the present study, both SFC and LC were utilized, with ESI serving as the primary ionization method. ESI is a soft ionization technique which generates gas phase ions from analytes in solution with little to no fragmentation[78, 79]. ESI operates at atmospheric pressure and involves the formation of charged droplets, followed by solvent evaporation in these droplets, leading to formation of gas phase ions.

A high voltage (around 3-5kV) is applied between the spray capillary and a counter electrode, creating an electrostatic field that causes accumulation of the charged liquid at the capillary tip. This results to the formation of a Taylor cone. As the electrostatic forces increase, they overcome the surface tension of the liquid, leading to the emission of a fine liquid jet[79]. This liquid jet consists of charged droplets that continue to shrink as the solvent evaporates, increasing the charge density until the droplets break into smaller ones or individual ions[78, 79]. Once the droplets reach the Rayleigh limit, they undergo fission, breaking into smaller droplets as the Coulombic force exceeds the surface tension of the liquid. This process repeats several times, resulting in tiny, highly charged second-generation droplets. Gas phase ions are formed when these droplets shrink to a sufficiently small size[78, 79].

There are two primary theories used to describe the transfer of solvated ions to gas-phase ions in ESI. These are the charged droplet model (CDM), also known as Coulomb fission and the ion evaporation model (IEM). The CDM suggests that ionization occurs through the fission of charged droplets as the solvent evaporates[78, 79]. As the fission continues, the droplets shrink in size, and their charge density increases. Once a critical size is reached, where the Coulombic repulsion overcomes the surface tension of the droplet, resulting in the emission of smaller charged ions. The continuous process of droplet shrinkage leads to the formation of gas-phase ions[78, 79]. IEM, on the other hand, considers that the ion itself is evaporating from the droplet. When the charged droplet shrinks to a size wherein the electrostatic energy of the ion overcomes the droplet's surface tension, the surrounding solvent molecules are removed as the ions move into the gas phase[78, 79]. Both models provide some ideas into the mechanisms underlying ion formation in ESI, though the precise dynamics may involve contributions from both processes.

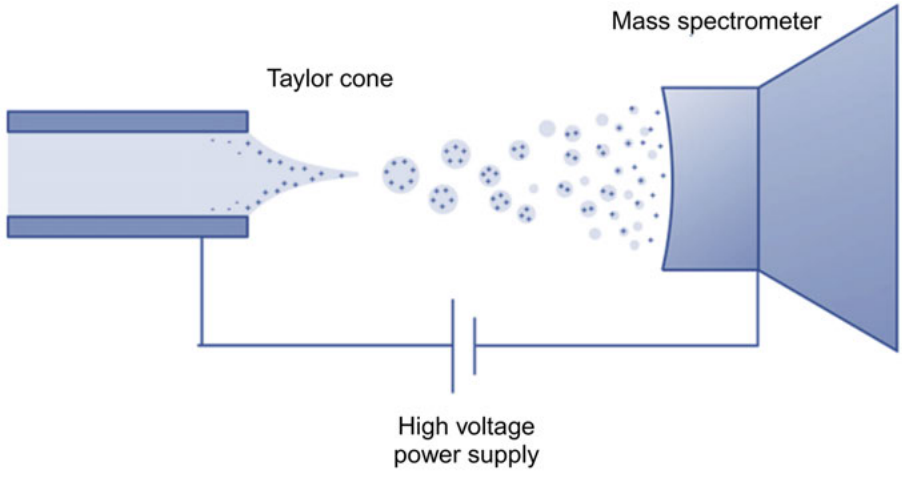


Figure 5. The process of electrospray ionization in positive mode (ESI^+). (Created in BioRender. Retrato, M. (2025))

Several factors influence the transition of charged droplets to gas-phase ions, including the physical and chemical properties of the solvent, the nature and concentration of the analyte, the ionizing agents used, applied voltage, and droplet surface tension[78, 79]. These considerations enable further optimization for tailored analyses, making ESI advantageous over other ionization techniques. However, ion suppression in ESI can occur due to several factors, including high concentrations of non-volatile compounds, co-eluting species, high salt concentrations, and matrix effects, which complicate the analysis. To overcome this, sample purification and separation methods are often employed before MS analysis. The compatibility of ESI with LC and SFC highlights its versatility in various method development applications, as demonstrated in **Papers II-IV**, where either positive or negative ions are generated depending on the selected ESI mode. **Paper II** used ESI^- for the detection of 31 FFAs in MS, while ESI^+ is used for the detection of cholesterol and 14 COPs in **Paper III** and 30 key lipid species in **Paper IV**.

Mass analysers

Among the components of a mass spectrometer, mass analysers are considered the most critical, as they utilize the mass-to-charge ratio (*m/z*) of ions for analyte detection and quantification. The primary role of a mass analyser is to discriminate between ions with closely related *m/z* ratios, enabling their resolution and focusing them at a single focal point. A quadrupole, the most commonly used mass analyser, consists of four parallel rods symmetrically arranged around a central axis. Opposite rods are electrically connected, mean-

ing they experience the same voltage at any given time[77, 79]. Phase-shifted radio frequency (RF) voltages are applied to different pairs of rods to guide the ion beam trajectory. As a result, one pair of rods experiences maximum positive voltage, while the other pair experiences maximum negative voltage. In principle, quadrupoles with RF voltages transport ions, but when direct current (DC) voltages are added, it becomes possible to isolate specific ions[77, 79]. Ion distinction and detection are achieved in a quadrupole by adjusting RF and DC voltages to suit the analytes of interest, as demonstrated in **Paper I**.

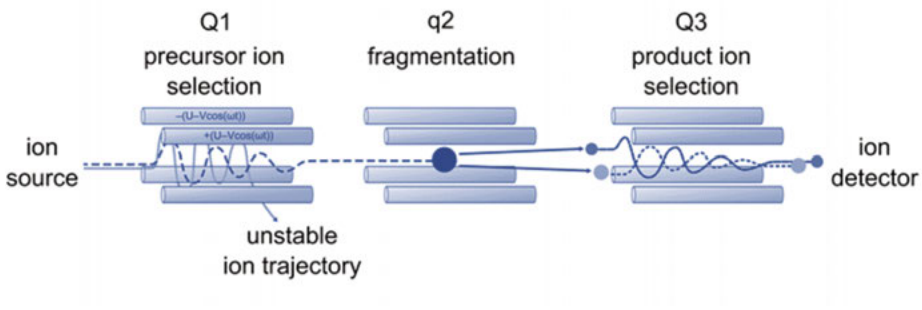


Figure 6. A triple quadrupole mass analyser, where Q1: first, q2: second, and Q3: third quadrupoles (Created in BioRender. Retrato, M. (2025))

The analytical power of quadrupole mass spectrometry is significantly enhanced in the triple quadrupole system, which improves both mass selection and fragmentation efficiency. The first quadrupole (Q1) selects the precursor ion, while the second quadrupole (q2) serves as an RF-only collision cell where fragmentation occurs, and the third quadrupole (Q3) filters the resulting product ions for detection. The use of triple quadrupoles in mass spectrometry setup improves the technique's resolution and sensitivity.

Selected ion recording (SIR) can be applied in both single and triple quadrupole MS systems, where only the parent ions selectively monitored in Q1 without undergoing fragmentation. This approach is particularly effective when analytes are chromatographically resolved prior to entering the MS, such as when baseline separation is achieved through a coupled GC-MS or SFC-MS system, as demonstrated in **Papers I** and **II**.

In multiple reaction monitoring (MRM) mode, tandem mass spectrometry (MS/MS) is performed using a triple quadrupole mass spectrometer (QqQ-MS). This technique provides high sensitivity, selectivity, and precision, allowing accurate quantification even in cases of incomplete chromatographic separation due to its highly specific parent-to-product ion transitions. The highly selective parent-to-product ion transitions in MRM allow precise detection and quantification of target analytes in complex matrices, as demonstrated in **Papers III** and **IV**.

1.4 Lipid classification

The LIPID MAPS classification system is a widely accepted framework for categorizing lipids based on their chemical structure and biosynthetic origin. This system organizes lipids into eight major categories, with each category further subdivided into classes, subclasses, and individual lipid species[80]. The structural diversity of lipids enables a wide range of targeted analytical approaches for their identification and quantification. As essential biomolecules, lipids play various physiological roles, influencing both therapeutic outcomes and the development of clinical conditions. Given their significance as key components of PLEs in the production of TPN formulations, understanding lipid properties is crucial for conducting stability studies of these vital pharmaceutical infusions.

1.4.1 Free fatty acids

Free fatty acids (FFAs) are classified as derived lipids, as they are hydrolysis products of simple lipids (*e.g.*, triacylglycerols) and compound lipids (*e.g.*, phospholipids) during cellular signaling, lipolysis, or other metabolic processes[81]. FFAs are unbound carboxylic acids with hydrocarbon chains of varying lengths, making them amphipathic molecules. Their amphipathic nature arises from the hydrophilic carboxyl group and the hydrophobic hydrocarbon chain. These molecules can exist within the intracellular environment or become protein-bound, circulating in the bloodstream[51]. FFAs are vital sources of energy, generating adenosine triphosphate (ATP) upon oxidation in the mitochondria, particularly during high-energy-demand activities such as exercise or fasting[82].

According to the LIPID MAPS classification, FFAs with 13 to 21 carbon atoms are categorized as long-chain FFAs (LCFFAs), which are the most abundant type in the human body[83]. These LCFFAs act as building blocks for cells in the body, as they are essential for cell membrane formation, along with energy production and other metabolic processes involving heart regulation, brain function, inflammation, and hormone balance[83]. Medium-chain FFAs (MCFFAs) which range from six to twelve carbon atoms, are more easily digested compared to LCFFAs, with common sources including palm kernel and coconut oil[84]. Short-chain FFAs (SCFFAs) consist of fewer than six carbon atoms, are particularly important in gut health and metabolism[85].

FFAs can also be categorised based on their saturation levels. Saturated FFAs, which are straight-chain fatty acids, have no carbon-carbon double bonds. These FFAs are typically found in animal fats and processed foods. In con-

trast, unsaturated FFAs contain one or more (polyunsaturated, PUFAs) carbon-carbon double bonds, and introduces kinks in the straight-chain structures of FFAs. The presence of these double bonds makes unsaturated FFAs and PUFAs more susceptible to oxidation, a process that plays a crucial role in cellular signalling, especially in inflammatory responses[86]. These unsaturated fats are predominantly found in plant and fish oils, which are known to support cardiovascular health. This is why fish and plant-based oils are often included in PLE formulations for TPN products[19]. They help regulate the proportion of saturated FFAs while providing the health benefits associated with unsaturated FFAs[86].

The key distinctions of FFAs were effectively utilized in **Papers I** and **II**, where these characteristics were leveraged to guide targeted analyses of FFAs across all types.

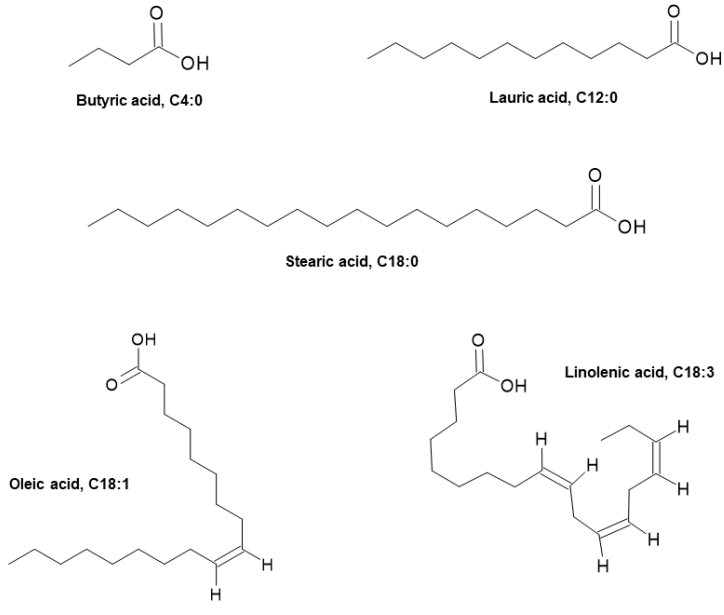


Figure 7. Chemical structures of saturated and unsaturated fatty acids across different chain lengths

At present, the analysis of FFAs is focused on chromatography-based mass spectrometric techniques, which offer enhanced sensitivity compared to other established analytical methods. GC-MS is conventionally used for FFA analysis. Since FFAs are non-volatile compounds, they must first be converted to thermally stable fatty acid methyl esters (FAMEs) through transesterification. **Paper I** optimised various derivatisation conditions and applied to three different TPN components. The analytical method developed in **Paper I** was able

to include 22 FFAs in a single run including C6:0 in the form of FAMEs, which is considered a first for GC-MS based techniques. For a long time, the integration of short-, medium-, and long-chain FFAs into a single analytical method has posed challenges in quantification, leading to segmented method development. While many studies have explored the potential of LC-MS for FFA quantification, this also typically requires analyte derivatisation to achieve satisfactory sensitivity. The use of SFC-MS for FFA quantification is relatively recent compared to GC- and LC-based methods. **Paper II** demonstrates the promising capabilities of SFC-MS, successfully analysing 31 FFAs ranging from C4 to C26.

1.4.2 Cholesterol and its oxidation products

Cholesterol is essential for maintaining the fluidity and structural integrity of cell membranes[26-28]. It serves as an important precursor for various bioactive molecules, including bile acids, steroid hormones, and vitamin D[26-28]. Dysregulation of cholesterol homeostasis is associated with cardiovascular diseases such as atherosclerosis and coronary heart disease. Cholesterol is susceptible to oxidation, which can occur via both enzymatic and non-enzymatic pathways, leading to the formation of cholesterol oxidation products (COPs) or oxysterols[30, 31, 76, 87].

Enzymatic cholesterol oxidation is catalysed by cytochrome P450 enzymes, which introduce a hydroxyl group to the isooctyl side chain at carbon-17 or the steroid nucleus[30, 31, 76]. Enzyme-mediated oxidation generates COPs with oxidized side chains, such as 20α-hydroxycholesterol, 22α-hydroxycholesterol, 22β-hydroxycholesterol, 27-hydroxycholesterol, 24,25-epoxycholesterol, and cholestanetriol[28, 30, 31, 45]. The B-ring of cholesterol, especially at the C7 position, is highly susceptible to free radical-induced oxidation leading to the formation of COPs such as 7-ketocholesterol and 7β-hydroxycholesterol[30, 31].

Non-enzymatic oxidation also leads to the formation of COPs, including 4β-hydroxycholesterol, 7α-hydroxycholesterol, α-epoxycholesterol, and β-epoxycholesterol[30, 31]. The addition of hydroxyl groups to cholesterol increases the polarity of these oxidation products, altering their biological properties. These structural modifications are associated with their roles in cell signaling and various metabolic processes[30, 31]. The association between COPs and the progression of cardiovascular diseases[34] and neurodegenerative diseases[32, 33] has driven targeted analysis to better understand how their levels contribute to these pathological conditions[32-34].

The analysis of COPs has been performed mainly using gas chromatography (GC) and liquid chromatography (LC), often combined with mass spectrometry (MS) or tandem mass spectrometry (MS/MS)[27, 67, 75, 88-91]. GC-MS requires chemical derivatisation, especially the conversion of hydroxyl-containing COPs to silyl ethers, which are more compatible with GC[31, 92]. However, EI for COPs analysis in GC-MS generates complex and inconsistent fragmentation patterns, complicating the accurate identification and quantification[67].

