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# Derivatization of selected lipid classes and their identification using RP-UHPLC/MS/MS method in human plasma

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# Derivatizace vybraných tříd lipidů a jejich identifikace pomocí RP-UHPLC/MS/MS metody v lidské plazmě

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# ZADÁNÍ DIPLOMOVÉ PRÁCE

(projektu, uměleckého díla, uměleckého výkonu)

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## Zásady pro vypracování

1. Proveďte literární rešerši zaměřenou na lipidomickou analýzu neutrálních lipidů, sterolů a vitamínů rozpustných v tucích s využitím chemické derivatizace.

2. V experimentální části zoptimalizujte derivatizační proces využívající 3-(chlorosulfonyl)benzoovou kyselinu jako derivatizační činidlo a RP-UHPLC/MSMS metodu pro analýzu výše uvedených lipidových tříd.

3/ Zoptimalizovanou metodiku aplikujte na lidskou plazmu a proveďte identifikaci lipidů z daných lipidových tříd.

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## ANNOTATION

This thesis was focused on the development of novel derivatization method for sterols, prenols, and neutral lipids using 3-(chlorosulfonyl)benzoic acid. The method using reversed phase ultrahigh-performance chromatography coupled with tandem mass spectrometry (RP-UHPLC/MS/MS) was optimized for the detection of these lipid classes in the negative ion mode. The fully optimized method was applied for the identification of sterols, prenols, monoacylglycerols, and diacylglycerols in human plasma.



## KEYWORDS

Chemical derivatization; mass spectrometry; liquid chromatography – mass spectrometry; lipids; lipidomics; sterols; prenols


## NÁZEV

Derivatizace vybraných tříd lipidů a jejich identifikace pomocí RP-UHPLC/MS/MS metody v lidské plazmě


## ANOTACE

Tato práce byla zaměřena na vývoj nové derivatizační metody pro stanovení sterolů, prenolů a neutrálních lipidů pomocí kyseliny 3-(chlorsulfonyl)benzoové. Byla optimalizována metoda vysokoúčinné kapalinové chromatografie s obracenými fázemi spojenou s tandemovou hmotnostní spektrometrií (RP-UHPLC/MS/MS) pro detekci těchto tříd lipidů v režimu negativních iontů. Zoptimalizovaná metoda byla použita pro identifikaci sterolů, prenolů, monoacylglycerolů a diacylglycerolů v lidské plazmě.



## KLÍČOVÁ SLOVA

Chemická deprivatizace; hmotnostní spektrometrie; kapalinová chromatografie s hmotnostní spektrometrií; lipidy; lipidomika; steroly; prenoly

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# The list of abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| APCI | Atmospheric Pressure Chemical Ionization |
| APPI | Atmospheric Pressure Photoionization |
| BuOH | Butanol |
| CE | Collision Energy |
| Chol | Cholesterol |
| CID | Collision-Induced Dissociation |
| Cl-SBA | 3-(Chlorosulfonyl)benzoic Acid |
| DB | Double Bond |
| DC | Direct Current |
| DG | Diacylglycerol |
| ECN | Equivalent Carbon Number |
| ESI | Electrospray Ionization |
| ESI-MS | Electrospray Ionization - Mass Spectrometry |
| FA | Fatty Acyls |
| FT | Fourier Transformation |
| FTICR | Fourier-Transform Ion Cyclotron Resonance |
| GL | Glycerolipids |
| GP | Glycerophospholipids |
| HR | High Resolution |
| HILIC | Hydrophilic Interaction Liquid Chromatography |
| HPLC/MS | High-Performance Liquid Chromatography - Mass Spectrometry |
| ICR | Ion Cyclotron Resonance |
| IEM | Ion-Evaporation Model |

| | |
|---|---|
| IPA | Isopropanol/2-Propanol |
| IS | Internal Standard |
| IS mix | Internal Standards Mixture |
| IT | Ion Trap |
| LC | Liquid Chromatography |
| LC/MS | Liquid Chromatography - Mass Spectrometry |
| LR | Low Resolution |
| MALDI | Matrix-Assisted Laser Desorption/Ionization |
| MeOH | Methanol |
| MG | Monoacylglycerol |
| MRM | Multiple Reaction Monitoring |
| MS | Mass Spectrometry |
| MS/MS | Tandem Mass Spectrometry |
| MW | Molecular Weight |
| NP | Normal Phase |
| RI | Refractive Index |
| PK | Polyketides |
| PB | Paternò-Büchi |
| PR | Prenol |
| RF | Radiofrequency |
| RP | Reversed Phase |
| SL | Saccharolipids |
| SP | Sphingolipids |
| SRM | Selected Reaction Monitoring |
| ST | Sterol |
| STD | Standard |

| | |
|---|---|
| STD mix | Standards Mixture |
| TG | Triacylglycerol |
| TOF | Time-of-Flight |
| UHPLC | Ultrahigh-Performance Liquid Chromatography |
| UHPSFC | Ultrahigh-Performance Supercritical Fluid Chromatography |
| Q | Quadrupole |
| QqQ | Triple Quadrupole |

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# 1. Introduction

Lipidomics is the study of the structure, function, and metabolism of lipids. Lipidomics involves the comprehensive analysis of all lipids present in biological samples, including their chemical properties, interactions, and modifications. Lipids are essential components of all living organisms and play critical roles in cellular processes, such as energy storage, signaling, and membrane structure. The dysregulation of lipid syntheses has an impact on health, like the mevalonate pathway has been linked to conditions, such as hypercholesterolemia, cardiovascular disease, and cancer. The mevalonate pathway is an important metabolic pathway that starts with the conversion of acetyl-CoA to mevalonate and produces isoprenoid molecules, such as cholesterol, dolichol, and farnesyl pyrophosphate as well as ubiquinone and other isoprenoids. Therefore, studying lipids like sterols and prenols can lead to a better understanding of the role of these lipids in disease pathogenesis and identify potential targets for therapeutic interventions.

Prenols are precursors for the biosynthesis of precursors of important biomolecules, such as vitamin K and coenzyme Q10, which have essential roles in blood clotting and cellular respiration. These lipids are crucial for the post-translational modification of proteins through the process of prenylation. Sterols are important components of biological membranes, where they regulate membrane fluidity, permeability, and stability. They are also involved in various cellular processes, such as signal transduction, cell proliferation, and apoptosis. In addition, sterols are precursors of steroid hormones and bile acids, which play critical roles in the endocrine and digestive systems. Furthermore, alterations in the sterol metabolism have been associated with a variety of human diseases, including hypercholesterolemia, atherosclerosis, and cancer.

Lipids are present in complex mixtures in a wide range of concentrations, making their identification and quantification challenging. Mass spectrometry coupled with liquid or gas chromatography is primarily used in lipidomics. This combination of techniques allows high-resolution separation of lipids and sensitive detection and identification of individual lipid species. tandem mass spectrometry (MS/MS) is preferred to achieve higher sensitivity, where two or more mass analyzers are used in series to isolate and fragment ions generated from the analyte. In lipidomics, mass analyzer may differ based on the application, however, the electrospray ionization (ESI) is the most common ionization technique.

Even though it is possible to identify many lipid classes or species using LC/MS, it is not achievable in some cases due to their complicated extraction, separation or ionization efficiency. Derivatization methods can improve these shortcomings and provide additional information, such as functional group analysis and double bond location. It can improve the detection and/or separation of lipid classes or specific lipid species.

In this thesis, I optimized the derivatization method using 3-(chlorosulfonyl)benzoic acid for sterols and prenols and applied the optimized method for human plasma. The method was performed on ultrahigh-performance liquid chromatography coupled with low resolution tandem mass spectrometry (UHPLC/MS/MS).

# 2. Theoretical part

## 2.1. Mass spectrometry

Mass spectrometry is an analytical technique that is used to measure molecular masses and their fragments after converting them to positively or negatively charged ions [1-6]. The principal of MS is to ionize molecules using an ion source, then during this process or after, these ionic compounds fragment into smaller pieces, so-called fragments, which are either positively or negatively charged or without charge. Individual charged particles separate from each other according to their mass to charge (*m/z*) ratio. Once the separation process is complete, charged particles are detected, and their mass spectrum is recorded as a relationship between the value of *m/z* and intensity [1-6].

The instrument used in mass spectrometry is called a mass spectrometer. As shown in *Figure 1* the mass spectrometer consists of sample introduction, ionization, mass analysis, and detection. There are at least three ways to do sample introduction: direct infusion, coupling with chromatography, and desorption ionization technique [1-6].

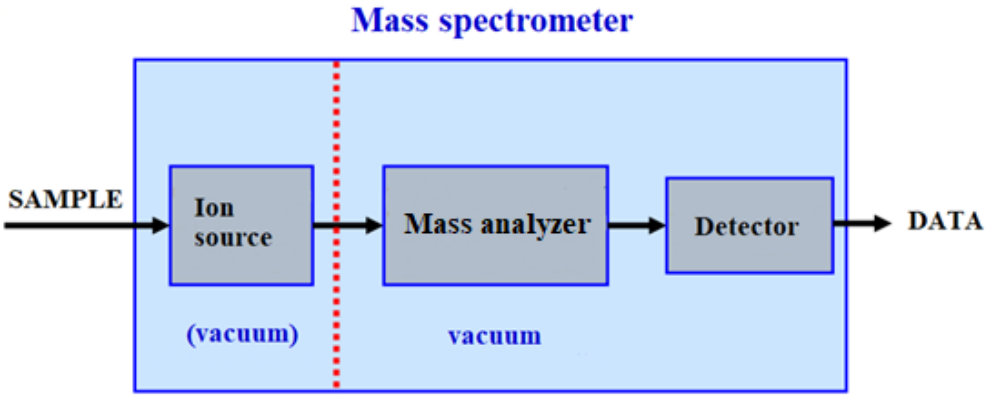



*Figure 1. Block scheme of the mass spectrometer [2]*

The mass spectrometer is very sensitive, very selective, and universal. Only a small amount of sample is required for both qualitative and quantitative analyses in mass spectrometry. However, this is a destructive method and can have high purchase and operating costs [2].

### 2.1.1. Ion source

The ion source is used to transform the analyzed substances into the ionic state and to accelerate the ions before entering the mass analyzer [1-3]. It is quite difficult to choose the universal ionization technique. That is why there are different methods for ionization and the type of ion sources depending on the analyzed material and the requirements for analyses. Electron ionization, chemical ionization, and field ionization are used for materials in a gas state. However, when dealing with a non-volatile substance, it is preferable to use techniques that ionize materials in a solid state of matter, such as field desorption, desorption chemical ionization, laser ionization, photoionization, etc. The resulting ionic compounds are then analyzed in the gas state [1-3].

For transforming analyzed substances into ionic state, a specific amount of energy is needed depending on a substance. Therefore, ionic techniques can be divided into two groups according to energy (the "hardness") [1-3]. Technique, where the excess energy given to ionized molecules is small and the probability of fragmentation is low, is called soft ionization. And vice versa when the given energy is enough for larger fragmentation of primarily created ion, is called hard ionization [3]. Electron ionization (EI) is the hardest ionization technique, which is used for analyzing low- to medium-polarity, non-ionic organic compounds [2,5]. When chemical ionization (CI) is considered as one of the soft ionizations, using a reagent gas (methane) at relatively high pressure (6 – 130 Pa) to analyze organic molecules [1-3,5]. EI and CI are both older techniques that have been largely replaced by newer ones, such as electrospray ionization and matrix-assisted laser desorption/ionization in many applications. However, EI and CI are still used and have some advantages over newer techniques, e.g., in GC/MS [1-3].

#### 2.1.1.1. Atmospheric pressure ionization

The techniques that use atmospheric pressure ionization (API) are electrospray ionization, atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). All these techniques belong to the soft ionization, which share similar setups.

In APCI (*Figure 2.A*) and APPI (*Figure 2.B*), molecules of analyte are ionized in a gas state, apart from supplied capillary, a heated block and a discharge needle (with a high voltage) or UV source, depending on the technique, are present. The solution of analytes is nebulized using dry nitrogen at the end of the capillary and quickly evaporated due to the heater. Hence,

ionization is done in a state of vapor. As a result of corona discharge, molecules of solvent are ionized in a gas state, and formed ions are used as the main source of protons for analytes. After that, ions are forwarded to the mass analyzer by accelerating electrode with suitable voltage polarity [1,2,6].

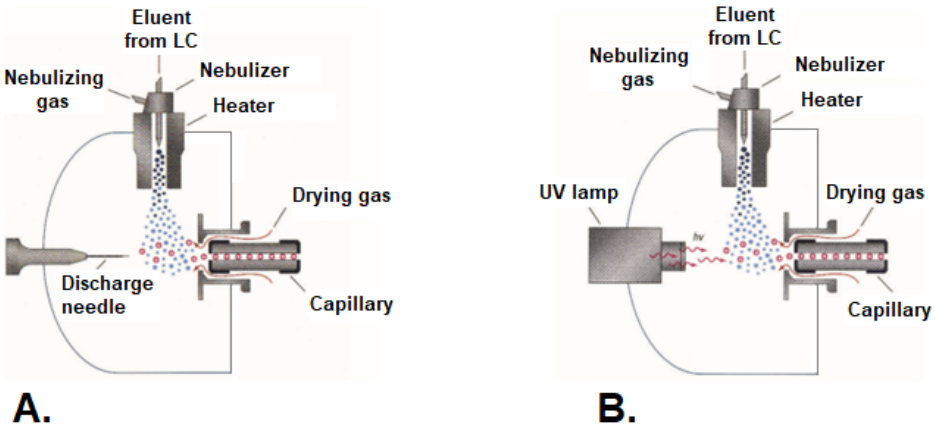



*Figure 2. Scheme of atmospheric pressure ionization (A.: APCI; B.: APPI) [2]*

### 2.1.1.2. Electrospray ionization

Electrospray ionization is considered the softest among common techniques [1-3,5,6]. Here, one up to multiply charged ions are obtained depending on the compound type and experimental conditions. ESI led to significant progress in analyses of large biomolecules (proteins, nucleic acids, lipids) without their further fragmentation [2,5,6]. It is by far the most frequently used ionization technique in HPLC/MS coupling [1,2,5,6].

ESI can be applied to a wide range of polarity. It is suitable mainly for medium polar to ionic compounds, but this range could be extended using a suitable way of cationization ESI [2,6]. This technique also forms multiply charged ions in the case of high-mass analytes. It is very beneficial, because it shifts the ions into *m/z* range that can be applied by most mass analyzers [5,13]. The following ions are typical for ESI spectra: $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, $[M-H]^-$, $[M+HCOO]^-$, *etc* [2,6].

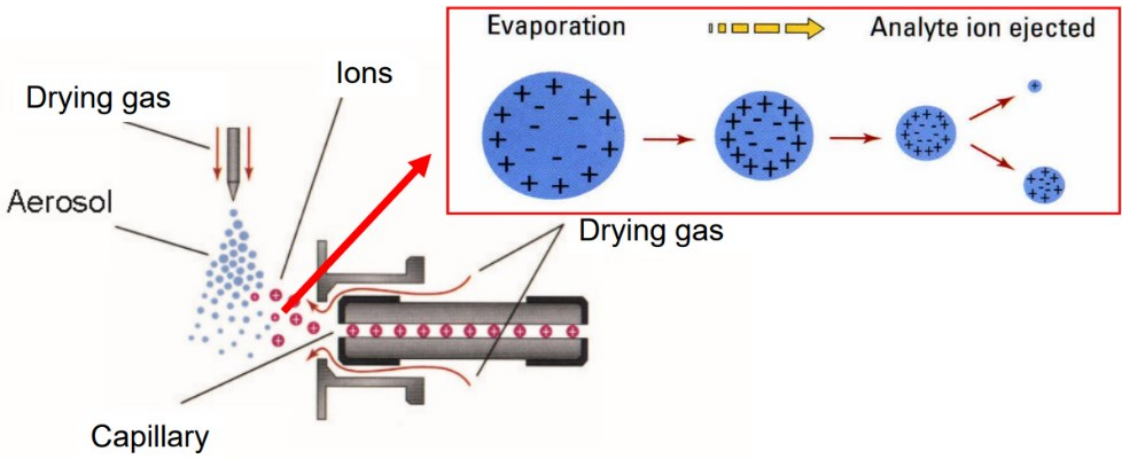



*Figure 3. Scheme of ESI [2]*

In *Figure 3*, the scheme of ESI is shown. Here, a sample solvent is continually infused to the ion source by a stainless-steel capillary, on which high voltage is applied (3 –5 kV in positive mode; approximately -2.5 kV in negative mode) [1,2,5,6]. The tip of the capillary creates an electrostatic field, in which a mist is formed, or rather an aerosol of droplets with a high charge. Droplets from the capillary are pushed to a space, which is rinsed by a counter-flowing drying gas, where they are quickly evaporated. It causes an increase in charge density on the droplet's surface until it reaches the critical value for Coulombic explosion, and then even smaller droplets are formed with the redistribution of initial charges. Newly formed droplets repeat the process of evaporation and Coulombic explosion. In the end, all generated ions enter the mass analyzer [1,2,6].