The direct analysis of COPs using LC-MS/MS is challenging because COPs do not readily form $[M + H]^+$, $[M + NH_4]^+$ or $[M - H]^-$ ions due to their lower ionization efficiency in ESI, leading to low and inconsistent signal intensities. As a result, most established analytical techniques for COPs analysis in LC-MS/MS utilize the less common atmospheric pressure chemical ionization (APCI) instead of ESI[67, 88, 91]. To enhance the ionization efficiency of COPs in ESI, various derivatisation strategies have been investigated. These include conversion to N,N-dimethylglycine esters[93], oximes[94], Girard hydrazones[45, 95], nicotinyl esters[96], and picolinyl esters[76]. However, derivatisation of COPs introduces additional sample preparation steps and longer analysis times, with a potential risk of analyte loss due to incomplete conversion.

Paper III explores the potential of SFC-MS/MS for the direct analysis of COPs applied in TPN components. While SFC has been less widely explored compared to GC- and LC-based methods, a previous study demonstrated its ability to separate eight oxycholesterols[97]. However, research in this area has not progressed significantly since then. The shift toward SFC is advantageous, as it enables the same analyses with reduced solvent usage, providing a more environmentally friendly and sustainable approach.

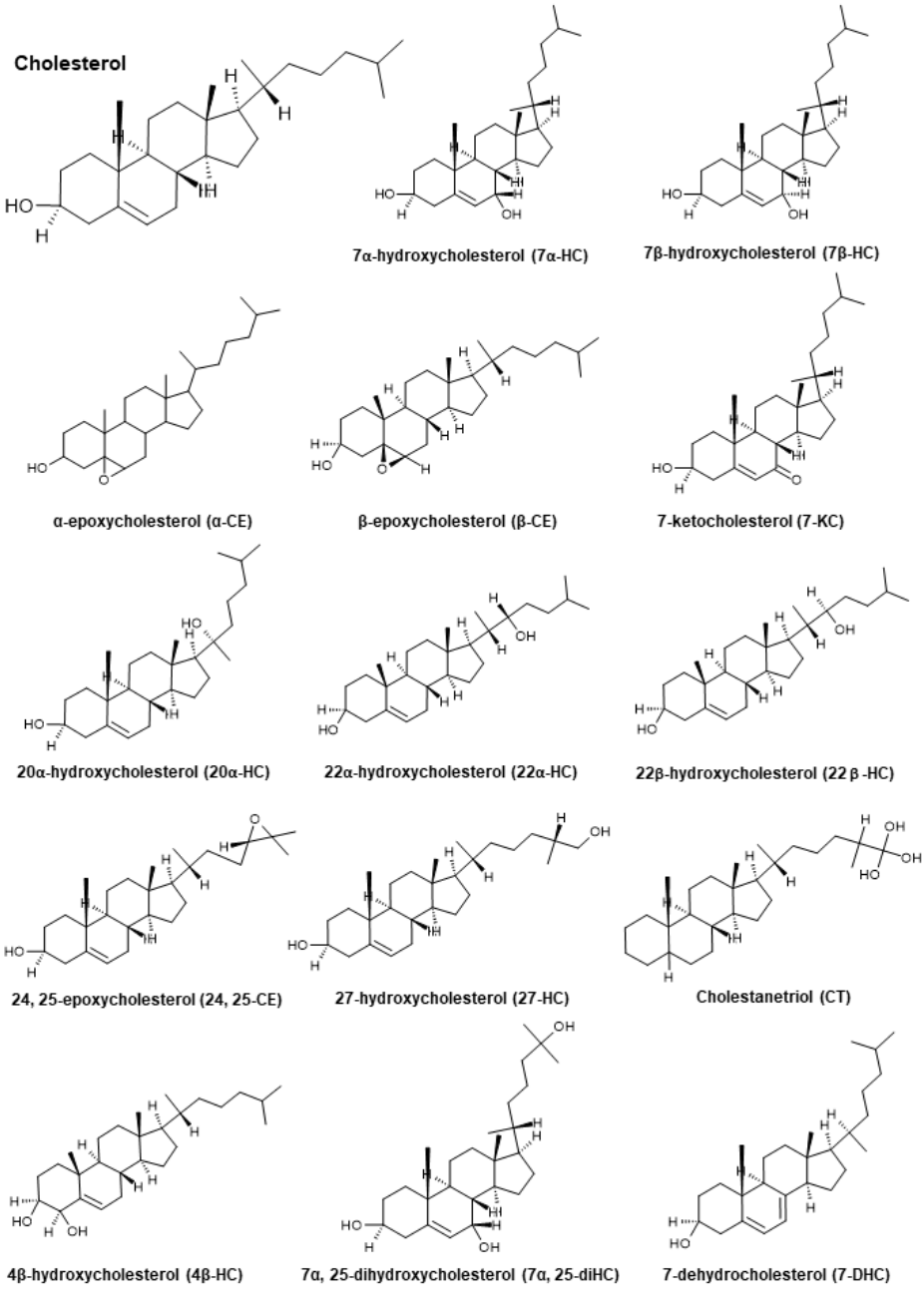


Figure 8. Chemical structures of cholesterol and selected cholesterol oxidation products (COPs)

1.4.3 Phospholipids and triacylglycerols

Triacylglycerols (TAGs) are complex lipids categorized as glycerolipids (GL), while phospholipids belong to the glycerophospholipids (GP) according to the LIPID MAPS classification[80]. Both of the lipid classes contain a glycerol backbone, modified with various functional groups attached at specific positions, as illustrated in Figure 9. These positions are known as *sn*-1, *sn*-2, and *sn*-3. The specific functional groups at these sites influence the chemical properties and physiological functions of the resulting lipids[80].

TAGs are formed by esterification of fatty acids to the three alcohol groups in glycerol. This structure serves as an efficient energy reservoir, storing fatty acids as fats in adipose tissues[17, 22]. During adipocyte lipolysis, TAGs are hydrolysed to release FFAs through the cleavage of ester bonds. These FFAs are subsequently utilized in energy-demanding processes, such as β-oxidation, which breaks them down into acetyl-CoA. Acetyl-CoA then enters the citric acid cycle where it contributes to ATP production, supplying energy for various metabolic activities[17].

PLs are formed by modification at the *sn*-3 position, either with a native phosphate group or additional side groups such as choline, ethanolamine, glycerol, or serine. These modifications constitute the polar, hydrophilic head group of PLs. In contrast, the *sn*-1 and *sn*-2 positions of PLs are occupied by fatty acyl chains, creating two hydrophobic tails. The duality of a PL structure containing a hydrophilic head and hydrophobic tails is linked to their amphipathic nature[17]. Further modification of PLs, such as the addition of the *sn*-1 position with a sphingosine and the *sn*-2 position with a fatty acid while retaining a phosphatidylcholine (PC) head group at *sn*-3, leads to the formation of sphingomyelin (SM). SM is a type of sphingolipid (SL) classified as one of the eight major lipid classes in the LIPID MAPS classification[80]. Aside from its major role in myelin sheath formation that surrounds nerve fibers, the biological functions of SMs are associated with the organization of plasma membrane lipids, formation of lipid rafts, and participation in cellular signalling processes[80].

Modifications to the functional groups attached to the glycerol backbone result in the formation of distinct lipid classes, each with unique chemical properties and biological functions. For example, lysophospholipids (LPLs) are generated when one fatty acyl chain is removed from phospholipids (PLs) through hydrolysis at either the *sn*-1 or *sn*-2 position of the glycerol backbone[80].

While appropriate levels of LPLs contribute to the stability of lipid emulsions in TPN products, excessive amounts can lead to formulation degradation and product instability. Excessive PL hydrolysis to LPLs disrupt the emulsion composition, resulting in increase in fat droplet size, separating the emulsion admixture into oil and water phases[41, 98]. Chromatography coupled with MS-based workflows, particularly LC-MS, is commonly used for targeted analysis of these lipid classes. LC-MS is favoured for its versatility in handling diverse sample types, its high sensitivity for analytes, and its suitability for routine analyses. This method allows for the separation of PL classes and other key lipid components, facilitating untargeted lipidomic profiling based on sample type or lipid source[72, 99, 100].

Despite its practicality, LC-MS-based targeted quantification has been limited to profiling and semi-quantitative approaches, primarily due to challenges in sample preparation and method validation[44, 68, 101-104]. **Paper IV** aims to provide targeted quantification for 30 distinct key lipid species, which includes specific TAGs, PLs, SMs, and LPLs in a single method using LC-MS/MS with minimal sample pre-treatment strategy suitable for high-throughput routine practices, applied in pharmaceutical-grade egg yolk powders, as a main raw material in PLE formulation necessary in TPN products.

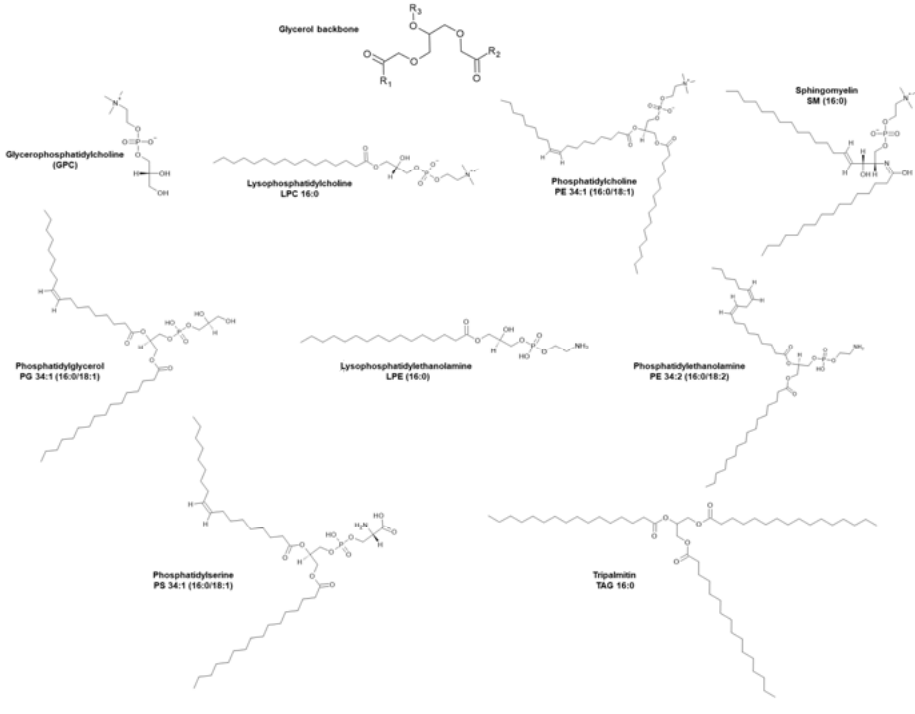


Figure 9. Chemical structures of complex lipid classes derived from the glycerol backbone

2. Aims

The aim of this thesis is to provide a better understanding of lipid components in pharmaceutical lipid emulsions (PLE), which are essential ingredients in total parenteral nutrition (TPN) formulations. Changes in their composition over time serve as indicators of formulation instability. Therefore, there is a significant need for lipidomic strategies that can perform efficient, reproducible, and high-throughput analysis of the raw materials used in TPN production.

To address this need, various chromatography techniques coupled with mass spectrometry (MS) were employed to investigate different lipid groups including free fatty acids (FFAs), cholesterol and cholesterol oxidation products (COPs), and complex lipids such as phospholipids (PLs) and triacylglycerols (TAGs). Three types of chromatography were employed: gas chromatography (GC), liquid chromatography (LC), and supercritical fluid chromatography (SFC). These methods were carefully optimized and validated to enable the targeted analysis of lipids based on their chemical characteristics. These analytical approaches uncovered essential compositional variations in PLE over time, demonstrating their direct influence on the stability of TPN formulations.

In addition to the method development strategies, this thesis also emphasizes method validation and the importance of analytical figures of merit to ensure the accuracy and reliability of lipidomic strategies. Given that TPN products are classified as pharmaceuticals, these must be analysed in accordance with stringent regulatory standards. Accordingly, each lipidomic strategy was validated following the ICH Q2(R2) guidelines, which serve as the foundation established by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

This thesis highlights the development of lipidomic strategies designed for quality control and routine analysis. Additionally, it explores the impact of matrix effects on lipid analysis and examines the role of internal standards in ensuring the accurate quantification of lipid species. These strategies were applied to pharmaceutical-grade egg yolk powders, a key component of PLEs, as well as other ingredients used in TPN formulations.

3. Method development

This thesis explores three chromatographic techniques in combination with mass spectrometry (MS): gas chromatography (GC), liquid chromatography (LC), and supercritical fluid chromatography (SFC) for analytical method development tailored for quantitative analysis of pharmaceutical lipid emulsion (PLE) components present in total parenteral nutrition (TPN) products. The advantages of each technique and their suitability for different classes of lipids were carefully considered for targeted analysis.

Chromatographic techniques aim to identify and separate analytes present in multicomponent mixtures, while MS detection enhance the overall method's selectivity and sensitivity for accurate quantification. In **Papers I-IV**, strong emphasis on the optimization of chromatographic and mass spectrometric parameters is given to ensure method efficiency without compromising accuracy and reliability of the results. This section also highlights the significance of the different lipid standards used at the frontline of method development and sample preparation workflows applied alongside tuning chromatography and MS conditions.

3.1 Targeted lipidomics

This thesis focuses on the targeted analysis of different lipid classes. Free fatty acids (FFAs) are quantified in **Papers I-II**, cholesterol and cholesterol oxidation products (COPs) are evaluated in **Paper III**, and glycerolipids, primarily phospholipids (PLs) and triacylglycerols (TAGs) are studied in **Paper IV**.

In **Paper I**, fatty acid methyl ester standards (FAMEs) were used for method development in GC-MS. Direct analysis of FFAs is not feasible in GC-MS. These compounds have poor volatility due to the presence of polar carboxyl group (-COOH). At high temperatures, FFAs can be unstable can become unstable due to thermal degradation, including oxidation, decarboxylation, and isomerization. A series of FAME standards and triglyceride (TG) standards are tabulated in Tables 1 and 2, which were prepared in MS-grade heptane. One of the highlights of the analytical method development in **Paper I** is the

inclusion of the C6:0 FFA as C6:0 ME in the method, since most of the currently available GC-MS methods can only analyse FFAs from C8:0 onwards[58, 105-109].

Table 1. Individual FAME and TG standards used in **Paper I**

	Systematic name	Common name	Purity
C20:0 ME	Eicosanoic acid, methyl ester	Methyl Arachidate	>99%
C22:0 ME	Docosanoic acid, methyl ester	Methyl Behenate	>99%
C24:0 ME	Tetracosanoic acid, methyl ester	Methyl Tetracosanoate	>99%
C17:0 ME	Heptadecanoic acid, methyl ester	Methyl Heptadecanoate	>99%
C18:3n-6 ME	cis,cis,cis-6,9,12-Octadecatrienoic acid, methyl ester	Methyl Gamma Linolenate	>99%
C20:4n-6 ME	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid, methyl ester	Methyl Arachidonate	>99%
C22:6n-3 ME	cis,cis,cis,cis,cis,cis-4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	DHA Methyl Ester	>99%
C18:1n-7 ME	cis-11-Octadecenoic acid, methyl ester	Methyl cis-Vaccenate	>99%
TAG C17:0		Triheptadecanoin	>99%
TAG C8:0		Tricaprylin	>99%
TAG C14:0		Trimyristin	>99%
TAG C18:0		Tristearin	>99%

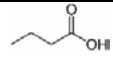
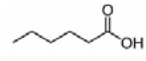
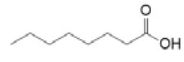
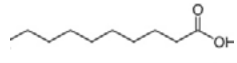
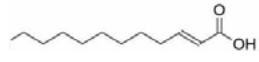
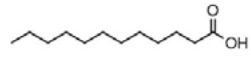
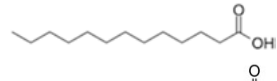
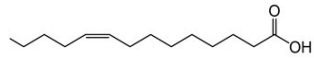
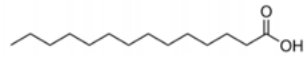
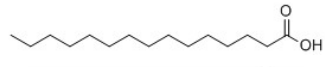
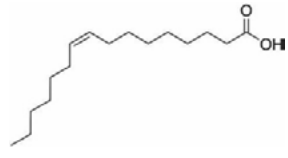
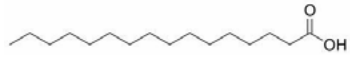
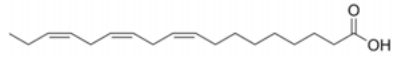
Table 2. Mixed FAME standards used in **Paper I**

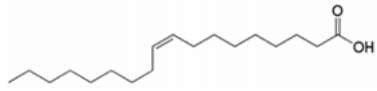
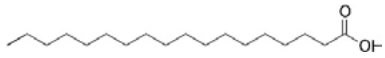
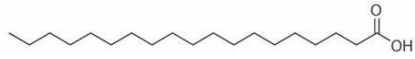
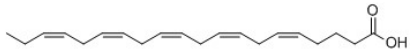
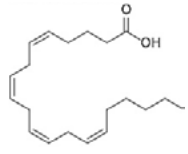
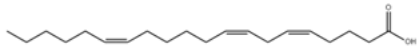
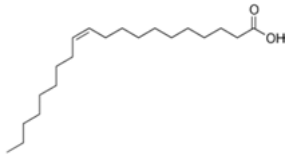
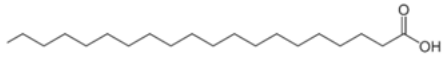
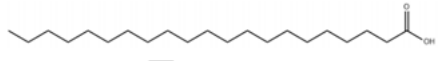
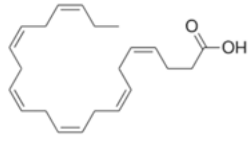
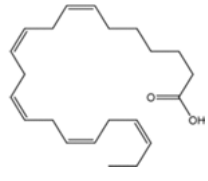
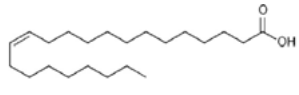
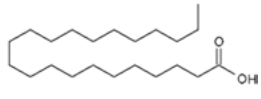
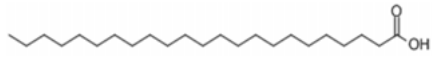
FAME mixture	Lipid number	Systematic name	Common name	FAME content [%]	Purity
ME 20	C6:0 ME	Hexanoic acid, methyl ester	Methyl Hexanoate	20.076	>99%
	C8:0 ME	Octanoic acid, methyl ester	Methyl Octanoate	19.885	
	C10:0 ME	Decanoic acid, methyl Ester	Methyl Decanoate	19.981	
	C12:0 ME	Dodecanoic acid, methyl ester	Methyl Dodecanoate	19.981	
	C14:0 ME	Tetradecanoic acid, methyl ester	Methyl Tetradecanoate	20.076	
ME 32	C16:0 ME	Hexadecanoic acid, methyl ester	Methyl Palmitate	20.040	>99%
	C18:0 ME	Octadecanoic acid, methyl ester	Methyl Stearate	19.940	
	C18:1n-9 ME	Methyl Oleate	Methyl Oleate	19.940	
	C18:2n-6 ME	9,12-Octadecadienoic acid, methyl ester	Methyl Linoleate	20.140	
	C18:3n-3 ME	9,12,15-Octadecatrienoic acid, methyl ester	Methyl Linolenate	19.940	
ME 33	C16:1n-7 ME	9-Hexadecenoic acid, methyl ester	Methyl Palmitoleate	20.000	>99%
	C18:1n-9 ME	9-Octadecenoic acid, methyl ester	Methyl Oleate	20.100	
	C20:1n-9 ME	11-Eicosenoic acid, methyl ester	Methyl Eicosenoate	19.900	
	C22:1n-9 ME	13-Docosenoic acid, methyl ester	Methyl Erucate	20.000	
	C24:1n-9 ME	15-Tetracosenoic acid, methyl ester	Methyl Nervonate	20.000	

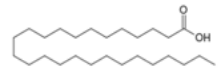
Note: In this labelling convention, 'C' represents carbon, the first number is the number of carbons, the second number is the number of double bonds, and the number after 'n' shows the position of the first double bond, with the methyl end as position 1.

Paper II, in contrast, focused on using free fatty acids (FFAs) instead of FAMEs, as the goal of this study was to perform direct FFA analysis without chemical derivatisation. A summary of the FFAs analysed in this study is presented in Table 3. To the best of our knowledge, **Paper II** has the most comprehensive direct FFA conducted analysis to date, spanning the analysis of FFAs from C4 to C26 in their native form. This work is novel, as no previous studies have systematically reported a comparable direct FFA analysis without derivatisation using SFC-MS, making it a significant advancement in lipidomics research.

Table 3. FFA standards used for method development in **Paper II**

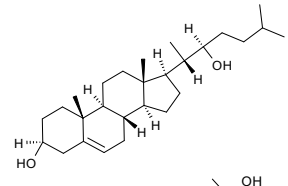
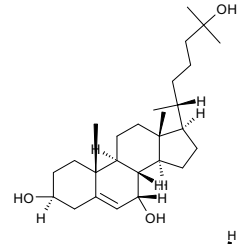
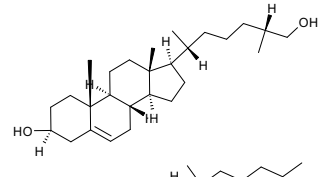
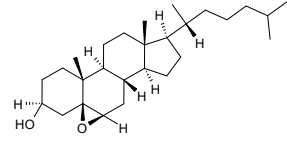
Name	Notation	Formula	Structure
Butyric acid	C4:0	$C_4H_8O_2$	
Caproic acid	C6:0	$C_6H_{12}O_2$	
Caprylic acid	C8:0	$C_8H_{16}O_2$	
Capric acid	C10:0	$C_{10}H_{20}O_2$	
Lauroleic acid	C12:1	$C_{12}H_{22}O_2$	
Lauric acid	C12:0	$C_{12}H_{24}O_2$	
Tridecylic acid	C13:0	$C_{13}H_{26}O_2$	
Myristoleic acid	C14:1	$C_{14}H_{26}O_2$	
Myristic acid	C14:0	$C_{14}H_{28}O_2$	
Pentadecylic acid	C15:0	$C_{15}H_{30}O_2$	
Palmitoleic acid	C16:1	$C_{16}H_{30}O_2$	
Palmitic acid	C16:0	$C_{16}H_{32}O_2$	
α-Linolenic acid	C18:3	$C_{18}H_{30}O_2$	
Linoleic acid	C18:2	$C_{18}H_{32}O_2$	

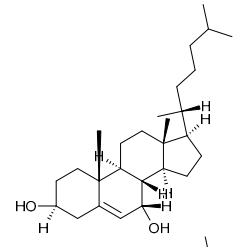
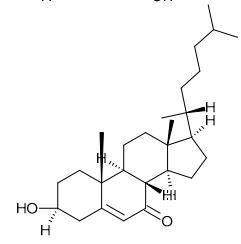
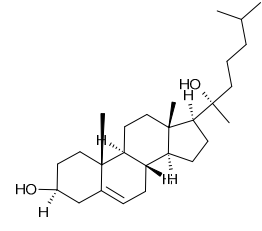
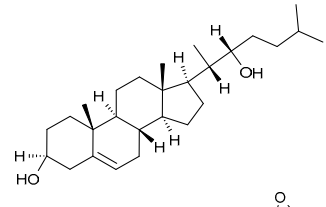
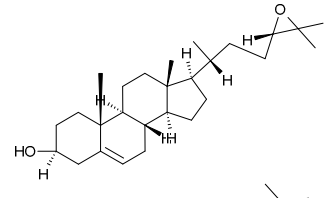
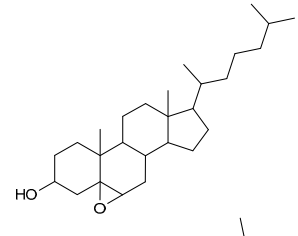
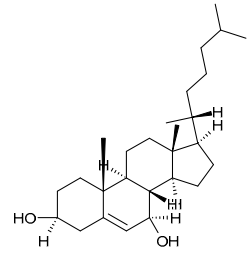
Oleic acid	C18:1	$C_{18}H_{34}O_2$	
Stearic acid	C18:0	$C_{18}H_{36}O_2$	
Nonadecylic acid	C19:0	$C_{19}H_{38}O_2$	
Eicosapentanoic acid	C20:5	$C_{20}H_{30}O_2$	
Arachidonic acid	C20:4	$C_{20}H_{32}O_2$	
Eicosatrienoic acid	C20:3	$C_{20}H_{34}O_2$	
Eicosenoic acid	C20:1	$C_{20}H_{38}O_2$	
Arachidic acid	C20:0	$C_{20}H_{40}O_2$	
Heneicosylic acid	C21:0	$C_{21}H_{42}O_2$	
Docosahexaenoic acid	C22:6	$C_{22}H_{32}O_2$	
Docosapentaenoic acid	C22:5	$C_{22}H_{34}O_2$	
Erucic acid	C22:1	$C_{22}H_{42}O_2$	
Behenic acid	C22:0	$C_{22}H_{44}O_2$	
Tricosylic acid	C23:0	$C_{23}H_{46}O_2$	
Nervonic acid	C24:1	$C_{24}H_{46}O_2$	

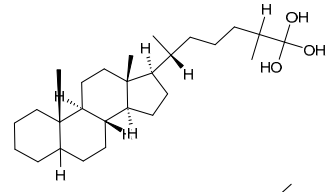
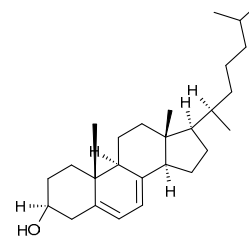
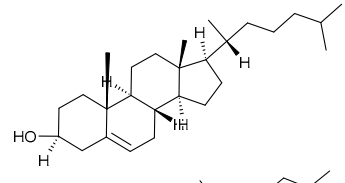
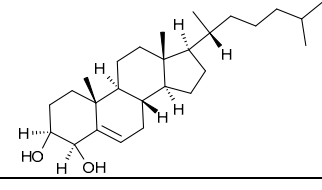
Lignoceric acid	C24:0	$C_{24}H_{48}O_2$	
Cerotic acid	C26:0	$C_{26}H_{52}O_2$	

Targeted analysis of cholesterol and COPs is given emphasis in **Paper III**. This study represents a groundbreaking advancement, as there are no existing SFC methods available for direct comparison. Notably, the method does not require derivatisation, utilizes electrospray ionization (ESI) in positive mode, and enables rapid analysis. This work is exceptionally novel, surpassing any previously reported approaches in terms of efficiency, speed, and direct COPs analysis in any chromatographic method.

Table 4. Cholesterol and COPs standards used in **Paper III** for method development

Name	Notation	Formula	Structure
22β-hydroxycholesterol	22β-HC	$C_{27}H_{46}O_2$	
7α, 25-dihydroxycholesterol	7α, 25-diHC	$C_{27}H_{44}O_3$	
27-hydroxycholesterol	27-HC	$C_{27}H_{46}O_2$	
β-epoxycholesterol	β-CE	$C_{27}H_{46}O_2$	

7α-hydroxycholesterol	7α-HC	$C_{27}H_{46}O_2$	
7-ketocholesterol	7-KC	$C_{27}H_{44}O_2$	
20α-hydroxycholesterol	20α-HC	$C_{27}H_{46}O_2$	
22α-hydroxycholesterol	22α-HC	$C_{27}H_{46}O_2$	
24, 25-epoxycholesterol	24, 25-CE	$C_{27}H_{44}O_2$	
α-epoxycholesterol	α-CE	$C_{27}H_{46}O_2$	
7β-hydroxycholesterol	7β-HC	$C_{27}H_{46}O_2$	

Cholestanetriol	CT	$C_{27}H_{48}O_3$	
7-dehydrocholesterol	7-DHC	$C_{27}H_{44}O$	
Cholesterol		$C_{27}H_{46}O$	
4β-hydroxycholesterol	4β-HC	$C_{27}H_{46}O_2$	

For **Paper IV**, Table 5 shows the standard phospholipids and triglycerols were utilized for method development aiming for targeted quantification of 30 key lipid species that are highly monitored in pharmaceutical-grade egg yolk powders used in PLE formulations for TPN products.

Table 5. Phospholipid and triacylglycerol standards used for analytical method development in **Paper IV**

Standard	Source	Analyte of interest
GPC	Egg	GPC
LPE	Egg	LPE (16:0, 18:1, 18:0)
LPC	Egg	LPC (16:0, 18:2, 18:1, 18:0)
PE	Egg	PE (34:2, 34:0, 36:1, 36:0)
SM	Egg	SM (16:0, 18:1, 18:0)

PC	Egg	PC (34:1, 34:0, 36:2, 36:1, 36:0, 38:2)
PS	Soybean	PS (34:1, 36:2)
PG	Egg	PG (34:1, 34:0, 36:1, 36:0)
TAG 16:0	Egg	TAG 16:0
TAG 18:1	Egg	TAG 18:1
TAG 18:0	Egg	TAG 18:0

In summary, this thesis successfully incorporated the analysis of 22 FAMEs (C6 to C24) in **Paper I**, 31 FFAs (C4 to C26) in **Paper II**, cholesterol and 14 COPs in **Paper III**, and 30 key lipid species in **Paper IV**. These analyses were performed using chromatography-MS-based methods for targeted lipidomics.

3.2 Sample preparation techniques

The incorporation of high-purity standards in method development is important and advantageous to maximize the sensitivity of the analytical instruments used. Certified reference materials (CRMs), primary and secondary standards, and ultra-pure reagents are the common examples of high-purity standards used for establishing an analytical method. However, when the developed method is applied to biological matrices, appropriate sample preparation procedures are required to eliminate analyte interferences and ensure accurate quantification.

Sample preparation schemes determine the analytical time required to perform targeted analysis in chromatography-MS-based workflows. Common interferences for lipidomics such as salts, proteins, and small metabolites that are present in biological matrices must be removed as thoroughly and efficiently as possible since these substances may affect chromatographic separation and MS detection of the target lipids. In this work, tailored sample preparation procedures were optimized and presented in **Papers I-IV** to ensure maximum analyte detectability, in line with the instrumental conditions established in the developed analytical methods.

3.2.1 Preparation of standards and blank matrix

Stock and working standard solutions were prepared for all analytes using the appropriate solvents prior to chromatography-MS-based method development. FAMEs in **Paper I** are all prepared in heptane, while the FFAs from **Paper II**, as well as the COPs from **Paper III**, and the glycerolipids from **Paper IV** were initially stored in chloroform but consequently diluted in methanol.