There are two models that describe the formation of ions from charged droplets: the charged-residue model (CRM) and the ion-evaporation model (IEM) [5,7]. CRM is an assumption that larger ions are formed by the subsequent evaporation of all solvent molecules and successive Coulomb fissions. The process is repeated until the solvent is dry, which leads to gas phase ions [7]. Using this method, it can be expected that biomolecules (proteins, nucleic acids, carbohydrates, lipids) can produce single charged ions [5]. When IEM, on the other hand, focusses on small inorganic and organic ions. This model describes the formation of ions on the surface of the very small droplets that creates the electric field that causes the evaporation of the droplets [5,7]. The higher the charge density, the more charges within the reach of an analyte molecule and the lower the charge of the surface is [5,8].

The physical and chemical properties of the liquid sample can affect the ESI process, as well as external parameters, such as liquid flow rate, electric field strength, *etc* [9]. The problem can be solved by using water-methanol or water-acetonitrile as solvents. There are two options

for solvent evaporation: a heated transfer capillary (introduced by Chait) or a countercurrent stream of hot dry nitrogen, also called curtain gas (introduced by Sciex) [5,9]. The variations in these factors can impact the robustness of a system and the extent of cluster ion formation when using a specific ESI interface [5].

There are other factors that can impact ESI analyses such as the pH of the sprayed solution, the flow of the sample solution, the flow (or pressure) of the nebulizing gas and the flow and temperature of the heating gas or the temperature of the heated capillary [5]. These parameters must be considered for the optimalization of MS parameters.

#### 2.1.1.3. Matrix-assisted laser desorption/ionization

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique [2,5,6]. Before ionization, the sample is mixed with a matrix (usually an organic acid) and then deposited on a MALDI target (*Figure 4*). In the ionization process, a brief laser pulse is utilized, which is absorbed by the matrix. This leads to the local desorption of the matrix, causing the appearance of analytes. The matrix molecules absorb the laser energy, are ionized, and transfer the charge to the analyte molecules in the gas phase. The analyte molecules are usually ionized by being protonated or deprotonated by nearby matrix molecules. Ionization takes place in vacuum but can also be carried out at atmospheric pressure [2,5,6].

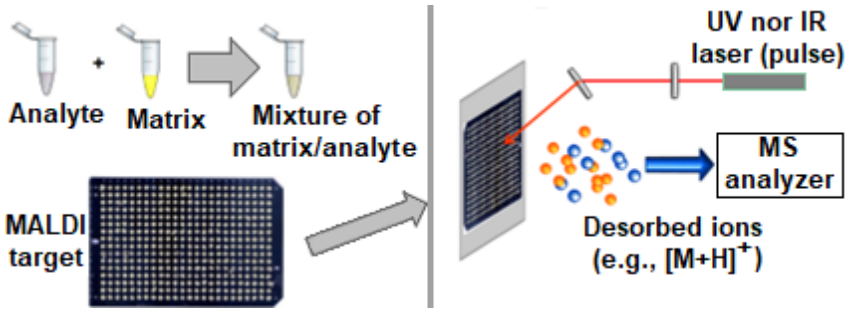



*Figure 4. Scheme of MALDI [2]*

The MALDI technique is suitable for the analysis of biomolecules, such as peptides, lipids, carbohydrates, or other organic macromolecules. Most often, MALDI is combined with a time-of-flight analyzer (TOF) but coupling with other mass analyzers is possible as well [2,5,6].

### 2.1.2. Mass analyzer

The mass analyzer is used for the ion separation produced in the ion source based on their *m/z* [1-3,6]. The whole process is under vacuum, and all analyzers use static or dynamic

electric and magnetic poles, alternatively, their combination. The mass analyzer is placed after the ion source, otherwise, the analyzer cannot properly work without any produced ions, and before the detector. The important parameters of the mass analyzers include resolution, accuracy, dynamic range, mass range, and speed of analysis (scanning per unit of time) [1-3,6].

General types of mass analyzers, which are most commonly used in practice, are time-of-flight (TOF), quadrupole (Q), ion-trap (IT), orbitrap, *etc*. Triple quadrupole is used for my experiments. Hence, this analyzer will be discussed in detail in the chapters below. However, I would like to explain principles of some other analyzers as well.

Ion trap (*Figure 5*) consists of three electrodes with a hyperbolic profile. The middle ring-shaped (circle) electrode and two convex ending electrodes, forming the bottom and top of ion trap, are isolated by sealing ceramic inserts and restricted space, where ions are trapped. It is used for capturing (trapping) ions and their subsequent analysis, using helium to focus ions in the center [10]. In addition, the operation of the IT is based on a pulsed mode, and only a limited number of ions can be accumulated, leading to a narrower dynamic range compared to quadrupole analyzers. Ion trap can be used to analyze a wide range of small to medium-sized molecules, including peptides, proteins, metabolites, and lipids. It is particularly useful for the analysis of complex mixtures due to its ability to perform multiple stages of mass spectrometry ($MS^n$), which can provide more structural information about the analytes. There are several types of IT, such as the 2D linear quadrupole trap and the 3D (spheric) ion trap [1,2,6].

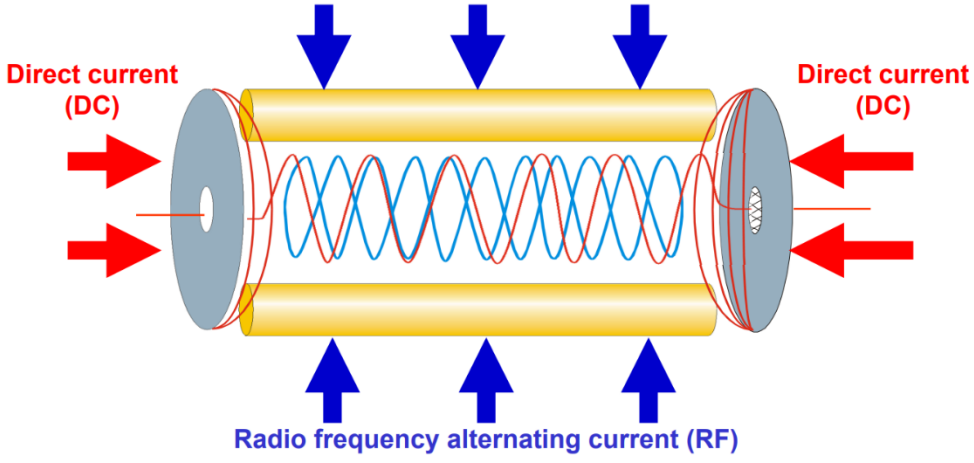



*Figure 5. Scheme of linear ion trap [6]*

Another type of ion trap device, is Orbitrap (*Figure 6*), described by Russian physician A. Makarov, which does not need to apply any radio frequency (RF) voltage and magnetic field. It consists of an outer and an inner spindle-like electrode, on which a voltage is applied. The

separation of ions of varying *m/z* values occurs as they oscillate at different frequencies [2,6,10]. Its high resolution (greater than 100,000) and mass accuracy make it particularly useful for the identification and quantification of complex mixtures of biomolecules. Orbital trap MS systems are widely used in shotgun lipidomics, which is typically performed by direct infusion of a sample into an ionic source without prior chromatographic separation [6,11].

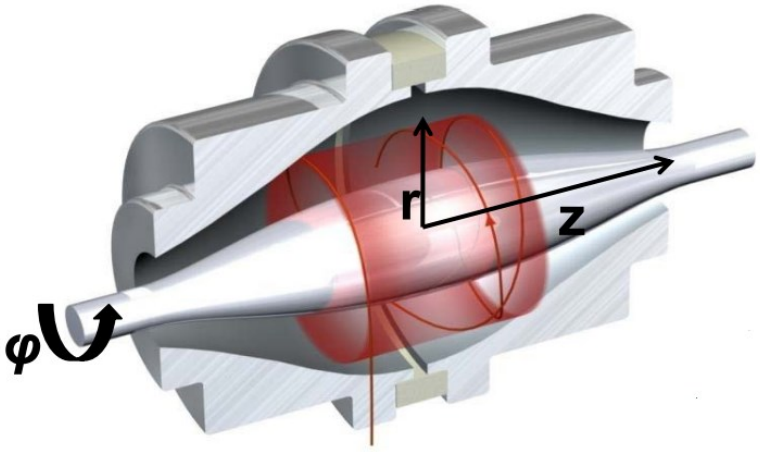



*Figure 6. Scheme of Orbitrap [2]*

The ion cyclotron analyzer resonance (ICR) with Fourier transform (*Figure 7*) is similar to orbitrap but uses the cyclotron frequency of ions in the crossing homogeneous magnetic and electric fields to separate them [10,12]. Here, the ions are trapped in a strong magnetic field in a cell that consists of two end electrodes, two opposite excitation electrodes and two opposite detection electrodes. ICR is the most advanced mass analyzer in terms of high accuracy of correct *m/z* measurement and resolution, which can exceed 10,000,000 [6].

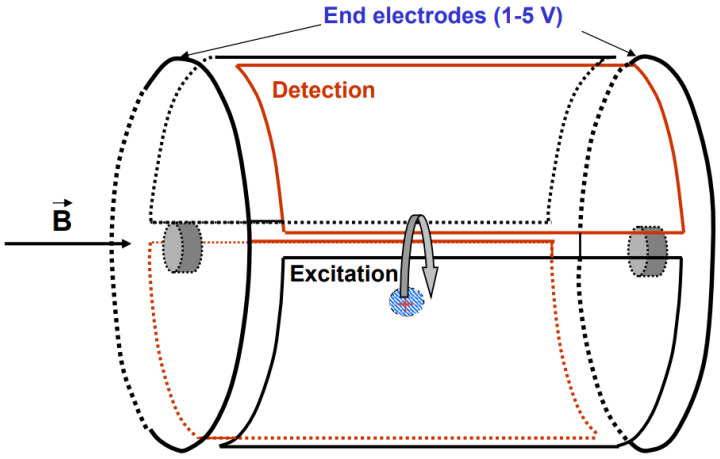



*Figure 7. Scheme of ICR [6]*

In conclusion, orbitrap and ICR with Fourier transform (FTICR) have the highest resolution, but they have disadvantages in operating costs and slower scan rate compared to

other mass analyzers [2]. The time-of-flight analyzer (*Figure 8*), on the other hand (resolution up to 60,000), is useful for the detection of trace amounts of analytes in complex samples, as well as for the detection of non-targeted compounds in environmental and forensic analysis. It uses short periodical pulses of ion collision, which after acceleration by electric potential (25-30 kV) to the same kinetic energy are entered through grounded grid to the evacuated flight tube [1,2,10]. TOF separates and detects ions of different *m/z* by measuring the time it takes for the ions to travel in the flight tube [1,10].

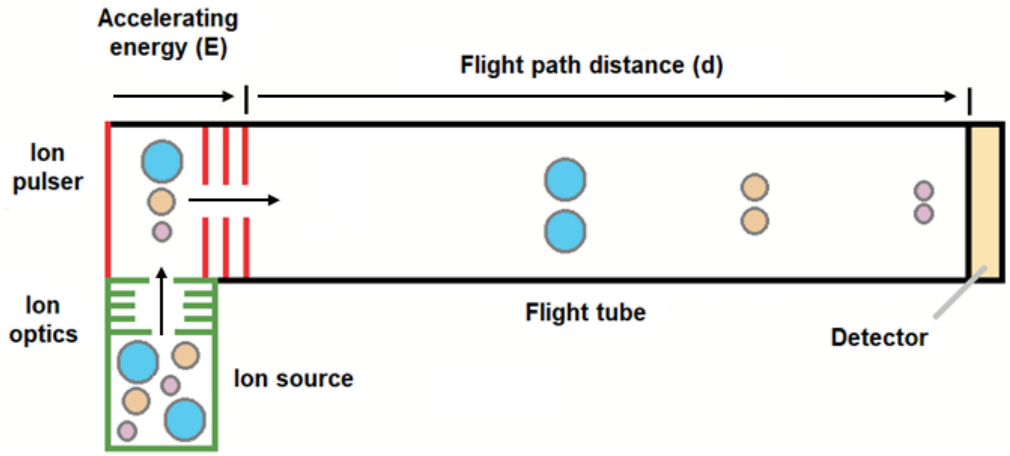



*Figure 8. Scheme of TOF [2]*

### 2.1.2.1. Quadrupole analyzer

The quadrupole analyzer (Q) is constructed out of four parallel hyperbolic or circle-shaped metal rods, which are connected to the sources of direct and high-frequency alternating current voltage (*Figure 9*). It is usually used in mass spectrometers designed for coupling with gas and liquid chromatography or capillary electrophoresis due to its simplicity and low price [1-3,6].

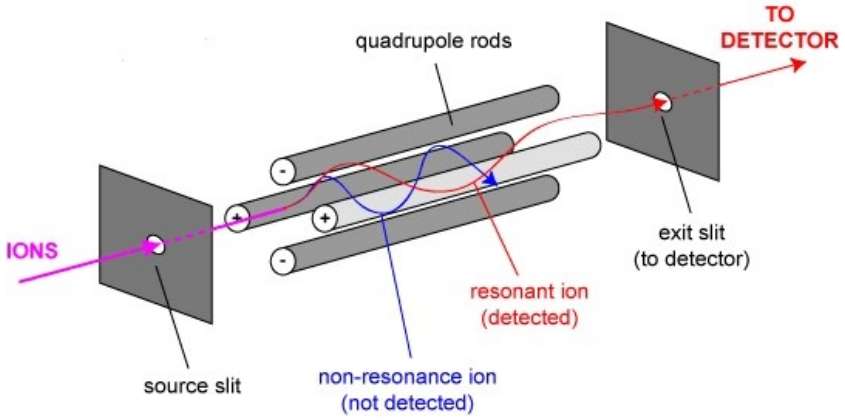



*Figure 9. Quadrupole mass analyzer [13]*

The principle is that the two positive direct voltage (+U) and the other two negative direct voltage (-U) are applied on two opposite metal rods. High frequency alternating voltage with the course of $V = V_0 \cdot \cos \omega t$ is put on all rods. Hence, the potential is made up of two components: voltage of direct current (DC) and radio frequency voltage. The alternating voltage has a higher amplitude $V_0$ than the positive or negative direct voltage of each rod pair, $V_0 > |U|$. The direct axis between four rods is kept at zero potential and along its direction are brought accelerated ions from the ion source [1,2,6].

The used ratio of voltage allows the ions to reach the detector with a specific range *m/z* because they will have a stable trajectory for the defined ratio of RF voltage and DC voltage. The remaining ions of different *m/z* have unstable trajectories and will be neutralized by collision with the rods. Hence, Q functions like a mass selective filter [1,2,6].

### 2.1.2.2. Triple quadrupole analyzer

The triple quadrupole analyzer (QqQ) consists of three quadrupoles in sequence. In *Figure 10*, the scheme of QqQ is shown. The first quadrupole (Q1) is a mass filter, which allows the transfer of selected ions (selection by defined *m/z*) to the second quadrupole and is run by DC and RF voltages. The second quadrupole (called q, Q2 or CID) is a collision cell, which is filled up by an inert gas like argon, helium, or nitrogen. The collision with gas molecules causes the formation of fragment ions, and these fragments are analyzed using the third quadrupole. Only the RF voltage is applied on Q2. The third quadrupole (Q3) is another mass filter, which carries selected fragmented ions to the mass spectrometer detection system, and is run by DC and RF voltages as Q1 [2,6].

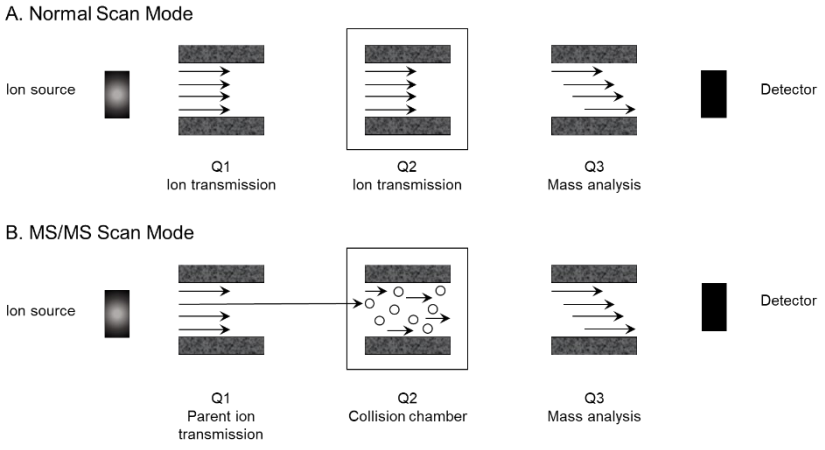



*Figure 10. QqQ scheme [2]*

QqQ allows to work with different scan modes like full scan product ions, precursor ion scan (PIS), neutral loss scan, and selected reaction monitoring (SRM) [2,6].