In **Paper II-IV**, analyte removal for matrix-matched calibration was achieved through activated charcoal treatment, followed by liquid-liquid extraction, which varied depending on the specific analyte to be removed from the matrix. The tailored solvent extraction procedures is presented in the next section. The analyte-free matrices from **Papers II-IV** were tested by analysing a small aliquot using the optimized GC-, LC-, or SFC-MS methods, ensuring that the analytes were undetectable, thus confirming their removal. The importance of creating appropriate analyte-free matrices is emphasized in **Papers II-IV**, ensuring the reliability of the analytical figures of merit essential for analytical method validation. This gives a strong advantage compared to similar lipidomic works which were not able to fully describe or emphasize the preparation of analyte-free matrices upon performing analytical method validation[27, 70, 75, 95, 110, 111].

3.2.2 Liquid-liquid extraction

Liquid-liquid or solvent extraction (LLE) is a convenient choice in sample preparation for bioanalysis. LLE offers benefits such as clean extracts, high analyte recovery, convenient removal of inorganic salts, and low-cost. Some of its drawbacks include the need for large quantities of organic solvents, difficulty in automation, and time- and labour-intensive. The choice of extraction solvent is crucial, as analyte partitioning between two immiscible solvents depends on their densities and the principle of "like dissolves like," affecting extraction efficiency.

A modified Dole extraction method was applied in **Paper II** for FFA extraction without derivatisation. This method effectively separates FFAs from other lipid components and impurities (such as salts) by combining an aqueous phase with an organic phase like heptane. The acid hydrolysis step releases FFAs from esterified forms (e.g., triglycerols or phospholipids), improving phase separation and enhancing FFAs recovery. This extraction method enables the efficient isolation of FFAs subject to direct analysis in SFC-MS in ESI^-.

In **Paper IV**, a liquid-liquid extraction method based on the Bligh and Dyer method was modified and performed. Lipids in pharmaceutical-grade egg yolk powders was extracted efficiently using this procedure. This direct sample preparation technique efficiently extracts lipids in their intact form, preserving their integrity. The introduction of an aqueous solvent to an organic mixture of chloroform and methanol mixture results in phase separation, partitioning the lipid contents from other egg yolk components, such as protein and salts, leading to improved analyte recovery and detection using the developed UPLC-MS/MS method.

3.2.3 Saponification

COPs are purified using saponification in **Paper III**. Acyl lipids and sterol esters are hydrolysed, yielding water-soluble FA salts and an unsaponifiable fraction that is soluble in organic solvent. This unsaponifiable fraction contains mainly sterols and COPs. There are two routes for saponification method: hot (60-100 ºC) or cold (20-40 ºC) method. However, COPs degrade during the hot saponification process, hence the cold process was utilized in **Paper III**. Ethanolic potassium hydroxide was used for the saponification process, which is stopped by the addition of saturated sodium chloride after 60 min, followed by liquid-liquid extraction with heptane.

3.2.4 Derivatisation

The conversion of FFAs to FAMEs was achieved through transesterification reactions to make the analytes compatible with GC-MS. In **Paper I**, five derivatisation methods were tested, representing different methyl esterification approaches: acid-catalysed methylation (sulfuric acid), base-catalysed methylation (sodium methoxide and potassium hydroxide), an acid-base combination (potassium hydroxide-sulfuric acid), and the acetyl chloride method. These five conventional yet different routes in transesterification were selected and modified aiming to provide a universal yet efficient chemical derivatisation method across the three TPN component matrices investigated in **Paper I**: fish and olive oils, and the unidentified lipid sample. Methyl esterification efficiency was assessed by monitoring spiked recoveries of TG 17:0 added to each TPN component.

This approach was possible because the test samples do not contain TG 17:0 or its fatty acid, C17:0. After derivatisation, the C17:0 methyl ester is well-mixed with other FAMEs in the sample, and its distinct chromatographic peak on the GC-MS allows for separation. By spiking the sample with TG 17:0 and measuring its esterification rate, matrix effects and other interferences can be accounted for, similar to a spiked recovery experiment for the pretreatment process. This approach in monitoring derivatisation rate was the first of its

kind across the different matrices, as usually a default FFA to FAME conversion rate is typically used to provide a quantitative estimate of FFAs using GC-MS[49, 112-114].

Based on the results from **Paper I**, the sodium methoxide method was chosen among the five derivatisation methods tested due to its efficiency and simplicity in methyl esterification, only utilizing 15 min, as compared to other chemical derivatisation techniques existing covering 30 min to overnight transesterification reactions[49, 56, 115, 116].

3.3 Mass spectrometric parameters

Electron ionization (EI) was applied for the analysis of FAMEs in GC-MS in **Paper I**, optimized using the selected ion monitoring mode (SIM). Once ionization occurs due to EI, the MS focuses on specific m/z values that are characteristic for each FAME standard. SIM only monitors this small set of target ions, allowing the MS to further scan these selected ions, significantly enhancing analyte sensitivity.

Electrospray ionization (ESI) was operated in positive (**Paper III** and **IV**) and negative (**Paper II**) modes. Its ease and compatibility with both LC and SFC methods makes this ionization technique more universal, expanding potential applicability of developed analytical methods. For the detection of FFAs in MS from **Paper II**, selected ion recording (SIR) was used, where only the first quadrupole is utilized for detection, thereby generating precursor ions without further fragmentation. FFAs, with their carboxylic acid group, readily generate intense and abundant deprotonated ions ($[M - H]^-$) in ESI^- mode. Generating fragment ions $[M - H - H_2O]^-$, $[M - H - 2H_2O]^-$, and $[M - H - 2H_2O - CO_2]^-$ can be challenging[84], and SIR dwell times were optimized to capture at least 12 data points per peak ensuring reproducible peak acquisition. The ionization of FFAs in ESI^- relies on the removal of a proton from the carboxylic group which is influenced by the fatty acid's chain length and acidity. Short-chain fatty acids (SCFFAs) often exhibit poor ionization efficiency due to their high pKa and volatility. On the other hand, the low solubility of long-chain fatty acids (LCFFAs) can also reduce ionization efficiency in ESI^-. Improving the ionization efficiency of FFAs in ESI^- was accomplished by optimizing the solvent system with additives like formic acid and adjusting source conditions, such as voltage and gas flow. Since the FFAs investigated have distinct precursor ions, SIR is already sufficient for their MS detection. This allows the direct MS detection of FFAs in **Paper II** without performing any derivatisation techniques, and it is considered a first in MS coupled to SFC as the main chromatographic technique.

Multiple reaction monitoring (MRM) was applied in **Paper III** and **IV** to enhance selectivity, especially for closely-related compounds. MRM monitors both the precursor ions and its corresponding product ions (Figure 6), creating specific transitions that are unique to the targeted analytes. As a result, employing MRM for analyte detection in the MS reduces background noise, thereby enhancing sensitivity. The sensitivity for detecting structurally similar COPs in **Paper III** was enhanced by employing unique MRM transitions, thereby enabling these analytes to be effectively distinguished in MS. COPs are neither acidic nor basic, hence monitoring $[M + H]^+$ in ESI^+ or $[M - H]^-$ in ESI^- is challenging and MS detection using these ions encounter issues in sensitivity. Instead of $[M + H]^+$ ions, COPs readily generate $[M + H - H_2O]^+$ ions, which are distinct and intense in ESI^+. The selectivity in the MS is further enhanced by optimizing collision energies, generating $[M + H - 2H_2O]^+$ fragment ions or neutral losses involving the side-chain or the sterol ring, as supported by other studies[26, 89]. These optimizations in the MS enable sensitive detection of COPs in **Paper III** without chemical derivatisation, a first in the analysis of these compounds in SFC-MS using ESI^+ instead of the conventional APCI for ionization[87, 91].

In **Paper IV**, unique MRM transitions were optimized for each targeted PLs and TAGs. These PL species are detected best in ESI^+ mode, generating abundant $[M + H]^+$ ions. However, further adduct formations were also observed, such as $[M + Na]^+$, $[M + K]^+$, and $[M + NH_4]^+$. The selection of precursor ion to monitor in each species depends on their abundance, and for most of the PL species, $[M + H]^+$ greatly exceeds the rest of the adducts. For lipids containing a phosphatidylcholine head group (PC, LPC, GPC and SM), their prominent $[M + H]^+$ parent ions and a significant daughter ion at *m/z* 184, corresponding to the intact PC head group, was monitored for their identification[117]. The loss of phosphatidylethanolamine (PE) head group corresponding to *m/z* 141 determined the daughter ions[117] for both PE and LPE, together with their parent $[M + H]^+$ ions. Phosphatidylserine (PS) species rely on the unique MRM transition between the precursor $[M + H]^+$ ion producing fragments corresponding to the the loss of PS head group with *m/z* 184 determined their product ions[117, 118]. Phosphatidylglycerol (PG) species, on the other hand, has more abundant $[M + NH_4]^+$, thereby utilized as the parent ion and the head group loss corresponding with *m/z* 189 determined their unique product ions. For the TAG species, $[M + Na]^+$ was used as both parent and daughter ions applied with minimum collision energy, since cleaving this stable structure has no distinct pattern and also requires a massive amount of energy for fragmentation. For the first time, these elaborate mass transitions for the detection and quantification of 30 key lipids were used and applied to pharmaceutical-grade egg yolk powders.

In summary, exploring different MS detection strategies was emphasized by this thesis. In **Papers I-IV**, various ionization techniques and modes were optimized for the analysis of lipids and fatty acid derivatives in MS. **Paper I** utilized EI in SIM mode to enhance sensitivity for FAMEs, focusing on specific *m/z* fragment ions for each standard. In **Paper II**, ESI^- was used for FFAs, with SIR ensuring effective detection by focusing on precursor ions and optimizing source conditions to improve ionization efficiency, especially for SCFFAs, which is a first for analyzing FFAs with no chemical derivatisation applied. **Papers III** and **IV** employed ESI^+ for more complex lipids like COPs, PLs, and TAGs, utilizing MRM to enhance selectivity by monitoring both precursor and product ions, improving sensitivity for closely related compounds. MRM transitions were tailored for each analyte, optimizing collision energies for enhanced detection of these complex lipids. These strategies enabled the sensitive detection of lipids and FFAs without the need for derivatisation, making the methods highly effective and versatile across different lipid classes.

3.4 Chromatographic parameters

The three chromatographic approaches used in this thesis: GC, LC, and SFC, were selected for tailored analytical development and refinement depending on the lipid class of interest. Analysis of FFAs using GC and SFC as chromatographic methods were highlighted on **Paper I** and **II**, respectively. **Paper III** utilized SFC for cholesterol and COPs separation, while **Paper IV** used the principles of LC to identify key PL and TAG species.

As mentioned earlier, while these chromatographic methodologies are well-established and widely used for analysing various biological matrices, there remains a significant need for further development and refinement to ensure efficient, robust, and reliable quantitative analysis. A wide range of parameters must be optimized, as highlighted in this thesis, and the robustness and accuracy of these developed strategies must be thoroughly validated.

3.4.1 GC instrumentation

The effectiveness of GC in separating FAMEs has been demonstrated in several studies, enhancing the resolving power of MS[53, 56, 57, 119-121]. However, to meet the needs of routine analysis of TPN components under pharmaceutical compliance, a tailored method must be developed. This method should ensure shorter analytical run times while enabling the quantification of both small- and medium-chain FFAs in a single analysis.

Paper I focuses on developing an analytical method for quantifying 22 FFA species, ranging from C6 to C24, in the form of FAMEs. The efficient and simultaneous analysis of these compounds is achieved through the carefully optimized GC parameters outlined in this study.

To achieve this goal, the optimization of GC injection type, split ratios, and temperature programming was given emphasis for FAME analysis. Initially, a splitless injection was used; however, poor peak shapes for C6:0, C8:0, and naphthalene (used as the surrogate internal standard) were observed. To address this, a 10:1 split ratio was implemented, resulting in improved peak shapes and better resolution of FAMEs.

Temperature programming in GC was time-consuming in the early stages of method development, as achieving a well-shaped chromatographic peak for C6:0 proved challenging. Initially, the starting temperature was optimized to 60°C, as temperatures above this value failed to produce a peak for C6:0. The final temperature was first set to 300°C, where 17 out of 22 analytes eluted before 20 minutes. However, the final standard C24:0 eluted around 41 minutes, with baseline drift that complicated analyte quantification (Figure 10A). To improve this, the final temperature was lowered to 275°C and the transfer line temperature increased from 105°C to 220°C, shortening the chromatographic run to 28 minutes with 20 analytes eluting before 20 minutes (Figure 10B).

Further optimization of the temperature programming rate to 25°C/min reduced the total analysis time to under 21 min, allowing all 22 analytes to elute within the chromatogram, which includes C6:0 which is a first of its kind in GC-MS methods analysing FFAs as FAMEs (Figure 10C). Compared with other GC-MS methods used for FFA quantification, this method is rapid, since existing methods currently have an analytical run time ranging from 25 to 60 min[49, 56, 112, 113, 122-124]. This optimized GC-MS operating procedure became the benchmark for establishing an analytical method suitable for FFA quantification in TPN products.

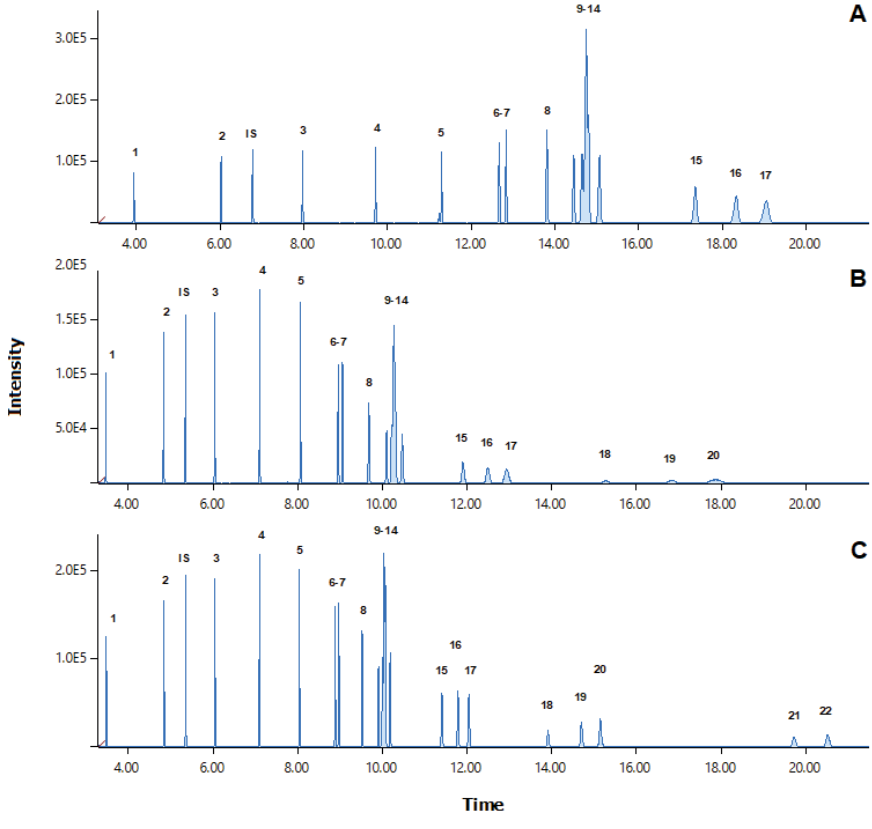


Figure 10. Optimization of gas chromatographic parameters for analytical method development for the identification of FAs in PLE components. (**A**) Chromatogram generated by setting the initial temperature programming from 60-300 ºC with 17 analytes eluting before 20 min. (**B**) Chromatogram generated by changing the transfer line temperature from 105 ºC to 220 ºC and temperature programming from 60-275 ºC. (**C**) Chromatogram generated by temperature programming from 60-275 ºC and adjusting the rate to 25 ºC/min.

In these chromatograms, the retention time of FAMEs is related to the carbon chain length and saturation levels. However, it is challenging to distinguish the FAMEs of isomers since their distinction is only based on spatial structure. Thus, the separation of these FAME species was achieved by employing MS for individual detection, using SIM as described in section 3.3. With the GC and MS parameters optimized for the FAMEs used, a suitable GC-MS method for differentiating 22 FAMEs from C6 to C24 for the first time under 21 min was established in **Paper I,** as also illustrated in Figure 11.

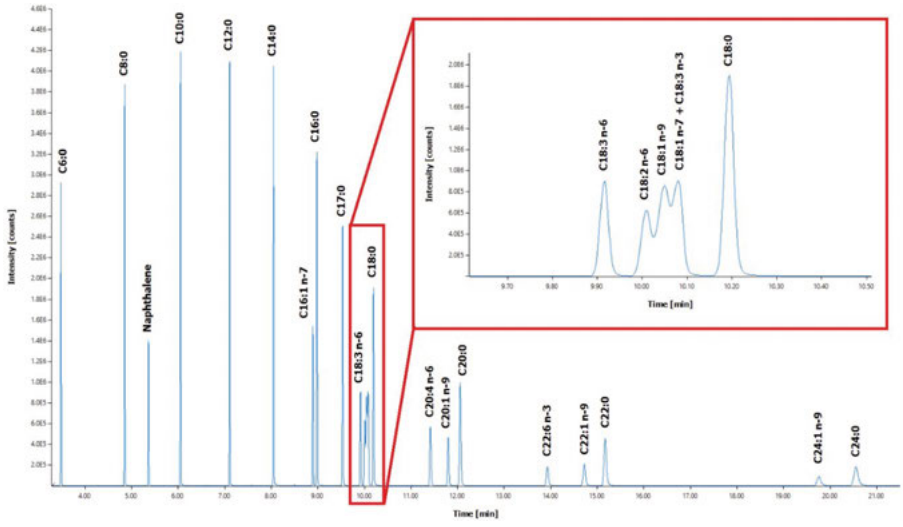


Figure 11. Chromatographic separation profile of 22 FAME standards and internal standard (IS) naphthalene in GC-MS. Inset: Zoomed chromatographic separation profile for C18:3 n-6, C18:2 n-6, C18:1 n-9, C18:1 n-7, C18:3 n-3, and C18:0. Reproduced from Ref. 38 with permission from the Royal Society of Chemistry.

3.4.2 SFC instrumentation

Application of SFC as the main chromatographic method is presented in **Paper II** and **III**. Since developed GC-MS method was able to quantify 22 FFAs from C6 to C24 in the form of FAMEs in **Paper I**, **Paper II** offers a different approach in FFA quantification—to perform direct analysis without derivatisation using SFC-MS.

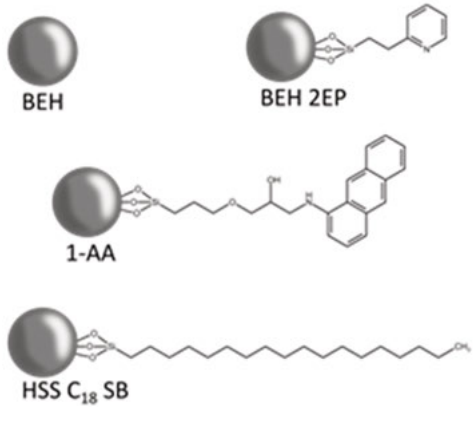


Figure 12. The chemistry of the stationary phases investigated for **Papers II** and **III**

Method optimization in SFC for **Paper II** involved selecting suitable stationary phases, starting with generic screening using methanol. The chemistry of

the stationary phase proved to be the most critical factor influencing FFA retention[125]. Despite well-characterized stationary phases, selecting the optimal phase remains challenging due to the diverse properties of FFAs. Two stationary phases, 1-AA and HSS C18 columns were selected in **Paper II** (Figure 12) for their potential to separate FFAs from C4 to C26[64]. These stationary phases were assessed for their ability to separate a broad range of FFAs, a key aspect of method development. This is the first time that these two stationary phases were used for column screening in SFC-MS aiming for direct FFA analysis, since most methods rely on LC-MS with chemical derivatisation applied on FFAs[59, 64, 66, 126-128].

FFA elution pattern in SFC is influenced by interactions with the stationary phase, along with the carbon chain length, degree of unsaturation, and spatial orientation [61, 64, 127]. The 1-AA stationary phase is less effective for FFA separation compared to HSS C18 due to differences in column chemistry as shown in **Paper II** (Figure 12). FFAs, being largely nonpolar, require strong hydrophobic interactions for effective retention and separation. The polar amine group of the 1-AA column does not provide the necessary hydrophobic environment, whereas the HSS C18 column, with its nonpolar C18 alkyl chains, facilitates strong van der Waals interactions[64]. This setup allows effective separation of FFAs across C4 to C26, following reverse-phase-like chromatography principles in SFC. This makes the HSS C18 column more suitable for comprehensive FFAs analysis, as it offers superior retention, resolution, and selectivity.

Under optimized chromatographic conditions, the HSS C18 SB column achieved near-baseline separation for both saturated and unsaturated FFAs, exhibiting symmetrical peaks and relatively short retention times, but unique $[M - H]^-$ ions of each FFA from the SIM mode in the MS will distinguish the species, hence performing accurate quantification. Figure 13 shows the elution of saturated FFAs in order of increasing alkyl chain length, with shorter-chain FFAs eluting earlier, likely due to weaker interactions with the C18-bonded phase. Interestingly, unsaturated FFAs showed longer retention times as the number of double bonds decreased, suggesting that FFAs with fewer double bonds interact more strongly with the HSS C18 SB phase, potentially due to reduced conformational flexibility and increased surface contact.

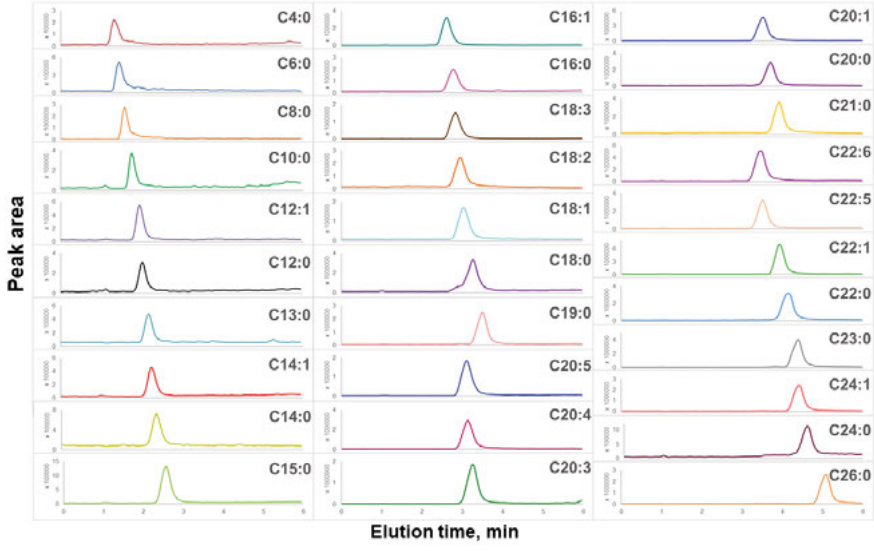


Figure 13. Elution profile of 31 FFA standards using ESI$^-$ in the optimized SFC-MS method within an analytical run time of 6 min. Adapted from Retrato, MDC, et. al., *Analytical and Bioanalytical Chemistry* **2025** © Authors 2025

In **Paper III**, the screening of stationary phases was performed using four different columns in SFC-MS/MS: 1-AA, BEH, BEH 2-EP, and HSS C18 SB **Paper III** (Figure 12). These columns were selected based on their distinct chemistries, which influence elution patterns for COPs separation. Some of these stationary phases have been previously used for COPs analysis in LC-MS/MS, either directly with APCI[28, 91] or after derivatisation with ESI[27, 76, 87, 129]. However, their application in SFC-MS/MS using ESI has not been explored. The elution pattern of COPs in SFC is primarily influenced by their interaction with the stationary phase, as well as their polarity. Polarity of COPs is determined by the type of chemical moiety they possess, its carbon position, and its spatial orientation. Since COPs are generally more polar than cholesterol, this information served as a guiding principle for chromatographic separation in SFC-MS/MS using the four columns investigated. So far, this is the most extensive column screening performed aiming for direct COPs analysis in SFC-MS/MS without any derivatisation, as exhibited with previous works mainly in GC- and LC-MS[28, 30-32, 76, 87, 129].

Among the four columns tested, HSS C18 SB outperformed the other stationary phases (Figure 14). The polar amine group in the stationary phase of the 1-AA column was able to distinguish some COPs of interest; however, it provided poor peak shapes for cholesterol, as it is less polar than the other analytes investigated. The BEH column presented a similar issue, where 19-hydroxycholesterol, used as one of the internal standards in this method, was difficult to identify due to a low signal-to-noise ratio. Lastly, the BEH 2-EP

column provided some resolution and distinction between cholesterol and COPs, but it was not as reproducible, and the peak intensities of the molecules on the HSS C18 SB column were higher at the same concentration. This can be attributed to the fact that the HSS C18 SB, being the least polar column among the four, was able to elute cholesterol and COPs from the most polar to the least polar, achieving near-baseline separations. This performance was further optimized by adjusting the organic modifier in supercritical CO_2, as well as testing different flow rates and make-up solvents. These further optimizations resulted in a 12-minute SFC-MS/MS method for separating cholesterol and COPs (Figure 15).

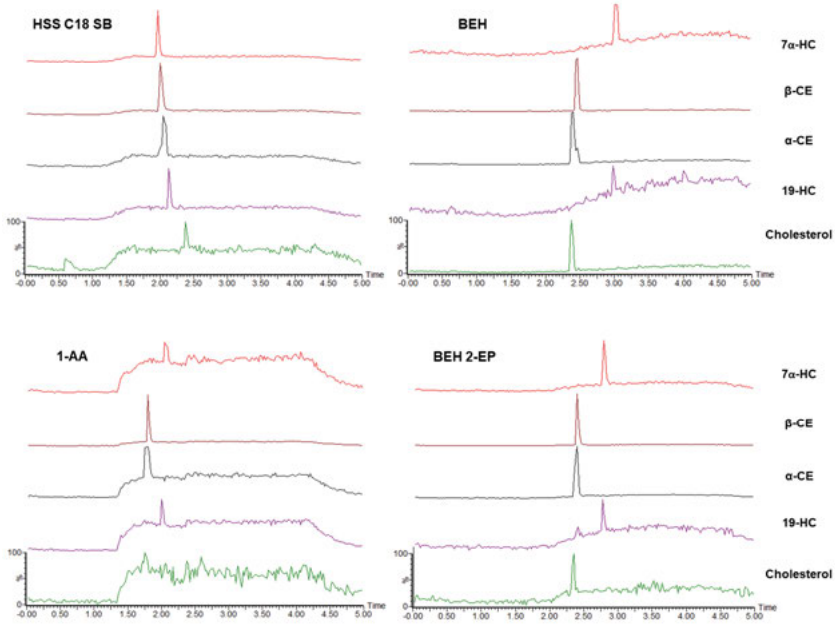


Figure 14. Column screening for COPs analysis in SFC-MS/MS

It was observed that most COPs with oxidized side chains elute earlier compared to COPs with oxidized sterol rings. The oxidized side chains likely interact less with the stationary phase, making them relatively more polar than their oxidized sterol ring counterparts. Since the added polar groups in the side chain contribute more to the polarity of the molecule, they elute earlier on a nonpolar C18 stationary phase. In contrast, COPs with an oxidized sterol ring are considered less polar due to the steric hindrance of the polar group attached to the ring structure. This results in these COPs eluting later on the HSS C18 SB column, approaching cholesterol, and creating a distinction between the COPs in both SFC and MS/MS.

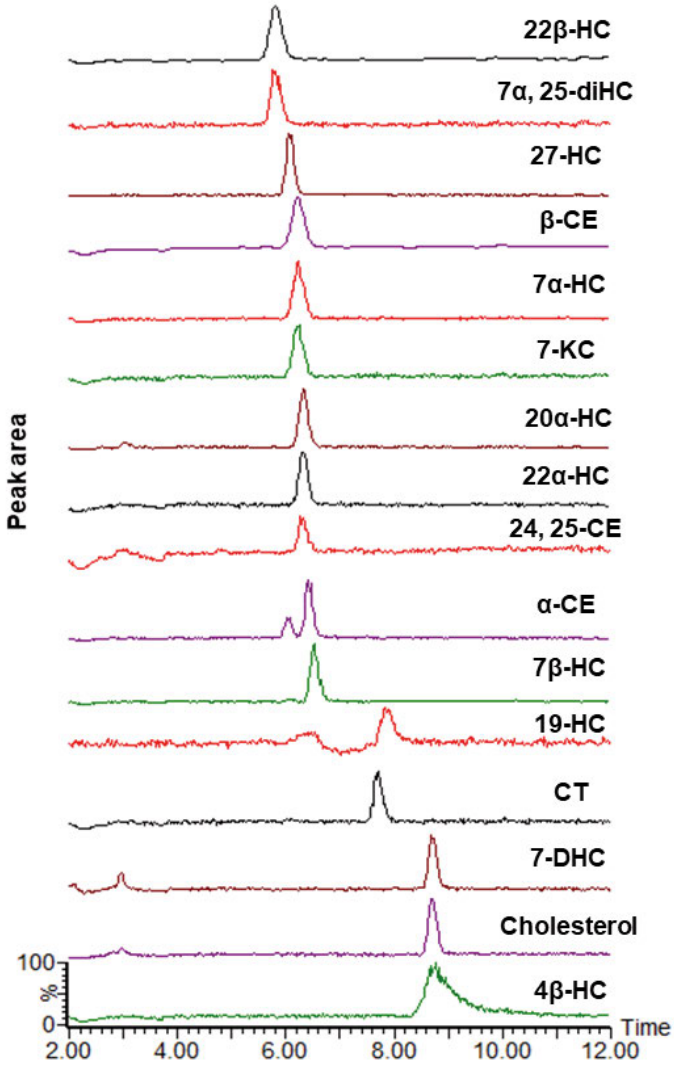


Figure 15. Elution profile of cholesterol and 14 COPs in SFC-MS/MS in HSS C18 SB column

From these two works in the thesis: **Paper II** and **III**, it is evident that the selecting the stationary phase is the most important aspect in analytical method development using SFC. Together with optimizing MS or MS/MS parameters, SFC methods are great alternatives for GC- or LC-based methods, offering direct analysis with minimal sample preparation, resulting in efficient chromatographic separations, without compromising analytical sensitivity.