### 2.1.2.2.1. Full scan of product ions

The full scan product ions (*Figure 10.A*) is a mode, where Q1 let the selected ion with the defined value of *m/z* through, in Q2 this ion goes under fragmentation and Q3 scans all product ions of the given precursor with the defined range of *m/z* [6,14].

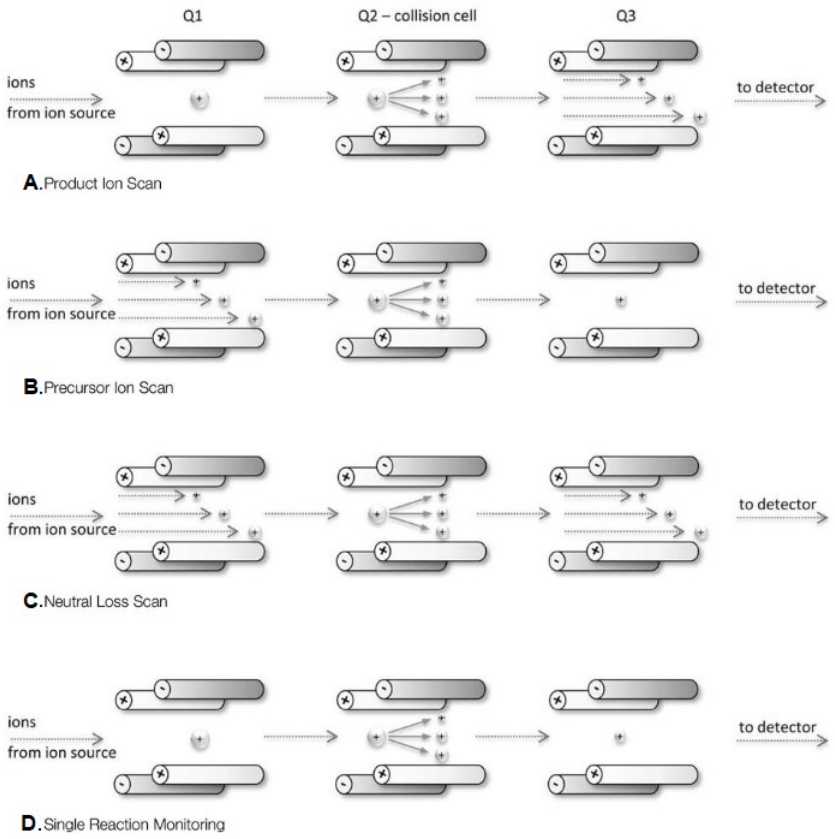



*Figure 11. Scheme of scans modes in QqQ [14]*

### 2.1.2.2.2. Precursor ion scan

The precursor ion scan (PIS) works in reversed mode comparing to the scan of product ions (*Figure 10.B*). Q1 scans the selected *m/z* range, in collision cell the fragmentation of ions happens, which are then brought to Q3. Q3 is set to monitor only selected fragmented ion [6,14].

### 2.1.2.2.3. Neutral loss scan

In this mode, Q1 and Q3 scan simultaneously ions with the same *m/z* difference. This difference shows neutral loss fragment produced in Q2 (*Figure 10.C*) [6,14].

#### 2.1.2.2.4. Selected reaction monitoring

In the selected reaction monitoring, the precursor ion (*Figure 10.D*) is chosen in Q1, which is in Q2 fragmented, and then Q3 selectively detects fragment ion. If two or more fragment ions of precursors are detected, then this mode is called multiple reaction monitoring (MRM) [6,14].

### 2.1.3. Tandem mass spectrometry

In the MS/MS setup, the selected ion is subjected to excitation (most often collisions with an inert gas, or so-called collision gas) in the analyzer or collision cell, where the ions decay into fragment ions, and then MS/MS spectrum is measured [2,15]. The MS/MS spectrum contains only fragment ions formed by the decay of the selected precursor and no impurities, unless the precursor is fragmented by close interference [2,15]. It is achievable by collision-induced dissociation (CID). Voltage difference must be increased in this region to increase ion fragmentation. CID is beneficial because it can present mass spectra with a variable degree of fragmentation from a single analysis [5,16,17].

There are also other possible fragmentation mechanisms (beside collision-induced dissociation), like fragmentation due to photodissociation (PD), fragmentation due to electron capture, and overexcitation during ionization (in MALDI) [2,18]. At the same time, MS/MS can be divided into techniques that allow fragmentation in space (isolation and fragmentation in another place) and time (isolation and fragmentation in one place, being trapped) [2].

MS/MS is often coupled with liquid and gas chromatography due to its high sensitivity, which is now a standard setup in many fields like food analysis, toxins and pesticide analyses and quality control, analyses of small molecules (amino acids, nucleotide, vitamins, *etc*.) [19].

### 2.1.4. Detectors

Detectors in mass spectrometry can be divided into two categories: detectors for direct measurement, detecting electric current occurring by direct impact of ions; multiplier detectors, which use the electron multiplication effect produced after ion impact and provide measurable signals even for single ions [3].

The selection of the detector type depends on the application. Detectors for direct measurement are essential for measuring the precise isotopic abundance of elements in determining the age of rocks. Multiplier detectors are normally used in common commercial instrumentation, including combined systems like GC/MS and LC/MS [3].

## 2.2. Liquid chromatography

Liquid chromatography (LC) is a separation technique conducted between two phases – stationary phase and mobile phase [20-25]. The mobile phase is a liquid, which can be an organic solvent, such as acetonitrile, methanol, or a combination of organic solvents and water depending on the polarity of an analyzed material. The stationary phase is usually the solid compound, which is packed in a column [21-25]. In liquid chromatography, stainless steel columns of a length of 5 – 15 cm and an inner diameter of 2 – 4.6 mm are used filled with particles of 2 – 5 μm in diameter for HPLC and sub-2 μm for UHPLC [20,26]. The columns may be filled with silica gel, alumina, graphitic carbon, or solid polymer, to which various stationary phases may be bonded. It is also possible to use hybrid stationary phases that are the combination of inorganic silica gel and organic organosilane, which surface is modified with trifunctional silanes. These phases combine the advantages of silica gel and polymer to provide mechanical, chemical, and thermal stability [20,21,23].

The mobile phase is pumped by a high-pressure pump [24,25]. Depending on the dimensions of the column, stationary phase particle size, mobile phase composition, and flow rate, it is possible to achieve operating pressures of approximately 1 000 bar [20]. A liquid chromatograph most often includes a six-way metering valve, which allows the manual or automatic injection of the sample to be analyzed in the mobile phase flow [20,23].

At the end of the chromatographic system, a detector is placed, which detects, based on physicochemical principles, the separated substances flowing out of the chromatographic column. The most commonly used detector is the spectrophotometric detector, which provides high sensitivity for routine analysis. The refractive index (RI) detector is the ideal choice for the analysis of substances with limited or no UV absorption. On the other hand, MS can be used as a detection method in LC instead of traditional detectors. LC/MS offers several advantages over traditional detection methods, such as increased sensitivity, selectivity, and specificity, and the ability to identify and quantify a wide range of compounds, including those that are not amenable to UV-Vis or fluorescence detection [24,25].

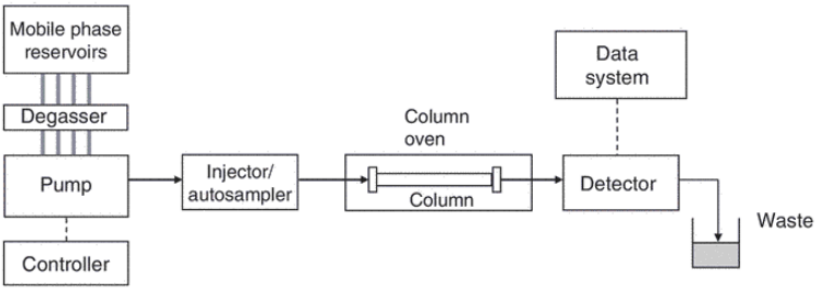



*Figure 12. Scheme of HPLC [21]*

*Figure 13. 1290 Infinity II LC System by Agilent [27]*

The current trend in HPLC (*Figure 12* and *Figure 13*) is towards miniaturization and shrinking column sizes and particle diameters. If the particle size in the column is less than 2 μm, it is ultrahigh-performance liquid chromatography (UHPLC), which operates at higher pressures, around 120 MPa, than conventional HPLC (40 MPa) and provides greater resolution, sensitivity, and speed of analysis [20,21, 26].

## 2.3. Coupling of liquid chromatography with mass spectrometry

The advantage of combining high-performance liquid chromatography and mass spectrometry is to allow the separation and identification of compounds in a single analysis, as it is shown in *Figure 14* [20-23]. “In contrast to GC/MS, which is limited to the analysis of volatile and thermally stable analytes, LC/MS is amenable to a wide range of involatile compounds including those with high molecular weights and polarities, ions, proteins, drugs, natural products, and biomolecules” [21].

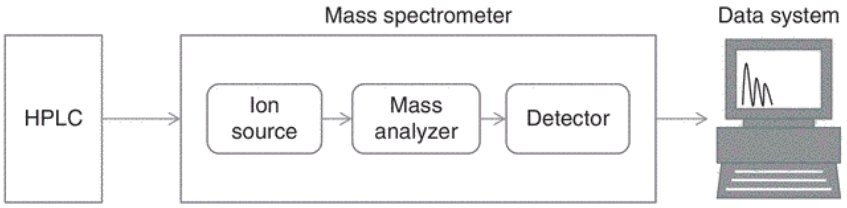



*Figure 14. Scheme of HPLC/MS [21]*

Combining LC with EI is technically much more demanding compared to GC/MS, instead of 1 mL/min of carrier gas (for GC/MS), 1 mL/min of liquid (for HPLC/MS) has to be split before entering to the mass analyzer. Ionization techniques operate at atmospheric pressure (ESI, APCI, and APPI) and the mobile phase directly participates in the ionization process. Nowadays LC/MS coupling is a routine matter. In *Figure 15* is shown the range applicable to LC analysis. The most used ion source in system LC/MS is ESI (80 – 90 % of LC/MS applications) [2,21].

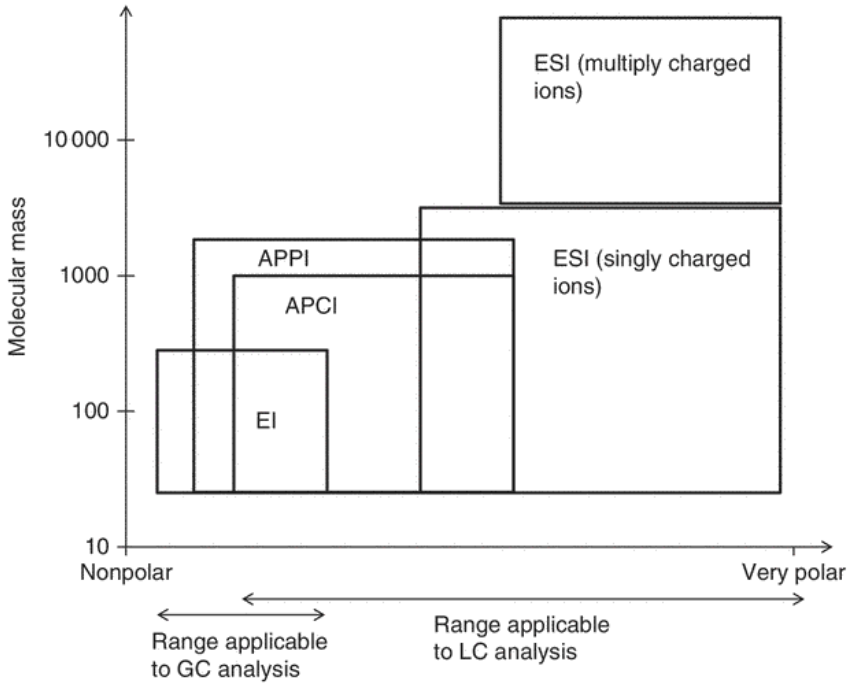



*Figure 15. Common ionization techniques and application areas in MS. Ranges applicable to GC and LC analyses [21]*

Libraries for LC/MS spectra are orders of magnitude smaller than those for GC/MS, spectra differ significantly depending on the used ionization technique, the operating conditions and the type of instrument (except EI) [22,23]. Spectra often must be interpreted manually. Libraries are not as extensive as EI with the exception of library for specific cases, such as proteomic libraries, laboratory libraries for a limited range of substances (e.g., groups of banned drugs, pesticides, or similarly defined group of known target analytes) [2].

HPLC allows the separation of complex mixtures using high-pressure pumps, hence, allows faster separation and higher resolution, identification of isomers, trace impurities, *etc*. Mass spectra of the HPLC eluate are measured at a certain frequency (rate), depending on the used analyzer (its scan rate), peak width, quality of separation, analysis requirements, e.g., additives, and others. Sensitivity of MS is affected by *m/z* range (or only by a certain interval) and the speed of spectrum acquisition [22,23]. After analysis, it is possible to recall the spectrum in a certain time, spectra can be averaged in the certain time interval (peak integration), and it is possible to recall a record signal intensity of certain *m/z* over time (reconstructed ion chromatogram) [2].

The HPLC conditions must guarantee good separation of compounds and at the same time not negatively affect ionization, and thus the sensitivity of detection; a suitable compromise is usually sought. Separation temperature has no effect on MS spectra [2,22,23]. However, in LC, generally, increasing the temperature can decrease the retention time of analytes, resulting in faster elution and reduced separation [28]. This effect is more pronounced for nonpolar compounds, which tend to have weaker interactions with the stationary phase and are more sensitive to changes in temperature. For polar compounds, it is vice versa – decreasing the temperature can increase the retention time of analytes, resulting in slower elution and improved separation [28]. Higher temperature is used for larger molecules to achieve better sorption and shorter retention times [29]. The mobile phase flow rate does not directly affect the signal quality of MS, but it is necessary to change the flow rates of drying and nebulizing gases, or use a flow divider. The composition of the mobile phase is crucial for the quality of the spectra. The mobile phase or rather solvents must dissolve the analyte and additives, easy to get nebulized, evaporate, allow ionization of the analyte, and must not provide intense background ions and contaminate the ion source. A chosen type of sorbent must not affect the MS spectrum. The column parameters (length, width, and sorbent size) affect only the value of the flow rate of mobile phase and therefore the flow rates of drying and nebulizing gases [2].

All HPLC systems (reversed phase (RP-HPLC), normal phase (NP-HPLC), and hydrophilic interaction liquid chromatography (HILIC)) are compatible with API techniques. The most common solvents are methanol, acetonitrile, ethanol and 2-propanol for RP-HPLC. NP-HPLC works much better with APCI or APPI than RP-HPLC that is used with ESI [2,23].

## 2.4. Lipids

Lipids are biomolecules that play important roles in the human and animal body [30,31]. Due to the numerous variations of lipids, they were divided into eight categories (according to the LIPID MAPS project): fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterols (ST), prenols (PR), saccharolipids (SL), and polyketides (PK). Each of these categories has classes and subclasses. For example, sterols, sterol esters, and steroids belong to ST, or monoacylglycerols (MG), diacylglycerols (DG) are subclasses of the GL [30,31].

Lipids are mostly hydrophobic nonpolar compounds because of the fatty acyls in their structures [31-33]. Fatty acids are separated into saturated (including only single bonds) and unsaturated (including one or more double bonds). Apart from FA, which has only fatty acyls and other functional groups, most ST and PR have a ring structure. Another example is in *Figure 16* showing a phospholipid and its moieties and how it can be bonded to other phospholipids creating a cell membrane (the lipid layer or phospholipid bilayer). In the image, it is also shown that those lipids are hydrophilic (thanks to phosphate group that is bonded to glycerol) and hydrophobic (thanks to the fatty acyl chain). These types of molecules are called amphipathic [30,34]. Based on these examples, it is coherent that lipid structures are diverse [31,32].