3.4.3 LC instrumentation

Two method development approaches were explored in **Paper IV**: (1) reverse-phase chromatography and (2) hydrophilic interaction chromatography (HILIC). HILIC, using an ethylene-bridged hybrid inorganic-organic (BEH) column as the stationary phase, was initially considered a promising approach for complex lipid separation. However, this method showed inconsistent retention times and poor reproducibility. Shifting the method development towards reverse-phase chromatography with a charged surface hybrid (CSH) C18 column improved reproducibility and sensitivity, enabling further optimization of lipid separation.

Multiple gradient elution conditions with various mobile phase compositions were tested, resulting in an optimized method within a 14-minute analytical run time with additional 4 minutes for clean-up. Figure 16 shows changes in the retention times of investigated lipids at the expense of gradient modifications of the mobile phases used.

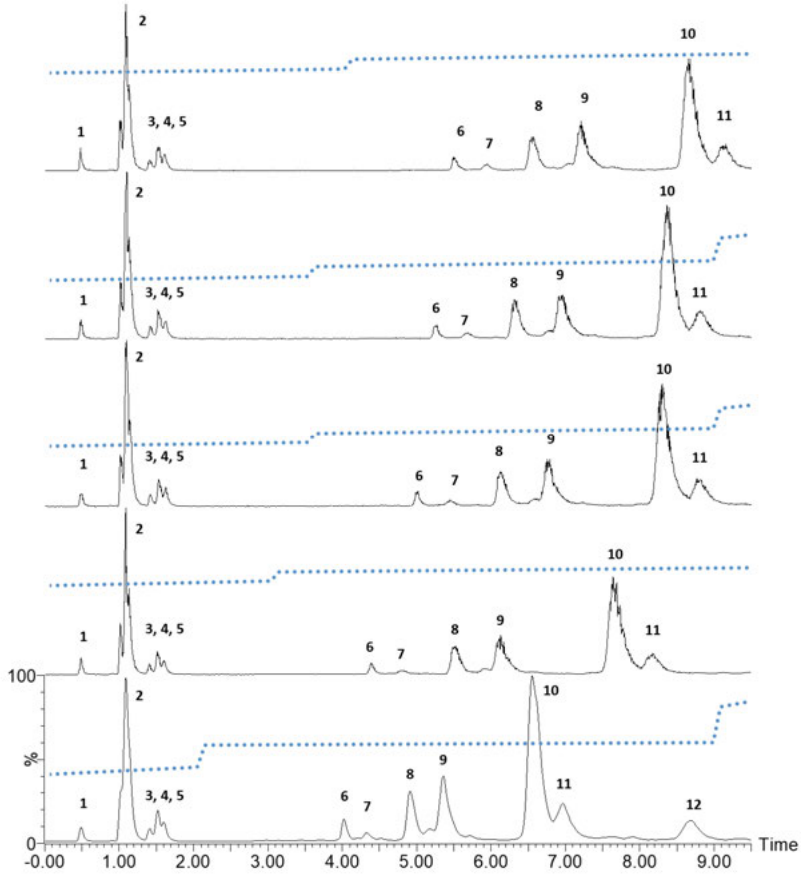


Figure 16. Optimization of gradient elution for the lipid classes investigated in **Paper IV**.

The elution profile of the targeted lipid species in **Paper IV** was influenced by differences in polarity. The most polar species: GPC, LPC, and LPE eluted first within the first 2 minutes of the analytical run. Despite multiple gradient elution iterations and solvent optimizations as seen in Figure 16, an elution delay between 2 and 4 minutes was observed before SM and PL species began to elute. This is common in lipidomic studies due to the substantial polarity difference between LPLs and PLs[44, 70, 104, 130]. This method achieved a shorter chromatographic gap between these lipid species compared to previous studies[103, 104]. For PLs, the hydrophobicity of the species increases with the number of carbons in the condensed structure, resulting in later elution times. The presence of unsaturation in the lipid groups leads to earlier elution compared to its saturated counterparts, as they are more polar and interact less with the nonpolar stationary phase[104, 117]. TAGs, being naturally non-polar and bulky, posed challenges for elution, especially when combined. Therefore, an internal standard (15:0-18:1(d_7)-15:0 TAG) was incorporated for peak assignment

and quantification. Its role in method optimization is further detailed in section 3.5, was used throughout the rest of the analytical method development.

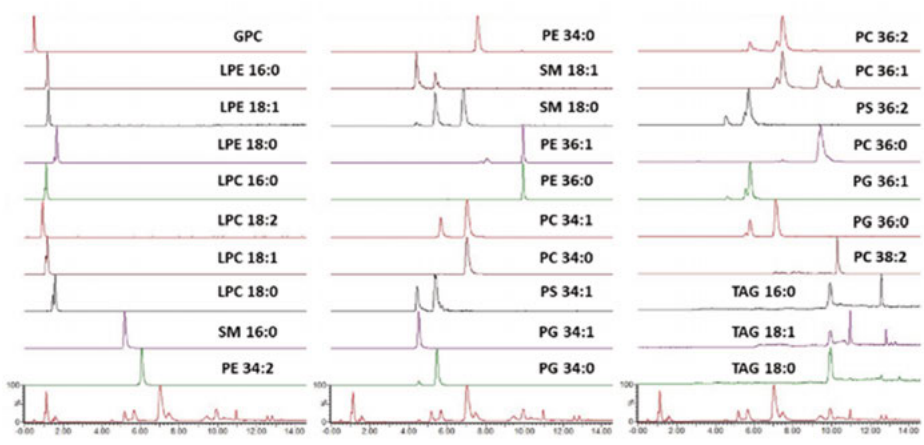


Figure 17. Chromatographic profile of the 30 key lipid standards in **Paper IV** using UPLC-MS/MS

Thirty (30) key lipid species were identified and separated in the optimized UPLC-MS/MS method, shown in Figure 17. Some of these lipid species, particularly PLs, exhibit multiple peaks at different retention times corresponding to these unique parent-to-daughter ion transitions elaborated in section 3.3. This can be explained by several factors, taking PC 36:2 as an example. Three distinct peaks were observed for this PL species at different retention times. The condensed formula of PC 36:2 indicates that the total number of carbons in the two fatty acyl chains is 36, with two double bonds between them. Therefore, multiple fatty acyl chain combinations can arise from this condensed structure. Structural isomerism (*cis*- or *trans*-) is possible, and if C18:2 (linoleic acid) constitutes one of the two fatty acyl chains, different double bond positions may be present for this species[131]. Another factor contributing to the multiple distinct peaks in PC 36:2 is the variation in fatty acid combinations[118], such as 18:2/18:0 or 18:1/18:1, which results in distinct peaks in UPLC-MS/MS. Lastly, the variability in fatty acid positions (*sn*-1 and *sn*-2) attached to the glycerol backbone of PC 36:2 (and generally for PLs)[102, 118] also contributes to these multiple chromatographic peaks. For example, in the 18:2/18:0 combination, *sn*-1 is assigned to 18:2 and *sn*-2 to 18:0, but this can be reversed. Even though the fatty acids are the same, the positions on the glycerol backbone affect the interaction with the stationary phase, leading to different chromatographic behavior. This concept can be extended to other key lipid species, explaining why multiple peaks are observed for some targeted analytes. However, GPC, LPCs, and LPEs, being hydrolysis products with none or only one fatty acid, do not exhibit multiple peaks, except for LPC

16:0, where a small twin peak suggests two possible *sn*-positions. The ultimate distinction of these species can be achieved by using specific standards with distinct chemical geometry and high purity. Since **Paper IV** aims to quantify these key lipid species, their quantification is expressed according to the condensed formula, using the sum of the chromatographic areas per lipid species.

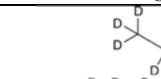
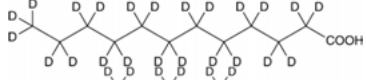
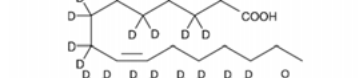
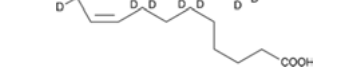
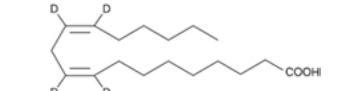
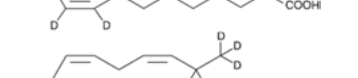
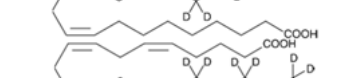
3.5 The internal standard method

The purpose of developing analytical methods resulting in **Papers I-IV** is enabling reliable quantification of pharmaceutical-grade egg yolk powders, among other PLE components used for TPN production. This is achieved by the internal standard (IS) method applied in constructing multipoint calibration curves. **Papers I-IV** stresses on the importance of using appropriate and elaborate internal standards and how their usage affect targeted lipid quantification.

For **Paper I**, naphthalene was used as a surrogate internal standard for quantification as it suits well within the retention times of the FAMEs from C6 to C24 during the process of the development for GC separation. Usually, an ideal internal standard should possess the similar chemical moieties compared to the analytes of interest. However in this work, it focuses more on the optimization of the transesterification of FFAs to FAMEs, therefore TAG 17:0 and C17:0 were used for this purpose. Since one of the limitations of GC-MS analysis of FFAs was the incomplete conversion through transesterification methods, the structural relevance of IS was rather applied here than the actual quantification after the conversion, hence introducing a surrogate IS that is consistent throughout the method development, and incorporating C17:0 and TAG 17:0 during the derivatisation process.

Paper II has overcome these limitations in **Paper I** by using direct analysis of FFAs in SFC-MS. Hence, the ease of using deuterated-FFA IS were maximized in this work. Fourteen (14) internal standards were used, and the 31 FFAs were matched according to their structural relevance. Among these IS, a comparison between deuterated-FFAs and C17:0 as the conventional IS for FFA analysis was made, in order to check its impact in quantification.

Table 6. Structural information of the deuterated FFAs and C17:0 used as internal standards for the SFC-MS method

Notation	Formula	MW	Structure	Monitored Analyte
C4:0-d_7	$C_4HD_7O_2$	95.10		C4:0, C6:0, and C8:0
C10:0-d_{19}	$C_{10}HD_{19}O_2$	191.38		C10:0, C13:0, and C15:0
C12:0-d_{23}	$C_{12}HD_{23}O_2$	223.50		C12:0 and C12:1
C14:0-d_{27}	$C_{14}HD_{27}O_2$	255.52		C14:0 and C14:1
C16:1-d_{14}	$C_{16}H_{16}D_{14}O_2$	268.44		C16:1
C16:0-d_{31}	$C_{16}HD_{31}O_2$	287.60		C16:0
C18:0-d_{35}	$C_{18}HD_{35}O_2$	319.68		C18:0 and C19:0
C18:1-*d*17	$C_{18}H_{17}D_{17}O_2$	299.57		C18:1
C18:2-d_4	$C_{18}H_{28}D_4O_2$	284.50		C18:2
C18:3-d_5	$C_{18}H_{25}D_5O_2$	283.51		C18:3
C20:4-d_{11}	$C_{20}H_{21}D_{11}O_2$	315.46		C20:0, C20:1, C20:2, C20:3, and C21:0
C20:5-d_5	$C_{20}H_{25}D_5O_2$	307.54		C20:5
C22:6-d_5	$C_{22}H_{27}D_5O_2$	333.52		C22:0, C22:1, C22:5, C22:6, C23:0, C24:0, C24:1, C26:0
C17:0	$C_{17}H_{34}O_2$	270.26		All of the 31 FA standards

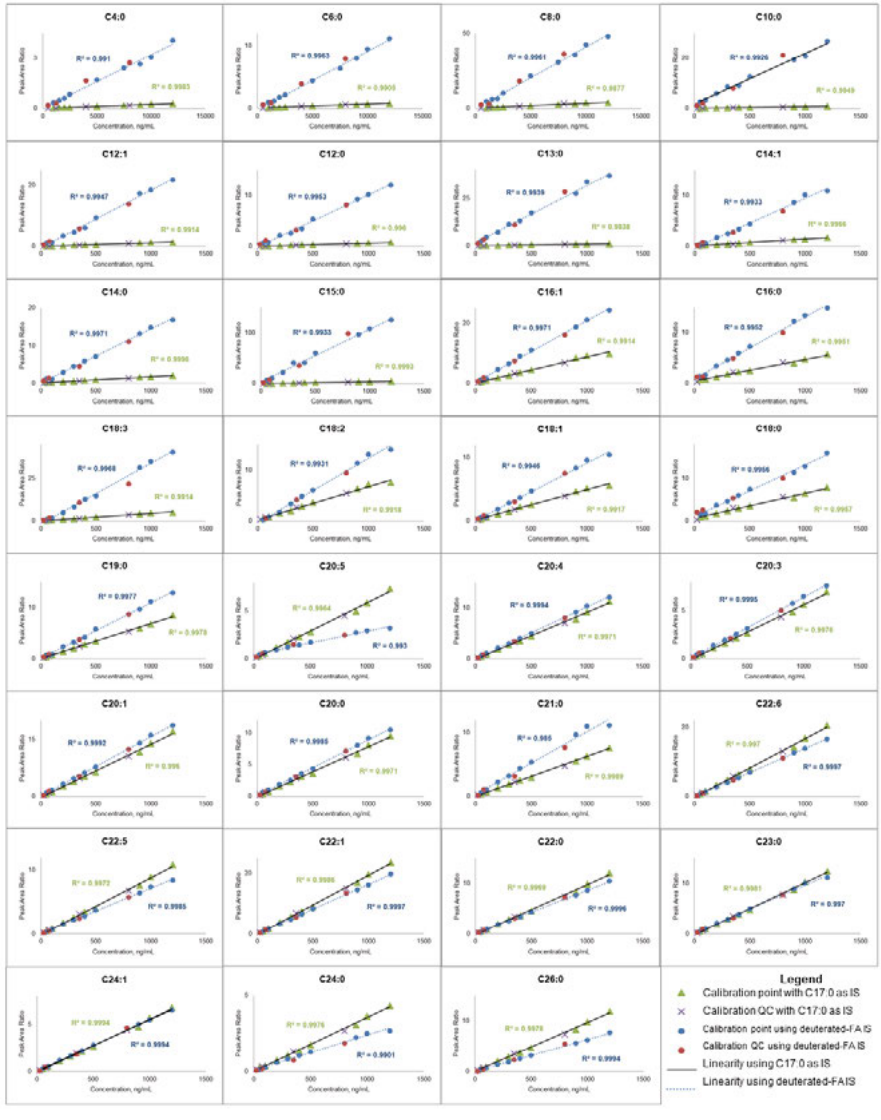


Figure 18. Construction of matrix-matched calibration curves using deuterated FFAs and C17:0 as internal standards in the validated SFC-MS method. Adapted from Retrato, MDC, et. al., *Analytical and Bioanalytical Chemistry* **2025** © Authors 2025

Figure 18 emphasizes the importance of using an appropriate internal standard in analytical quantification, as explored in **Paper II**. Using C17:0 across the entire FFA range gives different slopes as opposed to using structurally relevant deuterated-FFA IS. The striking differences in slope can be traced to the inherent differences in properties of C17:0, for instance in SCFFA such as C4:0. It can also be attributed to the differences in ionization efficiency of these compounds in the MS, hence the apparent differences in MS detection

producing significantly different signals were observed. More of this is explored in analytical validation.