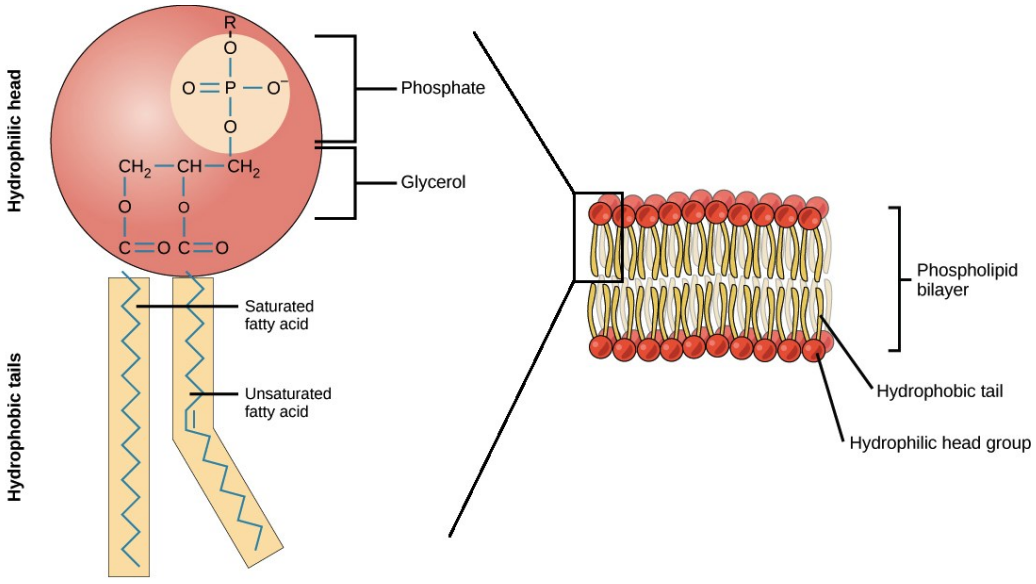



*Figure 16. Structures of phospholipids [34]*

Lipids are involved in cellular functions, such as building blocks of cellular and subcellular membranes (glycerophospholipids, sphingolipids, and cholesterol), signaling

molecules (glycerolipids and sphingolipids) and directing readout of cellular metabolic status [31,33,35]. They also participate in energy conversion, substance transport, cell growth, and apoptosis. When there is a disruption in lipid metabolism, resulting in the accumulation of lipids or altered levels of saturated and unsaturated fatty acids, it can contribute to the development of diabetes, obesity, Alzheimer's disease, tumorigenesis, and tumor cell metastatic capacity [35-38]. Now it is known that some lipids can be a tumor biomarker, which is a great opportunity for lipidomic analyses to identify tumors or cancer in the early stages according to the level of certain lipids [35-37].

### 2.4.1. Classification of lipids

The classification of lipids is based on physico-chemical properties at room temperature, their polarity, and their essentiality for humans [39]. The aim of establishing a unified language for the discussion of lipids across borders led to the development of a comprehensive lipid classification system. The International Committee on Lipid Classification and Nomenclature, in partnership with the LIPID MAPS consortium, created the system in 2005, based on established chemical and biochemical principles, with an ontology that is adaptable, pliable, and expandable [30]. This classification system is compatible with the requirements of the information science to handle the huge amount of data generated by the lipidomics community. The comprehensive classification system organizes lipids into eight well-defined categories that are mentioned in the previous paragraphs [30, 39-41].

The lipid categories include prokaryotic and eukaryotic sources of lipids. The system is also available online at the LIPID MAPS website (https://www.lipidmaps.org) and records more than 45,000 lipids [30].

#### 2.4.1.1. Fatty acyls

Fatty acyls (FAs) are a large category of lipids with over 10500 structures contained in the LIPID MAPS structure database [30]. They include several subgroups, such as fatty acids and conjugates; octadecanoids and their subclasses such as jasmonic acids; eicosanoids – prostaglandins, leukotrienes, lipoxins, *etc*; docosanoids and their subclasses such as neuroprostanes, neurofuranes; fatty alcohols; fatty aldehydes; fatty ethers; hydrocarbons and fatty acid derivatives such as esters, amides, nitriles and glycosides (*Figure 17*) [30,41].

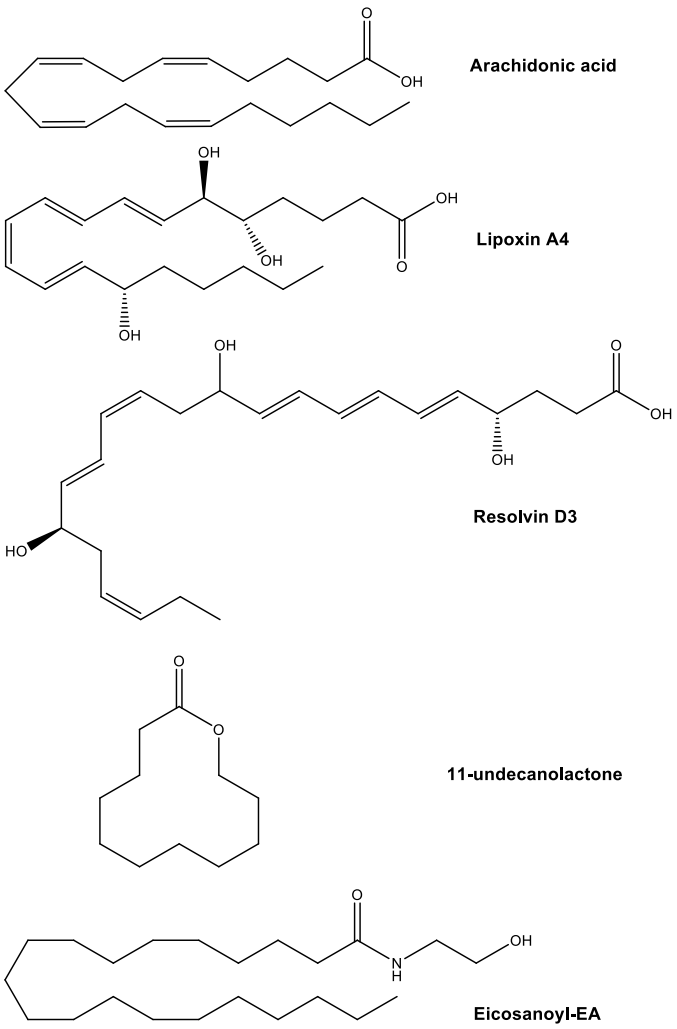



*Figure 17. Structures of different FA [30]*

The most common subgroup of fatty acyls are fatty acids and their conjugates. A common fatty acid consists of a long hydrocarbon chain that is terminated by a carboxyl group [33]. The classification of fatty acids is made with respect to the presence of branching or functional groups in the hydrocarbon chain or the degree of saturation [40,41]. They are also divided based on the length of the carbon chain into short-, medium- and long-chain fatty acids (SCFAs, MCFAs and LCFAs) [30,40]. In animals and humans, SCFAs are produced in the guts by microbiota that are influenced by fermentable carbohydrates (fruits, vegetables, bread, milk, *etc*.) [33,42]. Other important roles of SCFAs and MCFAs have yet to be discovered; however, as far as it is explored, MCFAs have antineoplastic properties and can induce apoptosis [33,43,44].

The biosynthesis of fatty acids is largely similar in plants and animals. Both organisms can produce fatty acids from acetyl coenzyme A (acetyl CoA) through the joint action of acetyl CoA carboxylase and fatty acid synthase[III]. The series of reactions culminates in the formation

of palmitic and stearic acids, which are further elongated and desaturated to form other fatty acids [39,41].

The fatty acyl group in the fatty acid and conjugate classes is characterized by a repeating series of methylene groups that give this category of lipids a hydrophobic character [40,41].

### 2.4.1.2. Glycerolipids

The LIPID MAPS structural database contains information on over 7500 glycerolipid structures [30]. The basic building unit of glycerolipids is glycerol, to which they attach one, two, or three fatty acids by ester linkage (mono-, di- and trisubstituted glycerols). In terms of the number of fatty acyls attached, the subcategories of glycerolipids are termed monoradenylglycerols, such as monoacylglycerols, monoalkylglycerols and mono-(1Z-alkenyl)-glycerols; diradylglycerols, such as diacylglycerols, dialkylglycerols, *etc*.; and triradylglycerols (TG), such as triacylglycerols, alkyldiacylglycerols, *etc*., which are the most important due to their presence in many human tissues and cells. MGs and DGs are also present in human tissues, and they are intermediates in the biosynthesis and digestion of TGs (by esterification). They most likely interact with various organic compound that can lead to different reactions to produce new products (derivatization) [6,30,41].

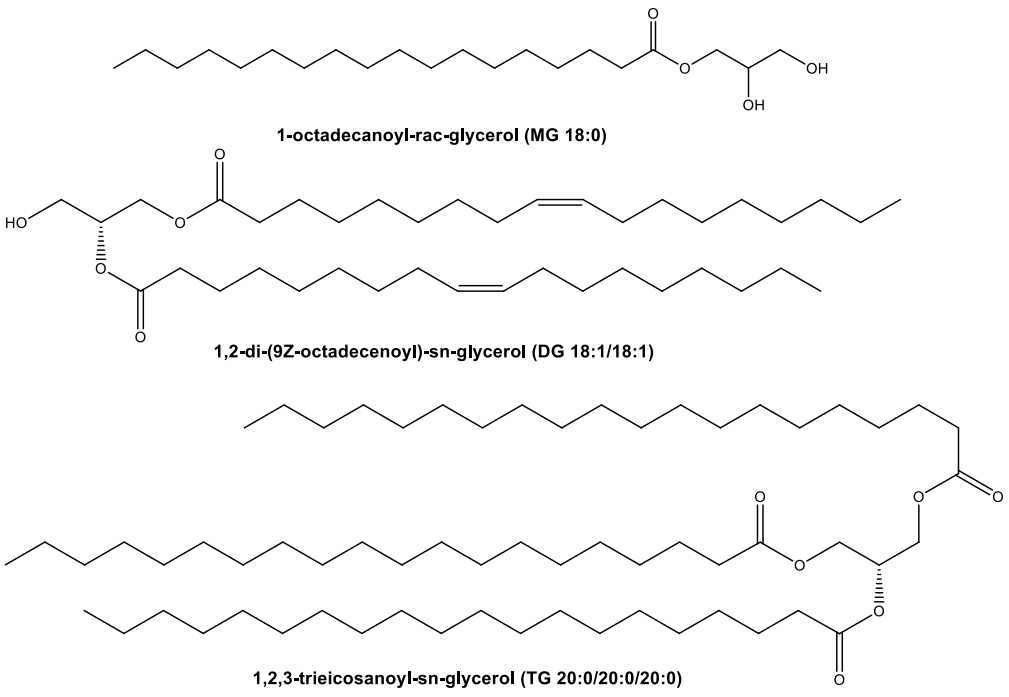



*Figure 18. Structures of different GL [30]*

In the nomenclature system, glycerolipids are labelled accordingly to their fatty acyl moiety. The backbone is glycerol (*Figure 19*); therefore, mono-, di- and triacylglycerols. Hence, they are coded by the ratio of the number of carbons in the FA moiety and the number of double bonds (*Figure 18*), for example, MG 18:0 means 18 carbons (octadecyl) and no double bonds. DGs and TGs can vary; for example, DG 18:1/18:1 and DG 36:2 are the same molecule but in the first abbreviation it is more detailed, and the second one informs about the total amount of carbons and double bonds. However, DG 36:2 does not necessarily mean DG 18:1/18:1, but also DG 14:0/22:2, DG 14:1/22:1, DG 16:0/20:2, *etc*. The same principle applies to TGs with the small difference in the substitution of the hydroxyl group, instead of two codes, they have three codes (TG 20:0/20:0/20:0). This system applies to glycerophospholipids and sphingolipids [30,31,45].

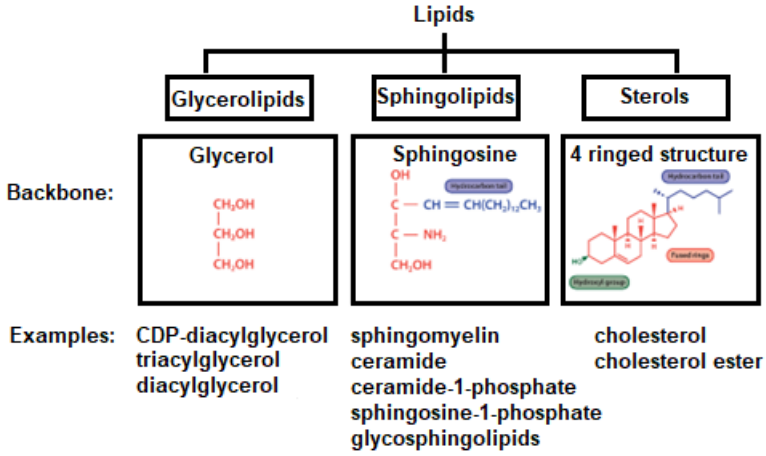



*Figure 19. Backbones of three lipid classes [45]*

Glycerolipids are a large class of biomolecules necessary for the formation of membranes, energy storage, and various vital intracellular processes [41,47,48]. To maintain energy homeostasis, it is necessary to synthesize and store glycerolipids. TGs are the main source of energy that is stored in adipose tissue and energy can be reserved for a long time, during which animals and humans can withstand food deprivation. The uptake of lipids in the diet occurs in the small intestine [6,46-48]. In the body, glycerolipids play an important role in fatty acid catabolism. TGs are hydrolyzed to free fatty acids (FFAs) and DGs by triacylglycerol lipases. By diacylglycerol lipase, DGs are further hydrolyzed to FFA and MGs that are in the final step hydrolyzed to FFA and glycerol by monoacylglycerol lipase [48]. The synthesis of these lipids are much complicated involving various enzymes and biotransformation. There is some evidence of growing cancer cells thanks to enzymes that participate in monoacylglycerol

synthesis and transformation, however, it is not yet clearly known why it is happening. Hence, these lipids, their derivates and enzymes can be targeted for cancer therapy [49, 50].

### 2.4.1.5. Prenol lipids

Prenol lipids are a class of compounds that are derived from the mevalonic acid pathway (specifically from mevalonate), which is a metabolic pathway that produces dimethylallyl diphosphates and isopentenyl diphosphates [31,51-53]. The mevalonic acid pathway is responsible for the biosynthesis of a wide range of important biomolecules, including cholesterol, steroids, and isoprenoids [31,51,53]. In LIPID MAPS database, there are more than 2400 PR and more than 3500 ST [30]. "Prenol lipids have the general structure H–[$CH_2CCH_3$=$CHCH_2$]n–OH, where the repeating unit $C_5H_8$ is called isoprene, from which the name of the group, isoprenoids, is derived (IUPAC-IUB, 1987a)" [51]. The simplest isoprenoids have the small chain (up to n = 4) and have their own specific names signed to the number of chains, however, long-chain isoprenoid alcohols are called terpenols, terpenoids, or polyterpenes. Prenols are also important components of essential oils, which are used in a variety of applications, including aromatherapy, cosmetics, and natural medicine [31,51].

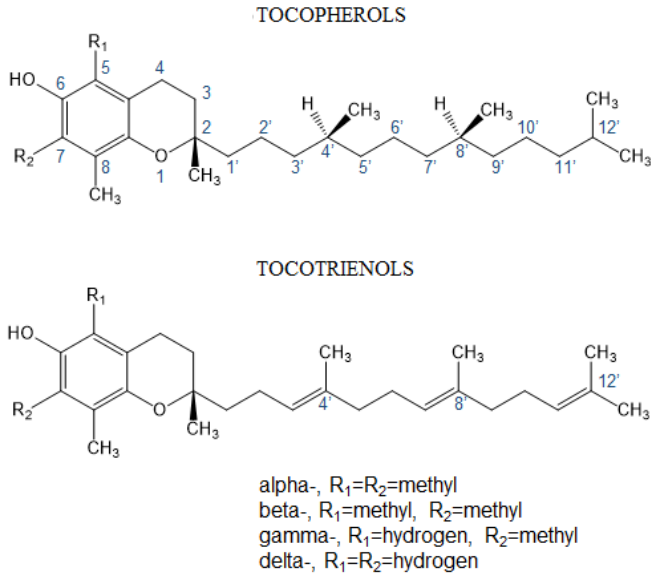



*Figure 20. Structures of tocopherols and tocotrienols (vitamin E) [51]*

Isoprenoids are the simplest form of prenols and are synthesized from the five-carbon isoprene unit. They are involved in a variety of cellular functions, including electron transport and membrane structure [51,53]. Vitamin A is a type of prenol, specifically a retinoid (cartenoids), and is derived from beta-carotene or other provitamin A carotenoids found in plant-based foods. It is a fat-soluble vitamin that plays an essential role in vision, immune

function, and cellular differentiation [51,54]. Another examples of carotenoids are vitamin E (tocopherols/tocotrienols (*Figure 20*)), vitamin K, and the phytol tails of ubiquinones like co-enzyme Q10, chlorophylls, and gibberellin [51].

Vitamin K is required for the formation of blood clots and bone metabolism. Vitamin K is found in a variety of foods, including leafy greens, and it can also be synthesized by the gut microbiota. Another important prenol is coenzyme Q10 (CoQ10), which is a crucial constituent of the electron transport chain in mitochondria. CoQ10 is a powerful antioxidant that helps to protect cells from oxidative damage, and it has been shown to have a number of potential health benefits, including improved cardiovascular health and increased energy production [51,53].

In conclusion, prenols are a diverse class of compounds that are involved in a wide range of important biological processes. They are important components of many plant and animal products, and they have a variety of potential health benefits. Further research on prenols and their biological activities is likely to lead to the development of new therapies and products for a variety of human ailments.

#### 2.4.1.6. Sterol lipids

Sterols are a class of lipids that are characterized by their fused ring structure, which is composed of four carbon rings [53,55-57]. They synthesized by the same biological pathway as prenols.[52] The most well-known sterol is cholesterol, which is found in high levels in animal cells and is an important component of cell membranes. Sterols play a variety of important biological roles, including serving as structural components of cell membranes, acting as precursors for the synthesis of hormones, and playing a role in the regulation of lipid metabolism. They are found in both plant and animal cells, and are essential for the growth and development of many organisms [53,55-57].

Cholesterol plays a crucial role in animal cells by regulating membrane fluidity and permeability, and also contributing to the synthesis of hormones like estrogen, testosterone, and cortisol. Cholesterol levels in the body are tightly regulated, and both high and low levels of cholesterol can have negative health consequences [53,56,57].

In plants, the most common sterol is sitosterol, which is involved in maintaining the integrity and stability of cell membranes. Plant sterols are also believed to have potential health benefits, including the ability to lower cholesterol levels and reduce the risk of heart disease [58].

Sterols have also been shown to have potential therapeutic applications in the treatment of various diseases, including cancer and Alzheimer's disease. Research has shown that certain sterols can inhibit the growth and proliferation of cancer cells, while others can help to prevent the formation of beta-amyloid plaques in the brain, which are a hallmark of Alzheimer's disease [59,60].

In conclusion, further research on the biological functions of sterols and their potential therapeutic applications is likely to lead to the development of new treatments for a variety of diseases.

## 2.5. Lipidomics

Lipidomics is a field of study that focuses on the identification and quantification of lipids, as well as their structural and functional roles in biological systems. It involves the comprehensive analysis of lipid species and their interactions with other molecules in complex biological matrices, such as cells, tissues, and biofluids [61-64]. The one of the first idea of lipidomics was proposed by Han and Gross in 2003. They used the ESI-MS technique to study lipids from biological samples on a large scale and at the system level [64-67]. Lipidomics has been made possible by recent advances in mass spectrometry and liquid chromatography technology, which enable the identification and quantification of hundreds or thousands of lipid species from a single sample. Lipidomics approaches are applied to a wide range of biological samples, including blood, tissues, and cell cultures, and provide insights into various biological processes, such as membrane dynamics, energy storage, and signaling pathways [61-63]. The lipidomic analysis uses three main approaches: chromatography coupled to MS, direct infusion (shotgun), and desorption ionization techniques MS [64-67].

There are many issues that can occur during the lipid analysis. The main problem is usually in the sample preparation. Robust, reproducible and rapid sample preparation protocols are essential for comprehensive, reliable and quantitative lipid analysis in biological samples. Great care must be taken when collecting samples, their preservation and extraction caution. These processes should always be repeated identically between samples and studies in order to obtain the highest reproducibility and data value. Careful consideration should be given to the appropriate selection of solvents, reagents, number of samples and other parameters. Proper lipidomic analysis involves the use of internal standards (IS) that are added to the sample prior to extraction in order to facilitate monitoring of yields and absolute quantification. It is best to use freshly collected samples, however, it is usually very difficult, e.g., taking blood samples

in a hospital and performing lipidomic analyzes elsewhere. Therefore, extracted liquid samples are stored at -80 °C prior to analysis to prevent their decomposition and to delay lipid oxidation [68,69].

TLC or GC was used for lipid analysis for a long time. However, it is now preferred to use either shotgun mass spectrometry or LC/MS techniques, such as ultrahigh-performance liquid chromatography (UHPLC) or ultrahigh-performance supercritical fluid chromatography (UHPSFC). The SFC has been used since the 1980s, and it is known for its physico-chemical properties (diffusion and viscosity comparable to gases, density to liquids). These properties allow for the use of high flow rates and good solubility [66,67]. In modern lipidomics various chromatography coupled with MS (or only shotguns) is used to separate and identify different lipids isomers and isobars. However, LC-ESI/MS is by far the most commonly used analytical technique due to its sensitivity, precision, low sample consumption, and good compatibility [65]. Therefore, cellular metabolism can be studied by analyzing the level of individual lipid classes, subclasses, and molecular species in the human body, which leads to the possibility of identifying diseases in the early stages [67].

The popularity of using liquid chromatography as a separation technique in modern lipidomics is explained by almost universal usability, high selectivity and a wide range of commercially available columns for various applications. Regarding columns for lipids identification, the liquid chromatography working in reversed phase using columns from C4 to C30 is preferred, but the most typical is C18. The retention mechanism is based on the interaction of hydrophobic fatty acyl chains with the stationary phase, which can be influenced by the composition of the mobile phase. Usually, the content of organic solvents (e.g., acetonitrile, methanol, or isopropanol) increases during the gradient, while the content of water or aqueous buffers decreases. This setting is most preferred due to interaction of nonpolar lipids with the nonpolar stationary phase and solubility in nonpolar solvents. To facilitate lipids elution, it is necessary to increase the elution power of the mobile phase and thus increase the proportion of organic components with a suitable gradient. Even though RP-LC enables identification of many lipids, this approach requires much more effort at quantitative measurements because it is practically impossible for such a large number of compounds achieve internal standards to coelute with individual analytes. The matrix effect that may vary between different samples, may not be fully compensated. This issue is solved by hydrophilic interaction liquid chromatography, where can be used one or two IS per lipid class, however, it

does not allow identification of lipid isomers. If we compared those two techniques, RP-LC would be more effective than HILIC in identifying a larger number of lipids [64,65,68,69].

LC/MS can not only be used for the identification of lipids, but also for their quantification. Quantification analysis typically requires the comparison to the internal standard with a similar structure to the analyte. Internal standards are added during the sample preparation and analyzed simultaneously with the analyte. It can be also done with external standards by establishing calibration curve at a series of concentrations under identical conditions. Accurate quantification using LC/MS requires meeting specific requirements. One of them is the ionization efficiency of each lipid species. It must be consistent under the experimental conditions, and the fragmentation kinetics of each species are identical when using tandem mass spectrometry techniques, such as selected reaction monitoring or multiple reaction monitoring. To use these modes, it requires information about fragment ions from precursor ions at a specified elution time [70]. Prediction of retention times of lipid species can be based on their carbon number and number of double bonds (DB), with equivalent carbon number (ECN) calculated as ECN = CN–2DB. According to ECN in RP-LC/MS systems, the higher carbon numbers leads to increased retention times and additional double bond leads to faster elution. Some overlap in retention times may occur, particularly for lipid species with many double bonds [71]. RP-LC/MS/MS can be applied to qualification as well. It is a complicated process demanding multiple steps from full scan mode to multiple reaction monitoring mode, however, it is a very sensitive method. After the necessary information is obtained, can be studied fragmentation and retention behavior of many lipids based on used standards [70].

## 2.6. Derivatization

Derivatization is a chemical process that involves modifying the chemical structure of a molecule. This technique is commonly used in analytical chemistry to enhance the sensitivity and selectivity of the analysis or stability of a molecule for detection by various analytical techniques, such as gas chromatography and liquid chromatography coupled with mass spectrometry. Derivatization can be performed on a variety of compounds including amino acids, peptides, nucleotides, carbohydrates, and lipids [72-74].

The process of derivatization involves the addition a functional group or a chemical moiety to the analyte molecule to improve its physico-chemical properties, such as polarity, solubility, and volatility, making it suitable for the analysis by different techniques [75,76]. The

functional group can be an alkyl or an aryl group, a fluorescent or a chromophoric group, or a reactive group, such as an amine or a carboxylic acid. The selection of the functional group depends on the nature of the analyte and the analytical technique used for detection [72,73,75]. It can also improve the stability of the analyte by protecting it from degradation or oxidation [75,76].

Derivatization can be performed in solution or on a solid support. In solution derivatization, the analyte is dissolved in the suitable solvent and the derivatizing reagent is added. The reaction is allowed to proceed for a specific time and under appropriate conditions. The excess reagent is removed by extraction, and the derivative is analyzed by the appropriate analytical technique [76-78].

On the other hand, solid-phase derivatization involves immobilizing the analyte on a solid support, followed by the addition of the derivatizing reagent. The reaction occurs on the surface of the solid support, and the derivative is released by elution or extraction. This technique is often used for high-throughput analysis of multiple samples [78].

### 2.6.1. Pre-column derivatization

Pre-column derivatization is a technique used in analytical chemistry to modify the chemical structure of an analyte before it enters a chromatographic column. This technique involves introducing a derivatizing reagent to the sample before the chromatographic separation step, which reacts with the analyte to form a derivative. The derivatized compound has different physico-chemical properties than the original analyte, making it more amenable to detection and quantification [75,79].

The derivatized compound can be separated by a chromatographic column and detected by a suitable detector, which is more sensitive to the derivative than the original analyte. Pre-column derivatization is typically used for the analysis of small molecules, such as amino acids, peptides, and carbohydrates, which are not easily detected by common detection techniques, such as UV-Vis or fluorescence [80,81].

The choice of derivatizing reagent depends on the nature of the analyte and the analytical technique used for the detection. The reaction conditions such, as temperature, pH, and reagent concentration need to be optimized to ensure efficient derivatization without interfering with the chromatographic separation [75].

Pre-column derivatization offers several advantages in analytical chemistry. It can enhance the detection sensitivity and selectivity of certain analytes, allowing for the detection of low-level analytes that would otherwise go undetected [79]. It can also improve the chromatographic resolution of the analyte by reducing interference from other compounds in the sample. However, it is experimentally demanding and requires a skilled operator (reproducibility of results) [75].

### 2.6.2. Post-column derivatization

The post-column derivatization is sometimes called on-line derivatization, when the reaction is in the flow reactor that is placed after the chromatographic column [79]. Advantages of this method are: the reaction can be non-selective (the reagent can react with several groups of substances), there is no need to look for new conditions for separation, minimization of manual operations, the reaction may not be completely quantitative. However, the method also has several disadvantages, including issues with the dissolution of analytes by the reagent, which can result in the detection of the derivatization agent as a peak, washing out the zone by passing through the reactor, which leads to lower separation efficiency, gradient problem - change of derivatization conditions with time. Therefore, there are a lot of requirements for post-column derivatization in liquid chromatography [75,82].

In lipidomics, pre-column derivatization is more commonly used for the analysis of lipids, as it offers several advantages over post-column derivatization. However, the choice of which approach to use ultimately depends on the specific requirements of the experiment and the desired analytical outcomes [71,83].

### 2.6.3. Derivatization of lipids

Neutral lipids, such as glycerolipids, prenols, and sterols typically have low ionization efficiency in mass spectrometry. Moreover, mixtures in biological samples can make their analysis challenging, as the presence of multiple lipid species can lead to overlapping signals and difficulty in distinguishing between them. As a result, they often require specific sample preparation techniques, such as derivatization or extraction methods, to increase their ionization efficiency and improve their detection by MS. To ensure accurate and reliable results, the derivatization procedure must be optimized to address potential challenges and achieve both quantitative and qualitative analyses [69,83-87].

There are many derivatization techniques that can be used depending on lipid class and species. The typical derivatized functional groups of lipids are carboxyl, hydroxyl, and amino groups [83-87]. There are several derivatization strategies that are applied to MS, GC, or LC and their combination, including silylation [88,89], alkylation [90,91], acetylation [92,93], and esterification [89,92]. These strategies involve the chemical modification of the functional groups to enhance their chromatographic and mass spectrometric properties [89,94].

There are many derivatization methods of GL. From this lipid class are usually analyzed certain lipid species: MG, DG and TG because most of them are present in human body and their synthesis is very important as it has been discussed in the previous chapters. Their analysis can be complicated due to their facile acyl migration. Therefore, one or two hydroxyl groups are substituted by various derivatization agents (benzoyl chloride [89], acetic anhydride [94], 3,5-dintrobenzoylchloride [95], 2,4-diflourophenyl isocyanate [96]), while TGs are often identified among others. However, triacylglycerols as well as MGs and DGs differ structurally, including their position on the glycerol skeleton (regioisomers or enantiomers), which can affect their biological properties [94-98]. Based on their chirality, they can be analyzed using, for example, chiral HPLC/APCI-MS [98]. The identification and separation of *sn* positions in MGs and DGs have become more feasible with the use of the derivatization method and tandem mass spectrometry [89,97].

Sterols are present in human plasma as free and fatty-acyl esterified forms. Cholesterol is highly abundant in the human body, hence, there are many studies on the identification of cholesterol and cholesterol esters. There are some derivatization agents used for cholesterol derivatization, like 4-(dimethylamino)phenyl isocyanate [99], 1-(carboxymethyl)pyridinium chloride hydrazide (Girard's reagent P) [100], even inorganic compounds (KOH followed by Dulbecco's phosphate-buffered saline [101]) can be used for hydrolysis. More importantly, cholesterol biotransforms by enzymes into other sterols like 24(S)-hydroxycholesterol, cholest-4-en-3-one, 3β-hydroxy-5-oxo-5,6-*seco*cholestan-6-al, 7-dehydrocholesterol, and others, which are called oxysterols. They play a crucial role in biological processes. Therefore, sterols and oxysterols can be analyzed simultaneously to provide more information about potential pathogens [99-102]. In some study, it was possible to convert 3β-hydroxy-5-ene (or 3 β hydroxy) to a 3-oxo-4-ene (or a 3-oxo group) to induce ionization using ESI, which is the most used ion source in investigation of derivatized ST [102]. For other research it was necessary to separate or transform esters into original sterols [101]. However, it is difficult to predict the retention behavior due to sterols unique structures: double bond inside the ring, double bond on

acyl chain, no presence of double bonds, oxo group, carboxyl group, one or multiple hydroxyl group on the ring or on the carbon chain, stereoisomers, *etc*. All additional functional group can possibly react with the derivatization agents. Hence, it is important to use standards for the identification and quantification of sterols [99-102].

Prenols play an important role in the human body as well. Typically, they are analyzed by LC/MS. However, not many species can be transformed using the derivatization method. Ubiquinone, such as coenzyme $Q_{10}$, and vitamin K ($K_1$ – phylloquinone) have oxo groups that is challenging to convert to hydroxyl group or to any other group, which would induce ionization [60,103,104]. Vitamin E is the most commonly targeted lipid species in its class for derivatization like oxidation of tocopherols to produce tocopherolquinone by with $FeCl_3$ and 2,2′-bipyridyl [105] or can be used 2-iodoxybenzoic acid in tetrahydrofuran to form 5,6-tocoquinone from δ-tocopherol and to subsequently synthesize phenazine-vitamin E hybrids [106].

To summarize all of these: some highly bioactive lipids are present in low concentrations and are prone to oxidation, resulting in the formation of unstable oxidized lipids. Additionally, sterol lipids have low ionization efficiency in mass spectrometry analysis and cannot be detected in regular lipid extracts. These factors collectively limit the accurate detection and analysis of these lipids [98-102,107]. Hence, the derivatization method should prevent the degradation or further transformation of targeted lipids, should be rapid, efficient, and yield stable products [83].

#### 2.6.3.1. Derivatization of double bond

Lipids contain fatty acyl chains in their structures, which have a long carbon chain (up to 26 carbon atoms) and may have up to six carbon-carbon double bonds (C=C bonds), resulting in the formation of lipid C=C positional and geometrical isomers. Research has shown that the isomeric transformation of lipid C=C bonds plays a vital role in the modified metabolic pathways, lipid interactions with cholesterol, protein binding, and pathologies of numerous diseases [108]. For instance, Xia and colleagues [109] discovered that the ratios of specific lipid C=C positional isomers were significantly different in patients diagnosed with breast cancer and type-2 diabetes. Her research team introduced an online method for determining the location of C=C bonds [110], which involved Paternò-Büchi (PB) reaction, as is the reaction of an excited carbonyl compound with an alkene to form a cyclobutane derivative [111]. They performed derivatization using acetone : water (1:1, v:v) as the reagent for the PB reaction a

co-solvent for nanoelectrospray ionization (nanoESI)-MS/MS. The team was able to identify and quantify carbon double bond location isomers for a range of lipids [110]. Tandem mass spectrometry is necessary to determine the location of double bonds, as it generates specific fragments that provide this information [109-111].