In **Paper III**, four IS were utilized for COPs analysis: 19-Hydroxycholesterol, a conventional internal standard used in COPs analysis due to the rare occurrence of cholesterol autooxidation at carbon-19, was compared against three deuterated internal standards to evaluate their effects on COPs quantification. Specifically, 27-hydroxycholesterol-d_4 was used for COPs with side-chain hydroxyl groups, 7β-hydroxycholesterol-d_7 for COPs with sterol ring hydroxyl groups, and cholesterol-d_7 for keto- and epoxy-containing COPs, regardless of carbon position. For most COP species, the slopes of the calibration curves (Figure 19) were almost identical with both internal standard approaches, although lower peak area ratios were observed when 19-hydroxycholesterol was used as the internal standard compared to the deuterated COPs. This can be attributed to the deuterated COPs being more structurally related to the analytes. The matrix effects appeared to become more prominent as standard concentrations increased, with lower signals at these concentration levels possibly indicating ion suppression due to the inherently lower ionization efficiency of COPs.

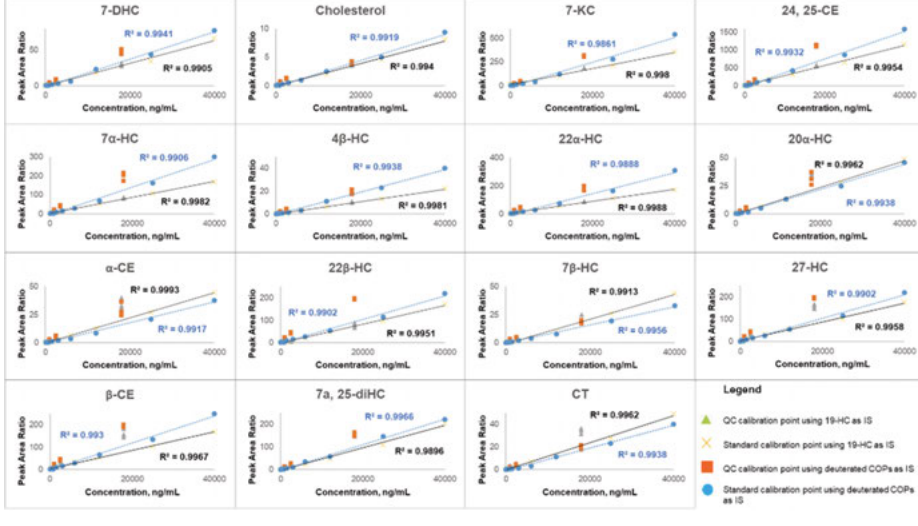


Figure 19. Comparison of standard calibration curves for COPs using 19-hydroxycholesterol against deuterated sterols as internal standards

Greater differences in calibration slopes were observed for COPs with oxidized sterol rings, such as 7α-, 7β-, and 4β-hydroxycholesterol. However, for COPs with oxidized side chains, such as 22β-, 20β-, and 27-hydroxycholesterol, only small differences were observed between the two internal standard approaches. COPs with keto- or epoxy-groups, such as 7-ketocholesterol, α-,

β-, and 24, 25-epoxycholesterol, also exhibited moderate differences in calibration slopes. These observations suggest that 19-hydroxycholesterol is best used as an alternative internal standard for COPs with oxidized side chains, while deuterated COPs may be more suitable for COPs with oxidized sterol rings.

For cholesterol calibration curves, no significant difference was found when using either cholesterol-d_7 or 19-hydroxycholesterol as internal standards. This implies that these two internal standards can be used interchangeably, or if an established method does not have access to a deuterated cholesterol species. Based on these findings, using a non-deuterated standard may be most effective for COPs with hydroxyl groups but less reliable for COPs with other polar groups.

Lastly, for **Paper IV**, considering the different lipid groups investigated, one IS was applied per group for quantification, as shown in Figure 20. The appropriate selection of deuterated-lipid internal standards in this work ensures the accuracy of quantification for the key lipid species investigated, even though it was expressed as a sum of the chromatographic peak areas, the IS used only appeared as one, sharp peak, attributing to its purity and reliability for quantification.

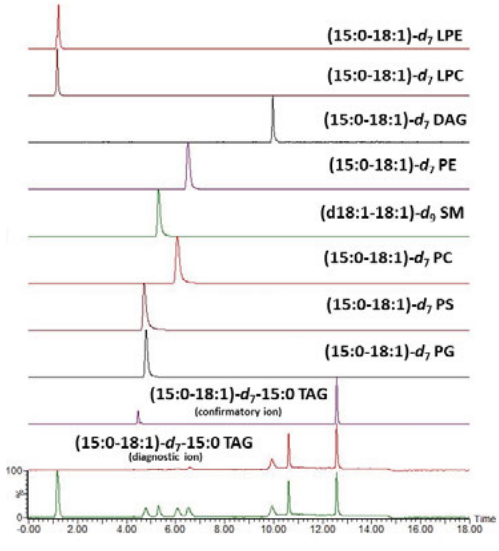


Figure 20. Chromatographic profile of the internal standards used for targeted analysis of 30 key lipid species in UPLC-MS/MS

In summary, **Papers I-IV** emphasized the importance of selecting an appropriate internal standard (IS) for lipid quantification. **Paper I** highlighted the role of IS in determining the best transesterification technique for GC-MS

analysis of FFAs as FAMEs. **Paper II** focused on the benefit of direct FFA analysis, using a deuterated-FFA IS specific to each analyte, and compared it to the conventional C17:0 IS. To date, this is one of the most elaborate work on FFA quantification using 14 internal standards from short- to long-chain FFAs. **Paper III** extended the approach on the IS method by comparing 19-HC to a deuterated-COP IS with hydroxyl groups in the B-ring or isooctyl side chain of COPs, along with cholesterol-d_7. Finally, **Paper IV** discussed the use of deuterated lipid standards tailored for key lipid species quantification, emphasizing the importance of head group relevance for accurate results. These quantification strategies were integral in developing methods combining chromatography and MS techniques for lipid analysis, particularly targeting lipid components in raw materials for PLE formulations. Method validation was conducted using these IS strategies to assess the reliability and accuracy of the analytical quantification.

4. Method validation

This section provides a description of the in-depth method validation procedures applied in **Papers I-IV**, demonstrating how the developed methods are reliable for lipid quantification aiming at TPN components, as well as checking how these specific components might affect stability of future TPN products by performing stability studies, which can provide more insights in emulsion retention and product degradation.

4.1 The ICH Q2(R2) guidelines

The ICH Q2(R2) guidelines serve as one of the central themes of this thesis, aside from utilizing and refining chromatography-MS-based techniques for method development in **Papers I-IV**. This document serves as a guideline for validating analytical procedures suitable for method development applied in pharmaceuticals, and the basis for analytical compliance of FDA and EMA. This thesis presents how the analytical figures of merit for each method is obtained, and how this affects the quantification of the lipid groups included in this study and its possible implication when applied to real pharmaceutical samples. The extensive method validation rubrics set in **Papers I-IV** exhibits how elaborate analytical method development for pharmaceuticals ensuring product quality and safety.

4.2 Linearity and working range

A calibration curve represents the relationship between the instrument response and the known concentration of the analyte. It should be constructed for each analyte using the same biological matrix as the samples. The curve should include at least five points, created by spiking matrix-matched samples at five different concentration levels in triplicate. Deviations should not exceed 20% at the lowest concentration and 15% for other calibrators. Matrix-matched zero samples (spiked with internal standard) and blank samples (without internal standard) should also be included. The working range of the curve corresponds to the expected concentration range for the study and typically determines the calibrator concentrations. Since the ICH Q2(R2) section

3.2.2.1 only contains minimum requirements for linear performance checks, some validation recommendations from Eurachem[132] with definite provisions have been applied.

For most lipid species analysed from **Papers I-IV**, their correlation coefficient, R^2 is equal to or greater than 0.9900, which is considered a strong measurement of linearity, showing stringent compliance in the ICH Q2(R2) guidelines, although it was not explicitly mentioned the ideal R^2 value for calibration curve constructions. The application of the IS method was applied in **Papers I-IV**. **Paper I** used a surrogate internal standard to establish the calibration curves for each FAMEs, while **Papers II-III** demonstrated solvent and matrix-matched calibration curves using deuterated IS compared to their conventional IS. **Paper IV** highlighted how its standard calibration curves are affected by matrix effects. The working range of each work is presented in **Papers I-IV**, which utilized a minimum of 7 calibration points, together with a minimum of 3 quality control (QC) samples, encompassing the working range of the constructed curves.

4.3 Sensitivity

The sensitivity is commonly expressed by the limits of detection and quantification (LOD and LOQ). **Paper I** highlights two types of LOD and LOQ—instrument and method. Estimation of these analytical figures of merit is important in establishing these analytical methods since these values signify at which concentration levels are considered reportable or acceptable values. These values indicate the establishment of an analytical result that is significantly different from zero[132]. These two approaches were applied in **Paper I** to obtain these two parameters. The first method derived from ICH Q2(R2) section 3.2.3.3 uses the standard deviation of the linear response divided by the slope from the generated calibration curves multiplied by 3.3 to determine LOD and the same value is multiplied by 10 to obtain LOQ. These were referred to in **Paper I** as the method LOD and LOQ since the measured values were obtained through the working range of the method being established. The second method as given in ICH Q2(R2) section 3.2.3.2 involves the concentration where the signal-to-noise (*S/N*) ratio is greater than 3 for LOD determination and 10 for LOQ. These values were referred to as instrument LOD and LOQ since the signal-to-noise ratios used in obtaining these values were derived from the instrumental conditions when the analytical method was tested. For the rest of the works in this thesis, **Papers II-IV** established the lowest calibration point as the lowest limit of quantification (LLOD), since the analytes involved in these studies are major components and are not present in trace amounts, such as performing analytical methods for impurities or

trace excipients. Since these lipid groups are considered as 'actives,' hence this approach is highly suitable for the fitness of its intended purpose.

4.4 Accuracy and precision

Accuracy in analytical measurement is defined as the difference of the measured value compared to its true (or nominal) value. Precision is defined as the proximity or the closeness of a series of replicate measurements with respect to each other. ICH Q2(R2) section 3.3.1.4 was used as a guiding principle for accuracy measurements and applied in **Papers I-IV** and reported as %bias. Compliance is considered if the %bias is less than or equal to 20% for lowest QC levels and less than or equal to 15% for other QC levels. Intraday (repeatability) and intraday (reproducibility) measurements were performed at all QC levels for all the developed methods in **Papers I-IV**. The ICH Q2(R2) section 3.3.2.1 was the basis for repeatability measurements, while sections 3.3.2.3 and 3.3.2.4 contain the fundamentals for reproducibility measurements. For both parameters, compliance is considered when the coefficient of variation (CV) is less than or equal to 20% for the lowest QC level and less than or equal to 15% for the other QC levels.

4.5 Recovery, carryover, and matrix effects

Analyte recovery from the sample matrix is evaluated by comparing the response from extracted samples to the response from non-extracted standards with the same nominal concentration as the extracted samples. The concept of recovery is an extension of accuracy, where matrix effects is investigated along with the trueness of the obtained analytical value in a measurement. The recovery experiments from **Papers I-IV** were performed according to the conditions based on the ICH Q2(R2) section 3.3.1.4.

Systematic carryovers were assessed by injection of a blank sample following injection of the highest concentration calibrator. This is important to check whether the blank contains some interferences that can affect analyte quantification. In **Papers II-IV**, carryovers were assessed by injecting blank matrices obtained from the activated charcoal treatment combined with tailored liquid-liquid extraction, serving as a reference reading for analytical measurements using the optimized methods.

Matrix effects were studied thoroughly in **Papers I-IV**, as lipid measurements were highly sensitive in the interferences introduced by the matrix composition. **Paper I** emphasized this by doing recovery studies for each type of biological matrices used in the analytical method development. In **Papers II-IV**,

a more intensive approach was utilized by comparing solvent calibrations to matrix-matched calibrations, and observe how their slope differs. The difference in analyte ratio signal corresponds to either ionization enhancement or suppression by the matrix.

4.6 Stability studies

This thesis highlights two stability studies applied in **Papers I-IV**: freeze-thaw stability and autosampler or ambient temperature stability. These investigations can provide information on how the lipid analytes behave and respond considering matrix effects under varying storage conditions. This aspect is critical for assessing retention and degradation of raw materials used in PLE formulations, the core component of TPN products.

4.6.1 Freeze-thaw stability

Three freeze-thaw cycles were performed for the lipid groups of **Papers I-IV** at a minimum of three QC levels. The capability of analyte retention is investigated in a freeze-thaw study is emphasized, since the extent of the developed analytical methods to detect compositional changes by repetitive cycling the optimum storage conditions, as mentioned in ICH Q2(R2) section 3.4. Analytical compliance is achieved when the coefficient of variation (CV) is less than or equal to 20% for the lowest QC level and less than or equal to 15% for the other QC levels.

4.6.2 Autosampler stability

Autosampler stability across lipid analytes were performed in **Papers I-IV** at a minimum of three QC levels. This study is similarly based with the freeze-thaw study derived from ICH Q2(R2) section 3.4. This on the other hand, observes the progression of possible analyte degradation upon a one-time change in storage conditions. The capability of the analytical method to detect analyte changes in this setup was assessed. Similarly with the freeze-thaw studies, analytical compliance is achieved when the coefficient of variation (CV) is less than or equal to 20% for the lowest QC level and less than or equal to 15% for the other QC levels.

4.6.3 Relevance of stability studies in PLE components

Freeze-thaw and autosampler stability measurements in **Paper I** highlight the importance of optimal storage conditions for FAMEs in heptane, as measurement variations were observed after 48 and 72 hours, exceeding the compliance %CV requirement. **Paper II** stresses the relevance of FFA concentration

and their sensitivity in matrix effects and temperature, as shown in both stability studies. SCFFAs generally more affected in the monitored stability experiments, primarily due to their volatility, sensitivity to phase-transitions, and susceptibility to thermal degradation. Through time, autosampler stability experiments for LCFFAs suggest possible chemical degradation, especially for unsaturated ones since they can undergo further oxidation.

For cholesterol and COPs investigated in **Paper III**, the measurement variation in autosampler stability studies were more prominent within the first 24 hours. However, in freeze-thaw studies, the measurement variability of COPs progressed as the number of cycles increased. This emphasizes the time-dependence of COPs, since they can be further oxidized or react, forming other COPs and other types of oxysterols. This is potentially useful for monitoring long-term stability of TPN raw materials, especially those who are kept in retention for a long period of time.

The stability of key lipid species investigated in **Paper IV** was observed to be analyte-sensitive, especially in LPC, PC and TAG species, highlighting their inevitable compositional change through time. This highlights the possibility of PC and TAG hydrolysis, potentially resulting in the formation of FFAs and LPCs. This provides an insight on how useful these chromatographic-MS-based methods, not only for their simplicity and efficiency towards routine analysis, but also as fundamental methods to thoroughly investigate or check possible compositional ingredients in PLE components for TPN products. For the first time, this thesis demonstrates an in-depth study on the behaviour of lipids in optimized storage conditions, aside from developing targeted methods for their quantification.