The other favored derivatization method is ozonolysis, which is the reaction of carbon-carbon double or triple bonds with ozone. The reaction generates ozonides, which are unstable and subsequently decompose to produce aldehydes, ketones, and carboxylic acids, depending on the nature of the starting material. Ozonolysis is a useful tool in organic chemistry for identifying the position of double bonds in unsaturated compounds, as well as for functionalizing carbon-carbon double bonds [112]. Harries et al. developed the ozonolysis device, which consisted of a flow cell, where the analyte solution was exposed to a mercury lamp, resulting in the formation of ozonolysis products in the solution. They could identify double bonds position in the targeted lipid species using MS. Furthermore, the platform is both inexpensive and easy to use on any instrument, as it does not need any modifications to the instrument and does not require an ozone generator [113].

An alternative method for determining the location of double bonds, which does not use derivatization, is MS/MS method performing on ultraviolet photodissociation (UVPD). This technique has been used by Blanksby and colleagues to selectively generate radicals using non-covalently attached molecules upon 266 nm UVPD. This method differentiates isomeric lipids by using radical directed dissociation (RDD) reactions [114].

# 3. Experimental part

## 3.1. Chemicals and standards

- Acetonitrile (ACN) – CHROMASLOV™ LC/MS, ≥99.9%, Honeywell (Seelze, Germany)
- Ammonium carbonate – ACS Reagent, ≥ 30.0 % $NH_3$ basis, Honeywell (Seelze, Germany)
- Ammonium formate – for mass spectrometry, ≥99.0%, Sigma-Aldrich (St. Louis, MO, USA)
- 1-Buthanol (BuOH) – CHROMASLOV® Plus, for HPLC, ≥99.7%, Honeywell (Seelze, Germany)
- Chloroform – LiChroslov® Chloroform for liquid chromatography, Merck (Darmstadt, Germany)
- 3-(Chlorosulfonyl)benzoic acid – 95%, Sigma-Aldrich (St. Louis, MO, USA)
- Formic acid – Honeywell (Seelze, Germany)
- Methanol (MeOH) – CHROMASLOV™ LC/MS, ≥99.9%, Honeywell (Seelze, Germany)
- 2-Propanol (IPA) – CHROMASLOV™ LC/MS, ≥99.9%, Honeywell (Seelze, Germany)
- Pyridine – for HPLC, ≥99.9%, Sigma-Aldrich (St. Louis, MO, USA)
- Redistilled water
- Standards and internal standards of lipids – Avanti Polar Lipids (Alabaster, AL, USA), Nu-Chek Prep (Elysian, MN, USA) or Merck (Darmstadt, Germany)

## 3.2. Instruments and apparatus

- Analytical weight *ABT 120-5DM* – Kern & Sohn GmbH (Balingen, Germany)
- Centrifuge *Hettich EBA 20* – Hettich (Tuttlingen, Germany)
- Laminar box *MSC-Advantage II. class* – Thermo Fisher Scientific (Waltham, USA)
- Liquid chromatograph *Agilent 1290 Infinity series* – Agilent Technologies (Waldbronn, Germany)
- Mass spectrometer *QTRAP® 6500* – SCIEX (Framingham, MA, USA)
- Mechanical pipettes with adjustable volume – Sartorius (Goettingen, Germany)

- Pasteur pipettes, glass plugged *Fisherbrand*® – Thermo Fisher Scientific (Bremen, Germany)
- Repetitive pipettes *Multipette*® *M4* – Eppendorf (Hamburg, Germany)
- Sample concentrator *NDK200-2* – MIULAB (Hangzhou, Zhejiang, China)
- Shaker *Lab Dancer* – fixed speed (2800 rpm), IKA® (Staufen, Germany)
- Shaking water bath *WNB/WNE/WPE* – Memmert (Schwabach, Germany)
- Small shaker *KS 130 basic* – IKA® (Staufen, Germany)
- Syringe *Injekt*® – B|Braun (Hessen, Germany)
- Syringe filters *OlimPeak*® – cellulose 0.2 μm, 13 mm, pk/100, Teknokroma® (Barcelona, Spain)
- Ultrasonic bath *Fisherbrand*® *FB15061* – Thermo Fisher Scientific (Waltham, USA)

## 3.3. Annotation and nomenclature of lipids

Lipid annotation and nomenclature was guided by updated guidelines established by the LIPID MAPS®. Individual lipids were annotated to different levels. The first one is "molecular species level", which is applied to all categories based on the identification of alkyl/acyl residues, e.g., DG 18:0_18:0. The second one is "species level", which shows the sum of carbon atoms and double bond equivalents, e.g., DG 36:0. The third is "*sn*-position level", which is applied to GL and GP based on fragments in the MS or MS/MS spectrum, and where the slash indicates the known *sn*-position, e.g., DG 18:0/18:0. For sterols and prenols, "species level" or rather their common name were used [115].

## 3.4. Biological samples

In this thesis, pooled plasma was utilized for optimalization of derivatization and for the lipid identification, which was a mixture of 30 human plasma samples (ages of 40−60 years; body mass index (BMI) of 20−30). This representative sample was already prepared and was used without corrections or alterations throughout the laboratory work. All manipulations with plasma were performed in a laminar box (MSC-Advantage II. class).

## 3.5. Standards and internal standards mixtures

Throughout laboratory works, mixtures of standards (**Supplement A**) and internal standards (**Supplement B**) were prepared.

## 3.6. Sample preparation

### 3.6.1. Protein precipitation

The deproteinization of 10 µL of pooled plasma spiked with 30 µL of internal standard mixture (IS Mix) in a 1.5 mL glass vial was realized using 250 µL of BuOH : MeOH (1:1, v/v) [116]. The samples were then carefully transferred to ultrasonic bath (Fisherbrand® *FB 15061*) for 10 min at 30 °C, and then additional 500 µL of BuOH : MeOH (1:1, v/v) was added to prevent losses on the filter. Subsequently, the samples were centrifuged (Hettich *EBA 20*) at 6000 rpm for 3 min and filtrated with a 0.25 µm filter at ambient temperature. The extracts were evaporated using the sample concentrator (MIULAB *NDK200-2*) for approximately 10 – 20 min. The residues of samples were stored at −80 ˚C in a freezer for next experiments.

### 3.6.2. Derivatization

The residue was redissolved in 250 µL of pyridine (392.8 mg/mL in ACN) and was stirred slowly in the small shaker (320 rpm, IKA® KS 130 basic) for 5 min. Pyridine is essential for derivatization with 3-(chlorosulfonyl)benzoic acid to avoid unwanted hydrolysis and neutralize the formed HCl during the reaction. Afterwards, 250 µL of 3-(chlorosulfonyl)benzoic acid (50 mg/mL in ACN) was added right before placing sample(s) in the shaking water bath with the electronically controlled shaking device (Memmert *WNB/WNE/WPE*). The reaction mixture was stirred (150 strokes per minute) at 60 ˚C temperature for 60 min. Immediately after this, Folch extraction was followed.

### 3.6.3. Folch extraction

The derivatization reaction was terminated by applying organic solvents first and then water. The extraction protocol was a modified Folch extraction [117] that was used to minimize the level of contamination to the mass spectrometer. Water was added to hydrolyze any excessive derivatization reagents. The organic phase, which mainly consisted of lipids and related hydrophobic compounds, was extracted using 2 mL of chloroform, 1 mL of methanol, and 600 µL of redistilled water. The water phase, which was on top of organic phase, was removed. Subsequently, the organic phase was evaporated under a stream of nitrogen in the sample concentrator (MIULAB *NDK200-2*). The residue was redissolved in 250 µL of a $CHCl_3$/MeOH (1:1, v/v) mixture and stirred on vortex (fixed speed 2800 rpm), IKA® *Lab Dancer*. Aliquot of 50 – 100 µL of the sample was transferred to insert in the vial for LC/MS analysis. The lipid derivatives were stored at −80 ˚C in the freezer.

### 3.7. UHPLC conditions

Analyses were performed on a liquid chromatograph *Agilent 1260 Infinity series* (Agilent Technologies, Waldbronn, Germany) with a column Acquity UPLC® BEH C18 of 150 mm × 2.1 mm, 1.7 μm (Waters). The conditions for RP-UHPLC: flow rate of 0.35 mL/min, injection volume of 1 μL, column temperature of 55 °C, and autosampler temperature of 4 °C. The gradient program was the following: 0 min–35% B; 15 min–80% B; 16 – 19 min–90% B; 20 min–35% B, where phase A was ACN/$H_2O$ (6:4, v/v), phase B was IPA/ACN (9:1, v/v), and both phases contained 5 mM ammonium formate and 0.1% formic acid [70,82]. Standard mixtures and plasma samples were replicated three times and injected one time due to the relative standard deviation (RSD) between injections was less than 10 – 15 %.

### 3.8. MS conditions

All experiments were performed using the *QTRAP® 6500* triple quadrupole (QqQ) mass spectrometer (SCIEX, Framingham, MA, USA). The following instrument parameter settings were used: ESI negative ion mode, drying temperature 600 °C, curtain gas pressure in ion source 10 psi, gas pressure 1 (nebulizing gas) in the ion source 70 psi, pressure of gas 2 (heating gas) in ion source 70 psi, *m/z* range 100 – 1000 at a scan rate of 200 Da/s, in MRM mode declustering potential (DP) range 20 – 270 V and collision energy (CE) range 50 – 75 V, exceptionally α-tocotrienol CE 25.9 (**Supplement C**). The optimalization of MS condition and derivatization reaction were performed using MRM mode. MRM mode and PIS were used for identification.

### 3.9. Data processing

All the data were acquired using Analyst Software from SCIEX for LC/MS/MS. Semi-automated identification was first performed by Skyline software and then manually were adjusted peaks area for all detected lipids.

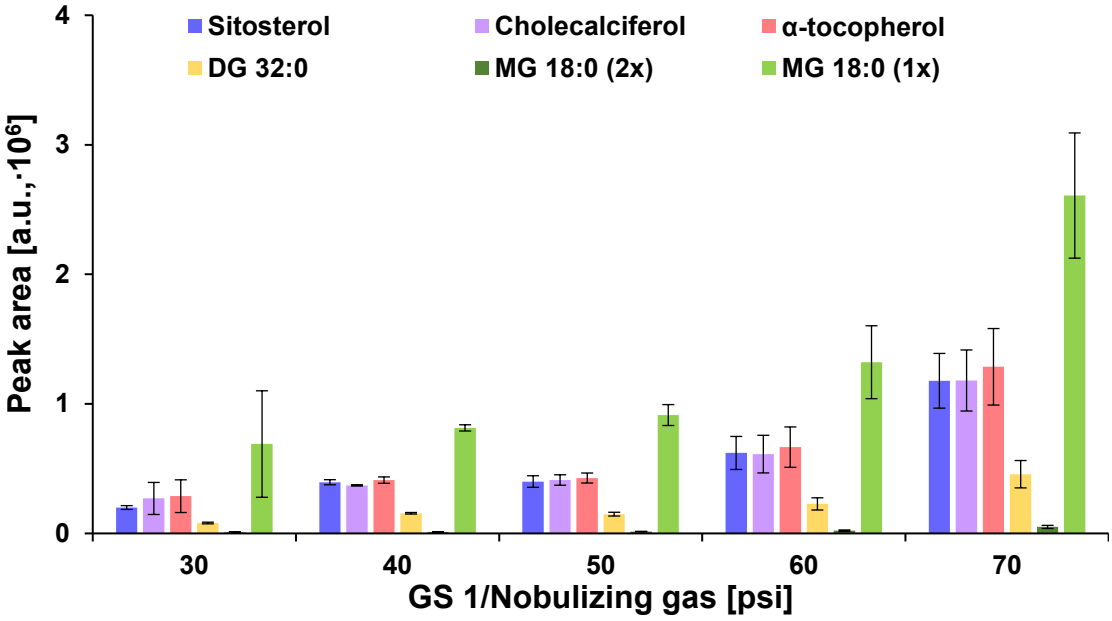
# 4. Results and discussion

As a part of the experimental part of this work, a suitable extraction protocol was first selected, and subsequently, the RP-UHPL/MS/MS and derivatization method were optimized for identification of diacylglycerols, monoacylglycerols, sterols, and prenols in pooled human plasma.

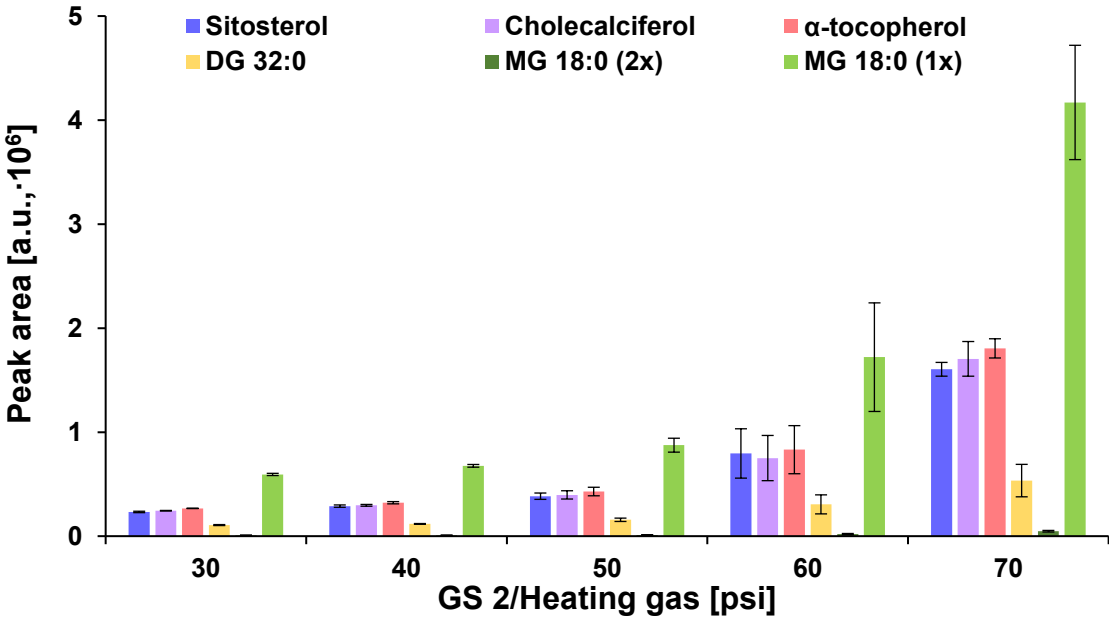
## 4.1. Optimalization of MS conditions

The optimalization of the mass spectrometer was performed using a mixture of standards (**Supplement A**). The starting conditions were suggested by Vaňková et al. [71]. The pressures and temperature for each optimalization are presented in the graphs below (dependency of the peak area). Data are presented as the mean value ± SD from three independent experiments. The graphs were prepared for one lipid from one lipid class, the others follow the same trend assuming that the other species in the corresponding class will behave similarly. Exceptionally, monoacylglycerols had two records due to their structure. They have two unoccupied hydroxyl groups on the glycerol backbone, therefore, derivatizing agent can interact either with one group or with both groups. Hence, in the graphs MG are represented as MG 1x and MG 2x.

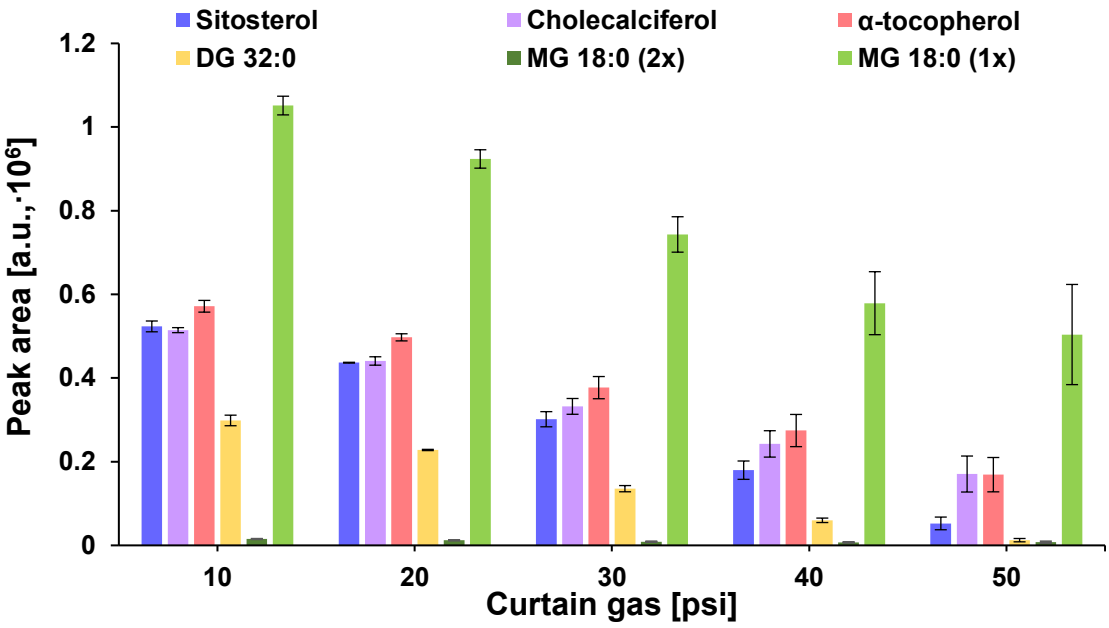
Firstly, the pressure of gas 1 (nebulizing gas) was corrected, where the other conditions were constant: LC flow rate 350 μL/min, pressure of gas 2 pressure (heating gas) 50 psi in ion source, pressure of curtain gas (CUR) 20 psi in ion source, drying temperature 500 °C. Afterwards, the pressure of gas 2 was optimized, where the best option for GS 1 (from the obtained data) was used, but the other conditions were identical as in the previous experiment. These steps were applied for all further experiments in this part of the work. Obtained data from Analyst Software were transferred to Skyline program, where areas peak were calculated. In Microsoft Excel data were evaluated.



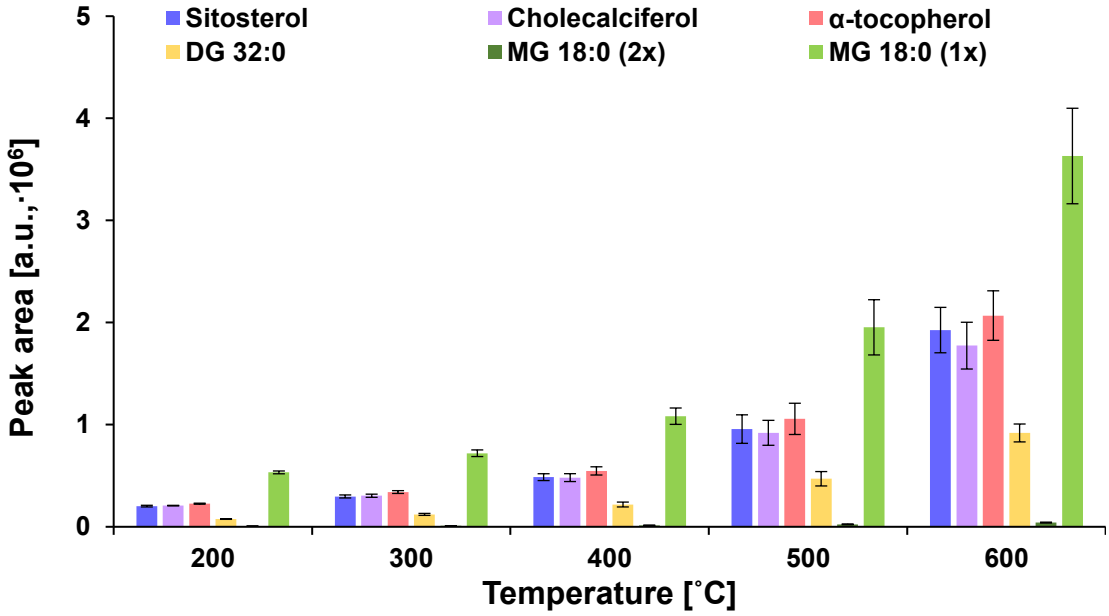

*Graph 1. Optimalization of GS 1*




*Graph 2. Optimalization of GS 2*




*Graph 3. Optimalization of CUR*

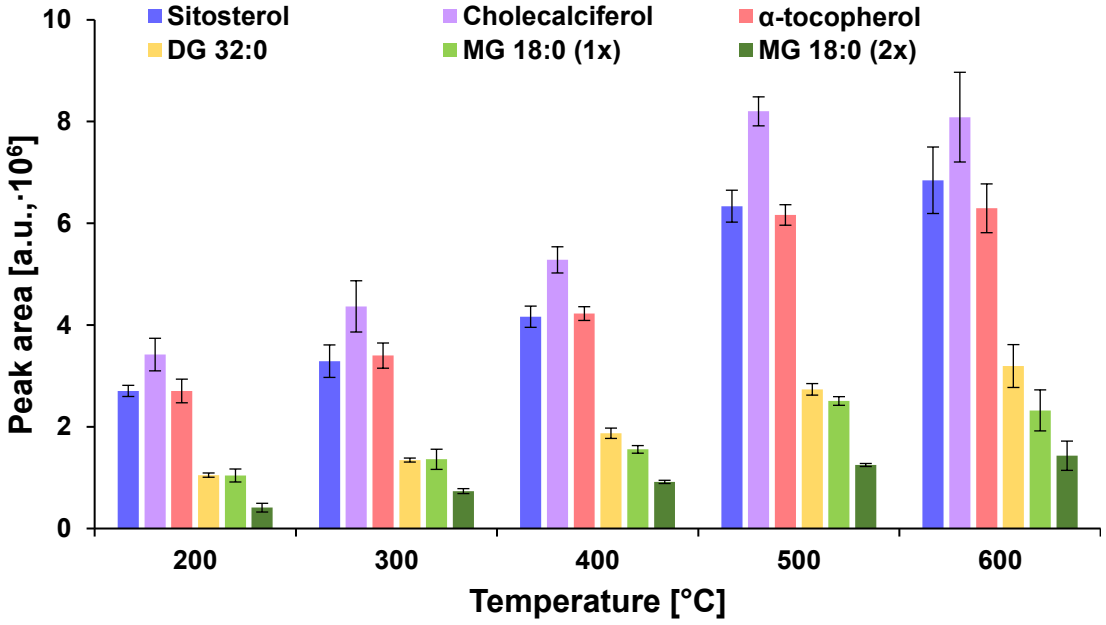



*Graph 4. Optimalization of drying temperature*

The measurement was performed three times for each individual parameter on the same sample containing the derivatized STD Mix (40 µL). Due to continuous work of the mass spectrometer, only two values were taken into consideration for the GS 1 70 psi, GS 2 70 psi, CUR 50 psi, T 600 °C due to measurement's error. The range of optimized values were selected based on the manufacturer's recommendation. Certainly, the best conditions were chosen, where the intensity or, rather, the area of peaks was the highest.

One occurrence happened during the analysis, when temperature was measured (*Graph 5*). The intensity of every lipid species was different, specifically increased. When the experiment was done again, the overall trend stayed the same (as the temperature increases, the intensity also increases). With the thorough investigation, it is unknown why intensity of MGs varies that much. This occurrence appears in other experiments, where I will discuss it in more details.

For all experiments, conditions of MS were used: source temperature 600 °C, curtain gas pressure in ion source 10 psi, gas pressure 1 (nebule gas) in ion source 70 psi, pressure of gas 2 (heating gas) in ion source 70 psi.




*Graph 5. Optimalization of drying temperature*

## 4.2. Optimalization of UHPLC conditions

The optimalization of ultrahigh-performance liquid chromatography was performed on the *Agilent 1290 Infinity series* (Agilent Technologies, Waldbronn, Germany) with the column Acquity UPLC® BEH C18 of 150 mm × 2.1 mm , 1.7 µm (Waters) using derivatized mixture of standards. The starting conditions were again suggested by Vaňková et al. [71]. I kept the flow rate at 0.35 mL/min, the column temperature at 55 °C and mobile phases: phase A – ACN/$H_2O$ (6:4, v/v), phase B – IPA/ACN (9:1, v/v), and both phases had 5 mM ammonium formate and 0.1% of formic acid. The mobile phases and their additives remained the same without any optimalization, since this setting worked for the lipid classes in other works. Thus, the only gradient was optimized with the base gradient shown in the *Table 1*.

*Table 1. Starting gradient parameters*

| t [min] | A [%] | B [%] |
|---|---|---|
| 0 | 65 | 35 |
| 21 | 5 | 95 |
| 23 | 5 | 95 |
| 24 | 65 | 35 |
| 25 | 65 | 35 |

Based on the retention behaviour was determined volume percentage (%) of mobile phase B at 15 min, which was approximately 80 %. The time was chosen after experimenting the starting gradient and the last lipid species (DG 40:0) was eluting before 15 min. Then the gradient was optimized using the obtained data, thus, was used 80 %, 85 %, 90 %, 95 % of mobile phase B. The higher percentage was taken into consideration due to the solubility of nonpolar lipids. The presence of water in the mobile phase can reduce the solubility of these lipids, leading to shorter retention times and poorer separation.

The first option (80 %) showed better separated peaks from each individual peaks, and the analysis time was reduced from 20 min to 16 min (chromatogram in **Supplement D**). The other reason why higher percentage of mobile B was added, was due to elution of nonpolar lipids (triacylglycerols and cholesterol esters) from the column. The final gradient was set:

*Table 2. Final gradient parameters*

| t [min] | A [%] | B [%] |
|---|---|---|
| 0 | 65 | 35 |
| 15 | 20 | 80 |
| 16 | 10 | 90 |
| 19 | 10 | 90 |
| 20 | 65 | 35 |

## 4.3. Optimalization of derivatization method

### 4.3.1. Molar ratio of pyridine and derivatizing agent

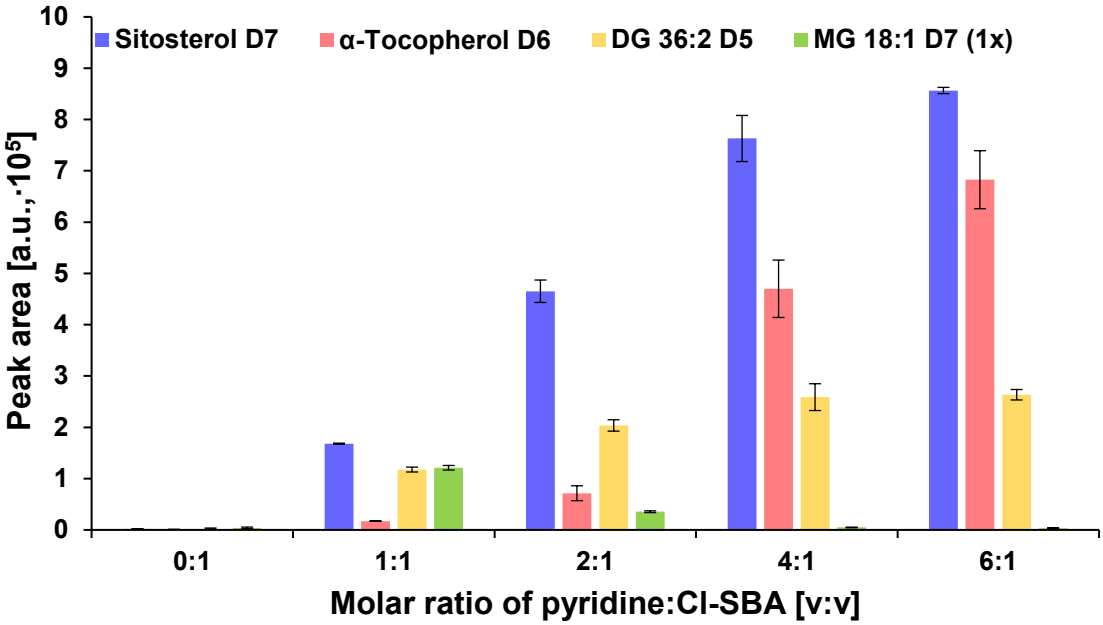
For this parameter, the different molar ratio of pyridine and derivatizing agent was used. The ratio corresponded to 250 µL of pyridine (diluted in ACN) and 250 µL of Cl-SBA at constant concentration 50 mg/mL in ACN and was optimized accordingly: 0:1, 1:1, 2:1, 4:1, 6:1 (*Table 3*).

*Table 3. Chosen molar ratio for optimalization*

| **Molar ratio** | **Volume [µL]** | |
|---|---|---|
| Pyr:Cl-SBA | Pyridine (Pyr:ACN) | Cl-SBA |
| 0:1 | 250 (0:1) | 250 |
| 1:1 | 250 (1:20) | 250 |
| 2:1 | 250 (1:10) | 250 |
| 4:1 | 250 (1:5) | 250 |
| 6:1 | 250 (1:2.5) | 250 |

As shown in the *Graph 6*, there are individual lipid species as representatives of each lipid class (DG, MG, ST, and PR) as it was mentioned before. For diacylglycerols, DG 36:2 D5 or DG 1,3-18:1/18:1 D5 was chosen, for monoacylglycerols – MG 18:1 D7, where MG 1x means derivatization was only on one -OH group, for sterols – sitosterol D7, for prenols – α-tocopherol D6. Graphs of other individual internal standards are in the supplementary part (**Supplement E**).

The targeted lipids (sterols and prenols) show a rising trend: the higher pyridine ratio means the higher area/intensity, unlike monoacylglycerols (1x) that showed the opposite trend. In the supplementary part, there are MG 2x, and they behaved differently from MG 1x, in a way that with the addition of more pyridine, was preferred substitution on both -OH group. And vice versa with less amount of pyridine, the substitution on one -OH group was likely to happen. This assumption is proved in the next optimalization. Diacylglycerols had small differences in changes. Addition to that, the experiment proves that without pyridine as a base it is nearly impossible to derivatize lipids using 3-(chlorosulfonyl)benzoic acid.

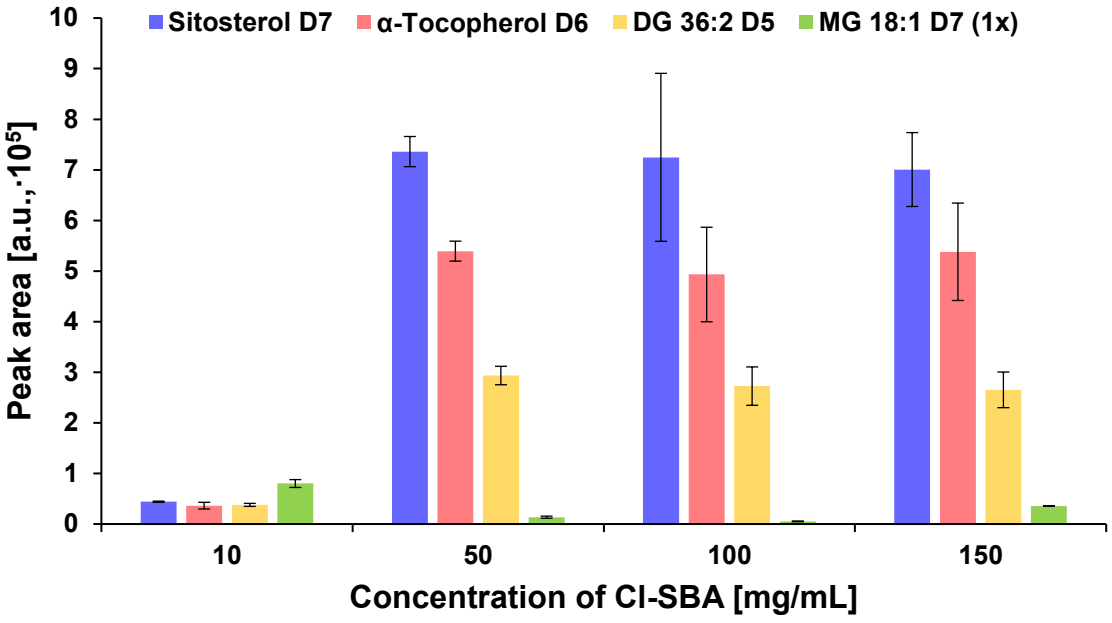



*Graph 6. Optimalization of the molar ratio of reagents*

For all further experiments the molar ratio of pyridine : 3-(chlorosulfonyl)benzoic acid as 6:1was used, in terms of practice, 250 µL of pyridine solution (diluted 2.5 times in acetonitrile) and 250 µL of Cl-SBA (50 mg/mL in acetonitrile) was used, in regards to sterols and prenols. If this thesis work was orientated on MG 1x the optimized parameter would be 1:1 of pyridine : 3-(chlorosulfonyl)benzoic acid (as for pyr:ACN 1:20).

### 4.3.2. Concentration of derivatizing agent

For this parameter different concentrations of derivatizing agent was used. The alternation was made during preparation: 12 samples residue was dissolved in 250 µL of pyridine (392.8 mg/mL in ACN) and then was added 250 µL of Cl-SBA with the concentration: 10, 50, 100, 150 mg/mL in ACN (three samples for each concentration) at ambient temperature and reaction time – 1 hour, followed by Folch extraction.