5. Method application in TPN raw materials

Application of the validated methods from **Papers I-IV** enables the identification and quantification of the lipid groups of interest. **Paper I** was able to analyse three pharmaceutical matrices: fish and olive oils, and an unidentified TPN component using the validated GC-MS method using the sodium methoxide method for transesterification of FFAs to FAMEs. Most FFAs obtained from the measurements were saturated FFAs, to which C16:0 and C18:0 were prominent, with C18:1 as the most abundant unsaturated FFA. **Paper II** was able to detect and quantify 15 FFAs in pharmaceutical-grade egg yolk powders using the validated SFC-MS method, with C16:0, C18:1, and C18:0 exceeding the rest of the FFAs, which is found consistent with **Paper I**. Cholesterol and COPs detection in pharmaceutical grade egg yolk powders was feasible using the tailored SFC-MS/MS methods, with cholesterol exceeds the rest of the 14 COPs investigated in **Paper III**. Lastly, **Paper IV** was able to detect and quantify the 30 key lipid species present in pharmaceutical-egg yolk powders, with PC and PE as predominant phospholipid species.

These findings emphasize the effectiveness of each method when applied to the target lipid groups in pharmaceutical-grade egg yolk powders. The developed chromatography-MS-based methods offer a comprehensive analysis of the composition and any changes occurring in the raw materials used in TPNs. The rigorous development of these analytical techniques, conducted under strict conditions and compliance standards, ensures their reliability and validity for both pharmaceutical and industrial applications.

6. Conclusions and future perspectives

This thesis, as presented in **Papers I-IV**, investigated specific lipid groups by developing tailored chromatography-MS-based methods. These methods were utilized not only for targeted lipid quantification, but also to provide insights into how matrix effects and storage conditions might affect the compositional changes of the targeted lipids potentially present in PLE raw materials. These methods are intended for long-term complementary use, providing a more comprehensive understanding of lipid composition and real-time quantitative levels, which are essential for assessing PLE raw material quality in TPN production.

In **Paper I**, the quantification of 22 FFAs was achieved using GC-MS, with chemical derivatisation converting FFAs to FAMEs via sodium methoxide. This work highlights the investigation of five selected transesterification methods, modified and tested in three TPN component matrices, offering versatility and efficiency. This study is the first to incorporate C6:0 into a GC-MS based method with an analytical run time under 21 minutes, making it more efficient than existing methods using the same instrumentation.

Paper II addressed the limitations of **Paper I** by developing a direct analysis method for 31 FFAs using SFC-MS with ESI^- without performing any chemical derivatisation, and reducing the analytical run time to 6 minutes detecting C4 to C26. **Paper III** enables direct quantification of underivatised cholesterol and 14 selected COPs using an SFC-MS/MS approach in ESI^+, which is a first in this field, since current methods rely on GC-MS or LC-MS/MS, aside from using APCI instead of ESI for COPs ionization.

Lastly, **Paper IV** details the development of an LC-MS/MS method for the analysis of 30 key lipid species across a broad range, including PLs, TAGs, LPLs, and SMs under 14 minutes. These four methods were validated according to ICH Q2(R2) guidelines, demonstrating their potential applicability to other lipid-containing raw materials or pharmaceuticals. When applied to TPN components, particularly pharmaceutical-grade egg yolk powders, this work underscores the methods' potential for assessing long-term raw material stability and degradation.

In conclusion, the work in this thesis sets a benchmark for more efficient, targeted lipidomics with minimal sample preparation and pretreatment. This approach facilitates easier technological transfer, making it suitable for pharmaceutical quality control and routine practice.

This thesis, through **Papers I-IV**, advances chromatographic-MS-based strategies for method development applied to TPN components. The applicability of these methods could be further expanded by applying them to other biological matrices, such as pharmaceutical raw materials, food components, and clinical samples. However, to analyse clinical samples effectively, there is still a need to improve the sensitivity of these methods.

The incorporation of smaller FFAs, starting from C2, could further enhance the method development established in **Papers I** and **II**. **Paper III** could be improved, particularly in terms of quantification, by adding more internal standards and utilizing a wider range of chromatographic columns with different stationary phases to achieve higher resolution in separating COPs. In **Paper IV**, the identification of specific lipid species could be strengthened by incorporating stereospecific lipid internal standards, providing a more definitive identification especially for lipids in condensed formulas with multiple peaks and distinct retention times.

Finally, parallel method development for phytosterols and phytosterol oxidation products (POPs) would be a valuable addition to the existing methods, particularly for monitoring phytosterol sources in TPN formulations, such as soybean, olive, and MCT oils. Long-term and accelerated stability studies of TPN raw materials, incorporating the developed analytical methods from **Papers I-IV**, would offer valuable insights to their retention and degradation. Given that these methods support routine analysis, exploring their technological transfer would be beneficial, demonstrating their usability and versatility.
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7. Popular scientific summary

"Health is wealth."

This phrase rings especially true when someone cannot eat or process nutrients properly. For premature infants, whose bodies are too underdeveloped to digest food, and critically ill patients, who may be unable to consume food due to severe medical conditions, this poses a life-threatening challenge.

Thankfully, modern medicine has developed a remarkable solution: Total Parenteral Nutrition (TPN). This life-saving therapy delivers essential nutrients such as carbohydrates, proteins, fats, vitamins, and minerals directly into the bloodstream, completely bypassing the digestive system. Administered intravenously, TPN is designed to sustain life, promote recovery, and provide the nourishment these vulnerable patients need to survive and heal. Given TPN's vital role, rigorous research and strict quality control are essential to ensure its safety and effectiveness. For many premature infants and critically ill patients, TPN is more than a treatment and a lifeline.

Its purpose is to support recovery and restore strength in severely sick or near-death patients. Since TPN is delivered directly into the veins, it is classified as a medical treatment. Therefore, stringent measures in pharmaceutical manufacturing are essential to ensure its safety and efficacy intended for clinical subjects with severe ailments. TPN contains various components designed to support the recipient's recovery. A typical TPN bag includes carbohydrates in the form of glucose, proteins as amino acids, and fats in the form of a lipid emulsion. TPN lipid emulsions are crucial because they provide a range of lipids, including fatty acids (both essential and non-essential), phospholipids, triacylglycerols, cholesterol, and other fat-soluble components to meet the metabolic demands of the recipient. Each type of lipids plays a role in maintaining the effectiveness of a product intended for TPN. My research focuses on developing analytical methods to accurately quantify the composition of TPN ingredients. This is important because previous studies have suggested that changes in the composition of TPN ingredients, particularly lipid emulsions, may indicate spoilage or a complete loss of TPN efficacy.

To investigate this, the research combines two fascinating scientific tools: chromatography and mass spectrometry. Think of chromatography as a method to separate the lipid ingredients in a mixture, while mass spectrometry identifies each one by giving it a unique "molecular fingerprint." The research utilizes three types of chromatography gas chromatography (GC), supercritical fluid chromatography (SFC), and liquid chromatography (LC), each optimized together with mass spectrometry to analyse specific lipid components of TPN, such as fatty acids, phospholipids, triacylglycerols, cholesterol, and cholesterol oxidation products. The findings from this research will provide valuable insights into the stability of TPN formulations, helping to ensure their safety and effectiveness for patients, particularly those with critical medical needs who rely on this life-saving therapy.

8. Populärvetenskaplig sammanfattning

"Hälsa är rikedom."

Detta uttryck stämmer särskilt när någon inte kan äta eller bearbeta näringsämnen ordentligt. För för tidigt födda barn, vars kroppar är för outvecklade för att smälta mat, och för svårt sjuka patienter, som kanske inte kan konsumera mat på grund av allvarliga medicinska tillstånd, utgör detta en livshotande utmaning.

Lyckligtvis har modern medicin utvecklat en anmärkningsvärd lösning: Total Parenteral Nutrition (TPN). Denna livräddande behandling levererar viktiga näringsämnen som kalorier, proteiner, fetter, vitaminer och mineraler direkt in i blodomloppet, vilket helt undviker matsmältningssystemet. TPN administreras intravenöst och är utformad för att upprätthålla liv, främja återhämtning och ge den näring som dessa sårbara patienter behöver för att överleva och läka. Eftersom TPN har en så viktig roll, är noggrant forskningsarbete och strikt kvalitetskontroll avgörande för att säkerställa dess säkerhet och effektivitet. För många för tidigt födda barn och svårt sjuka patienter är TPN mer än en behandling – det är en livlina.

Syftet är att stödja återhämtning och återställa styrka hos svårt sjuka eller livshotade patienter. Eftersom TPN levereras direkt i venerna, klassificeras det som en medicinsk behandling och ett läkemedel. Därför är detaljkunskap om dess innehåll och beskaffenhet avgörande för att säkerställa dess säkerhet och effektivitet. TPN innehåller olika komponenter som är designade för att stödja mottagarens återhämtning. En typisk TPN-påse innehåller kolhydrater i form av glukos, proteiner som aminosyror och fetter i form av en lipidemulsion. TPN lipidemulsioner är avgörande eftersom de tillhandahåller en mängd olika lipider, inklusive fettsyror (både essentiella och icke-essentiella), fosfolipider, triacylglyceroler, kolesterol och andra fettlösliga komponenter för att möta mottagarens metaboliska behov. Varje typ av lipid spelar en viktig roll för att upprätthålla effektiviteten hos en produkt avsedd för TPN. Min forskning fokuserar på att utveckla och förfina analytiska metoder som möjliggör detaljerad förståelse och exakt kvantifiering av sammansättningen av TPN-ingredienserna. Detta är viktigt eftersom tidigare studier har visat att förändringar i

sammansättningen av TPN-ingredienserna, särskilt lipidemulsioner, kan indikera försämring eller en total förlust av TPN:s effektivitet.

För att undersöka detta kombinerar forskningen två fascinerande vetenskapliga analytiska verktyg: kromatografi och masspektrometri. Tänk på kromatografi som en metod för att separera eller dra isär lipidkomponenterna i en blandning, medan masspektrometri identifierar varje komponent genom att ge den ett unikt "molekylärt fingeravtryck". Min forskning använder tre typer av kromatografi: gaskromatografi (GC), superkritisk vätskekromatografi (SFC), och vätskekromatografi (LC), som var och en optimeras tillsammans med masspektrometri för att analysera de specifika lipidkomponenterna i TPN, såsom fettsyror, fosfolipider, triacylglyceroler, kolesterol och kolesteroloxidationsprodukter. Resultaten från denna forskning kommer att ge värdefulla insikter i innehåll och stabilitet hos TPN-formuleringar, vilket hjälper till att säkerställa deras säkerhet och effektivitet för patienter, särskilt viktigt för de med kritiska medicinska behov som är beroende av denna livräddande terapi.

9. Buod ng agham

"Ang kalusugan ay kayamanan."

Ang katagang ito ay higit pang makatotohanan kapag ang isang tao ay hindi makakain o magkapagtunaw ng sustansiya at nutrisyon nang maayos. Para sa mga sanggol na isinilang nang hindi husto ang gulang (*premature*), kung saan hindi pa lubusang sapat ang kakayahan ng kanilang mga katawan upang makatunaw ng pagkain, at sa mga pasyenteng nasa kritikal na kalagayan, na maaaring hindi makakain dahil sa mga malulubhang kondisyong medikal o iniindang karamdaman, ito ay nagiging isang malaking banta para sa kanilang buhay.

Sa kabutihang palad, sa tulong ng makabagong medisina ay napagtagumpayan ito sa pamamagitan ng isang kamangha-manghang solusyon: *Total Parenteral Nutrition* (TPN). Ang produktong ito ay naglalayong pagaanin o di kaya ay maibsan ang isang sakit o karamdaman sa pamamagitan ng paghahatid ng mahahalagang nutrisyon tulad ng asukal, protina, taba, bitamina, at mineral, na direkta sa daluyan ng dugo at hindi dumadaan sa bibig at sikmura. Ito ay idinudulot sa pamamagitan ng *intravenous* na pamamaraan at layunin nitong mapakapagpalakas at magbigay ng nutrisyon na kinakailangan ng mga pasyenteng ito tungo sa mabilis na paghihilom at tuluyang paggaling. Dahil sa mahalagang papel ng TPN, kinakailangan ang masusing pananaliksik at mataas na antas ng pagsusuri sa kalidad nito upang matiyak pagiging epektibo at kaligtasan ng kung sinuman ang makakatanggap nito. Para sa maraming premature na sanggol at pasyenteng nasa malubhang kondisyon, ang TPN ay higit pa sa isang uri ng panglunas– dahil ito ay ang kanilang dakilang saklolo sa gitna ng buhay at kamatayan.

Dahil ang TPN ay ibinibigay direkta sa ugat, itinuturing ito bilang isang medikal na paggamot o parmaseyotiko. Kaya’t mahalaga ang masusing pananaliksik at pagsusuri ng kalidad upang matiyak ang kaligtasan at pagiging epektibo nito. Ang TPN ay naglalaman ng iba't ibang mga sangkap na idinisenyo upang suportahan ang paggaling ng tumatanggap nito. Karaniwang naglalaman ang isang sisidlan ng TPN ng mga *carbohydrates* sa anyo ng *glucose*, mga protina bilang *amino acids*, at mga taba sa anyo ng *lipid emulsion*. Ang mga *lipid emulsion* sa TPN ay mahalaga dahil nagbibigay ito ng iba't ibang

uri ng mga lipido, kabilang ang mga *fatty acids* (parehong *essential* at *non-essential*), *phospholipids*, *triacylglycerols*, kolesterol, at iba pang mga *fat-soluble components* upang matugunan ang mga *metabolic* na pangangailangan ng pasyenteng nangangailangan nito. Ang bawat uri ng lipido ay may papel sa pagpapanatili ng bisa ng produktong ginagamit para sa TPN. Ang aking pananaliksik ay nakatutok sa paghinang (*develop*) ng mga analitikal na pamamaraan upang tumpak na masukat ang komposisyon ng mga sangkap ng TPN. Mahalagang gawin ito dahil ang mga naunang pag-aaral ay nagmungkahi na ang mga pagbabago sa komposisyon ng mga sangkap ng TPN, partikular ang *lipid emulsions*, ay maaaring magpahiwatig ng pagkasira o ganap na pagkawala ng bisa ng TPN.

Upang ito ay higit pang mapag-aralan, pinagsasama ng pananaliksik na ito ang dalawang kahanga-hangang siyentipikong prinsipyo sa sangay ng *analytical chemistry*: kromatograpiya at *mass spectrometry*. Isipin ang kromatograpiya bilang isang pamamaraan upang paghiwalayin ang mga lipidong sangkap sa isang pormulasyon ng TPN, habang ang *mass spectrometry* ay tumutukoy sa bawat isa sa pamamagitan ng pagbibigay ng isang natatanging "molekular na *fingerprint*." Ang aking pananaliksik ay gumagamit ng tatlong uri ng kromatograpiya: *gas chromatography* (GC), *supercritical fluid chromatography* (SFC), at *liquid chromatography* (LC), na bawat isa ay lubusang hininang kasama ng *mass spectrometry* upang suriin ang mga lipidong bahagi ng TPN, tulad ng *fatty acids*, *phospholipids*, *triacylglycerols*, kolesterol, at mga produkto ng kolesterol dahil sa *autooxidation* nito (*cholesterol oxidation products*). Ang mga natuklasan mula sa pananaliksik na ito ay magbibigay ng mahalagang kaalaman tungkol sa katatagan ng mga sangkap ng TPN formulations, na makakatulong upang matiyak ang kanilang kaligtasan at pagiging epektibo para sa mga pasyente, lalo na ang mga may kritikal na pangangailangang medikal na umaasa sa terapiyang ito na ang pangunahing layunin ay magligtas at magpalawig ng kalusugan—at buhay.

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