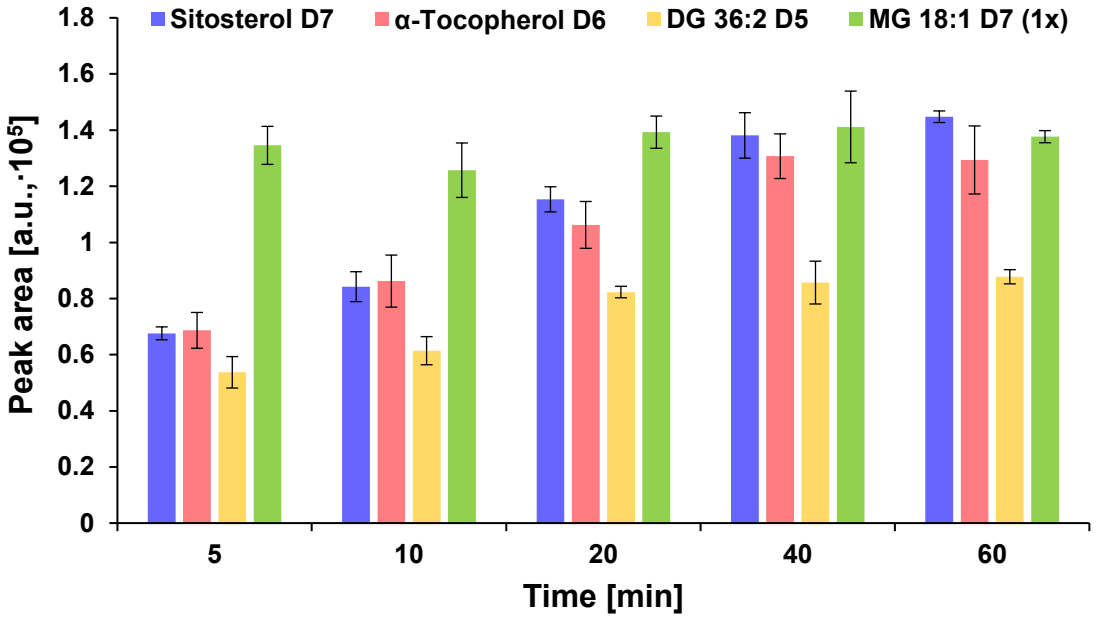



*Graph 7. Optimalization of the concentration of the derivatizing agent*

As it was mentioned in the previous experiment, with the higher amount of pyridine in the reaction, there is the higher chance of full derivatization MG 1x (**Supplement F**). MG 2x shows the rising trend, while MG 1x shows a high peak area/intensity at 10 mg/mL, then it displays a notable decrease over the measured interval between 50 mg/mL and 100 mg/mL. No significant differences were observed in the concentration change in the range of 50 – 150 mg/mL for other lipid classes. Therefore, the optimized condition was 250 µL of 50 mg/mL of 3-(chlorosulfonyl)benzoic acid.

### 4.3.3. Reaction time

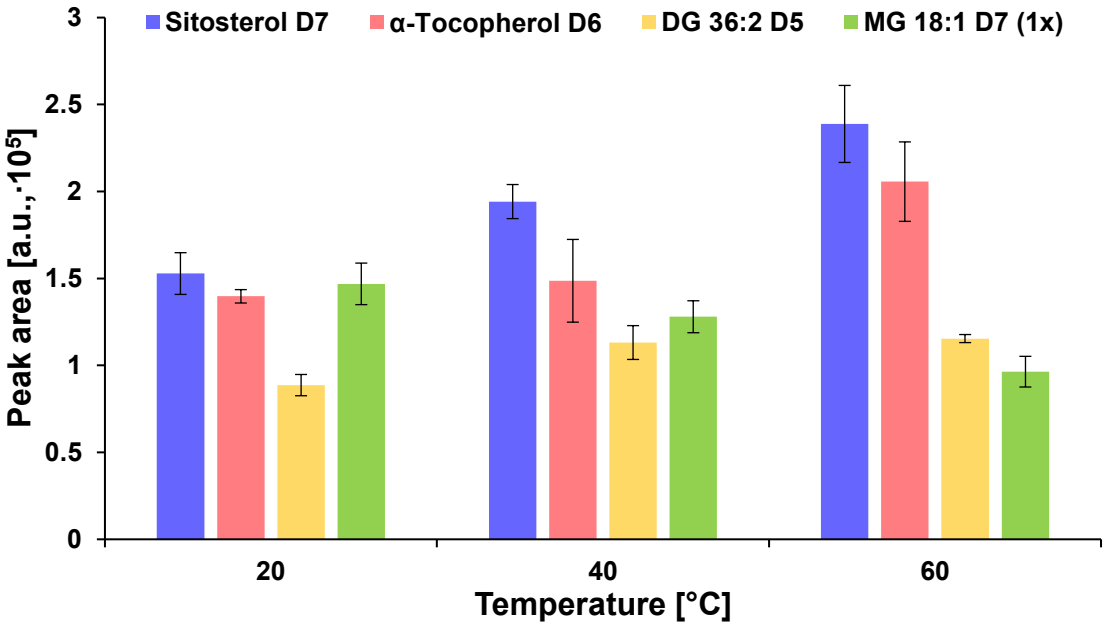
The goal for this parameter was to achieve an optimal time of reaction. The previous steps and optimized parameters were applied to this experiment. The experiment showed the best yield at 60 minutes (*Graph 8* and **Supplement G**) for targeted lipid classes. Here, the problem happened similar to the optimalization of MS. The results, which are shown in the graph below and in *Graph 9*, are like the other ones as before in MS parameters, when the other results of all derivatization optimalizations are related to the *Graph 5*. The only difference that for the MS optimalization was used 40 µL of STD Mix, thus, higher area of peaks. The experiment was done again, and it showed the same yields (the area of peaks was almost identical). MG 1x were still the highest, and the highest area was around $2 \cdot 10^5$. One of the reasons why it can happen is that MG 1x compete with MG 2x and hence shows different intensities/area of peaks. Despite the differences comparing other optimalizations was still considered 60 min as the optimized parameter.




*Graph 8. Optimalization of the reaction time*

### 4.3.4. Reaction temperature

Reaction temperature was the last parameter to optimize. Three different reaction temperatures were selected: 20, 40, and 60 °C. The data showed that the optimal option was the temperature reaction at 60 °C (*Graph 9*). The individual lipid species are shown in **Supplement H**.

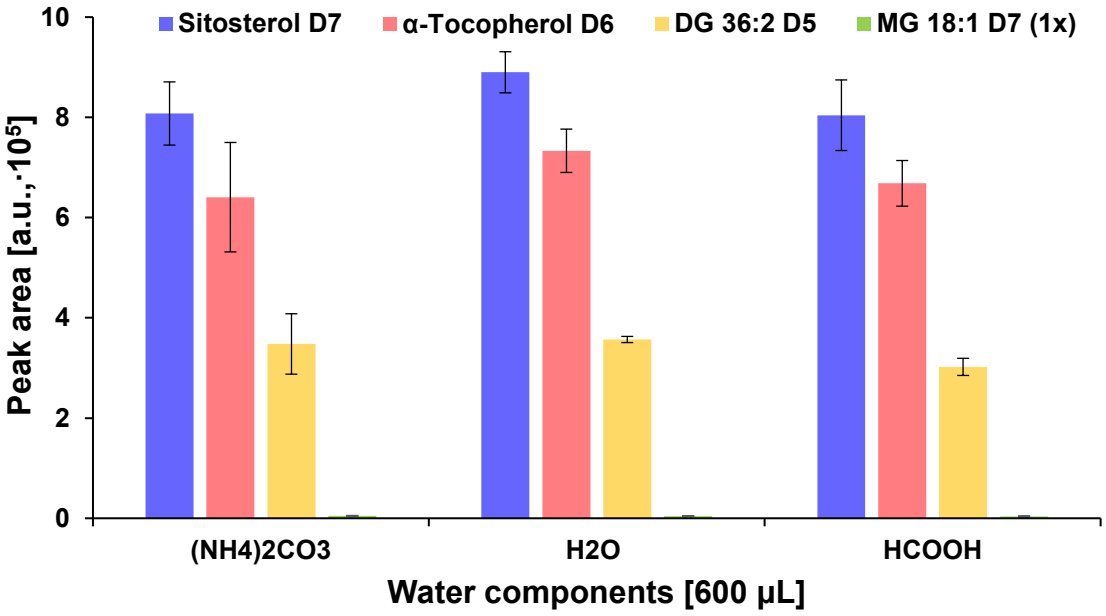



*Graph 9. Optimalization of the reaction temperature*

### 4.4. Optimalization of extraction

The Folch extraction was chosen for this method due to its simplicity, versatility, and high yields of total lipid species. The detailed procedure is described in **3.5.3. Folch extraction**. The starting conditions were: 2 mL of chloroform, 1 mL of methanol, and 600 µL of ammonium carbonate solution (250 mM), which were applied for optimalization of derivatization. To increase the solubility of lipids in the solvent, the alteration of pH of the water phase was suggested, thus, alkaline, neutral, and acidic media were used. For the experiment, 600 µL of 250 mM ammonium carbonate solution was added to three samples, to other three samples – 600 µL of redistilled water and the next three – 600 µL of 0.1 % formic acid. The organic components remained the same for all 9 samples.

No significant differences were observed in the change of pH. However, sterols and α-tocopherol had a very small improvement of area peak in redistilled water. Therefore, was used 600 µL redistilled water for the Folch extraction protocol. The individual results are shown in the **Supplement I**.



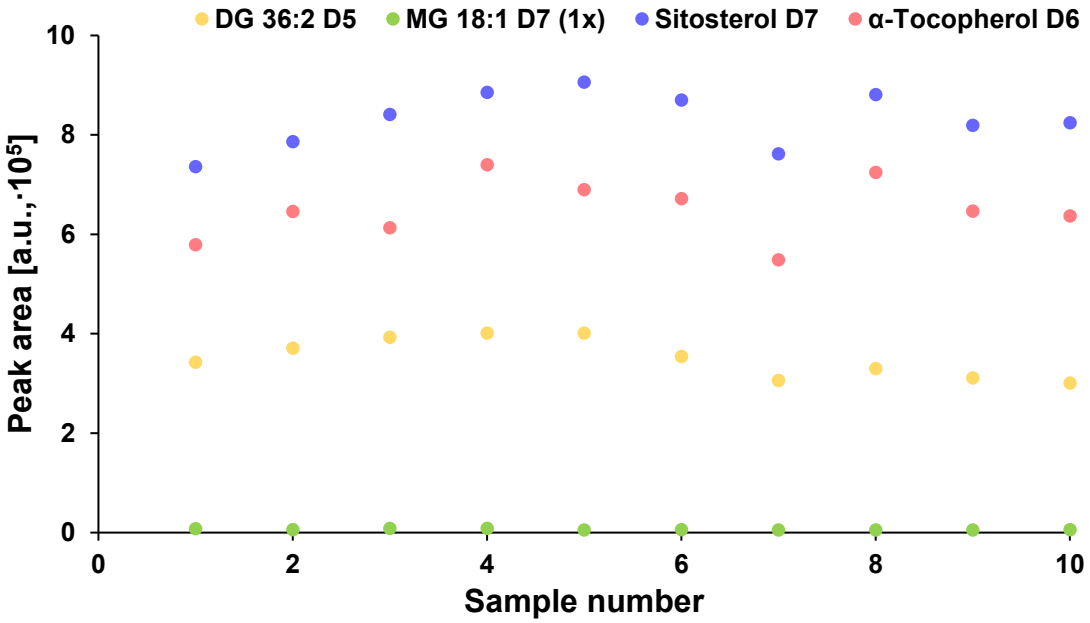

*Graph 10. Optimalization of extraction procedure*

## 4.5. Verification of derivatization method

### 4.5.1. Repeatability

For the repeatability test, I prepared ten independent samples (pooled plasma with spiked IS Mix) using the optimized conditions for the derivatization method and extraction,

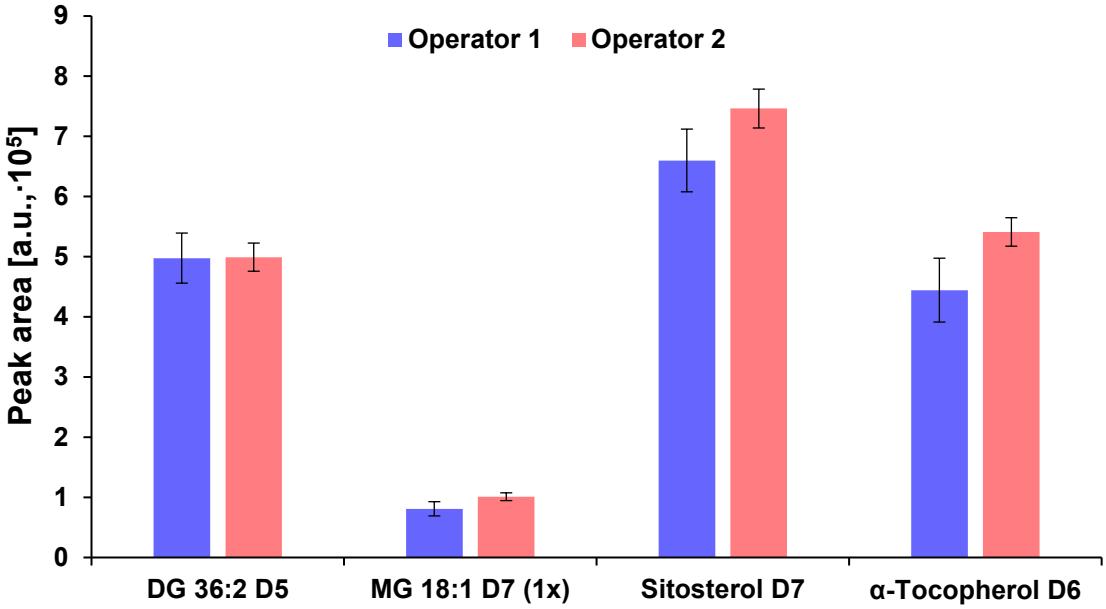
resulting in a relative standard deviation of less than 12% for all lipid classes, except for MG 1x (RSD ~ 20%). I assume that this high RSD is due to the chosen optimized parameters, which are optimal for sterols and prenols, as well as for diacylglycerols. This test also proves that this method works excellently for targeted lipid classes.




*Graph 11. Repeatability test*

### 4.5.2. Reproducibility

Two operators (I was operator 1) on the same day in the same laboratory prepared 5 samples each with the exact pooled plasma and IS Mix using the optimized derivatization method and extraction. Data showed RSD of both operators lower than 15% (the RSD of the experienced operator 2 was even less than 10%). There are some differences in area of peaks comparing both works. However, DGs, MGs 2x, and desmosterol reported almost the same values (by 0 – 3% different). The detailed comparison is in **Supplement J**.

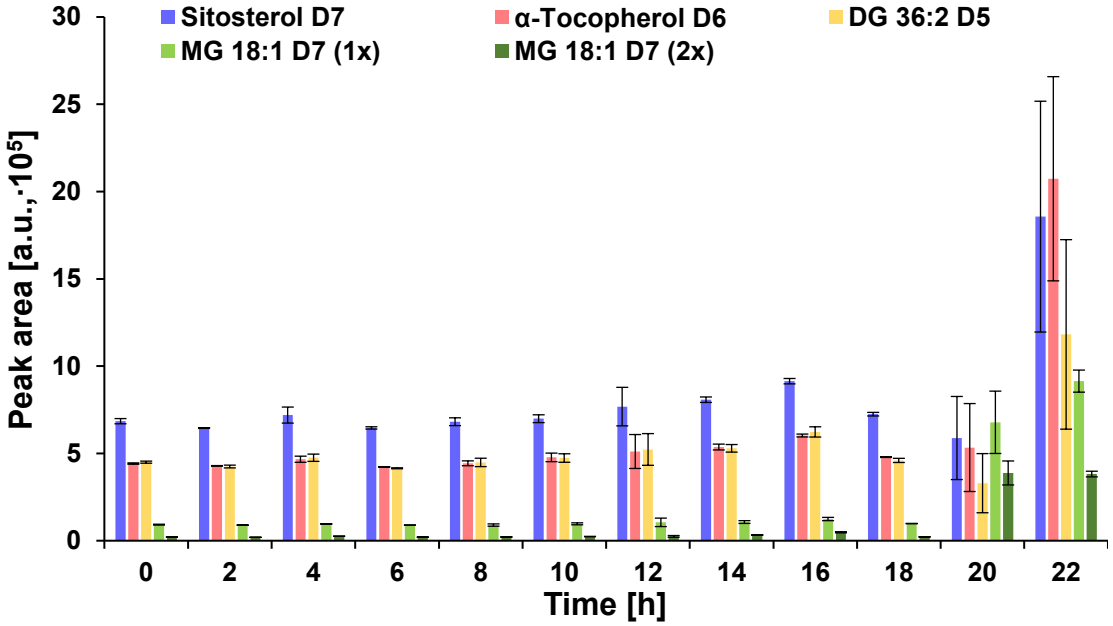



*Graph 12. Reproducibility test*

### 4.5.3. Stability

#### 4.5.3.1. Short-term stability

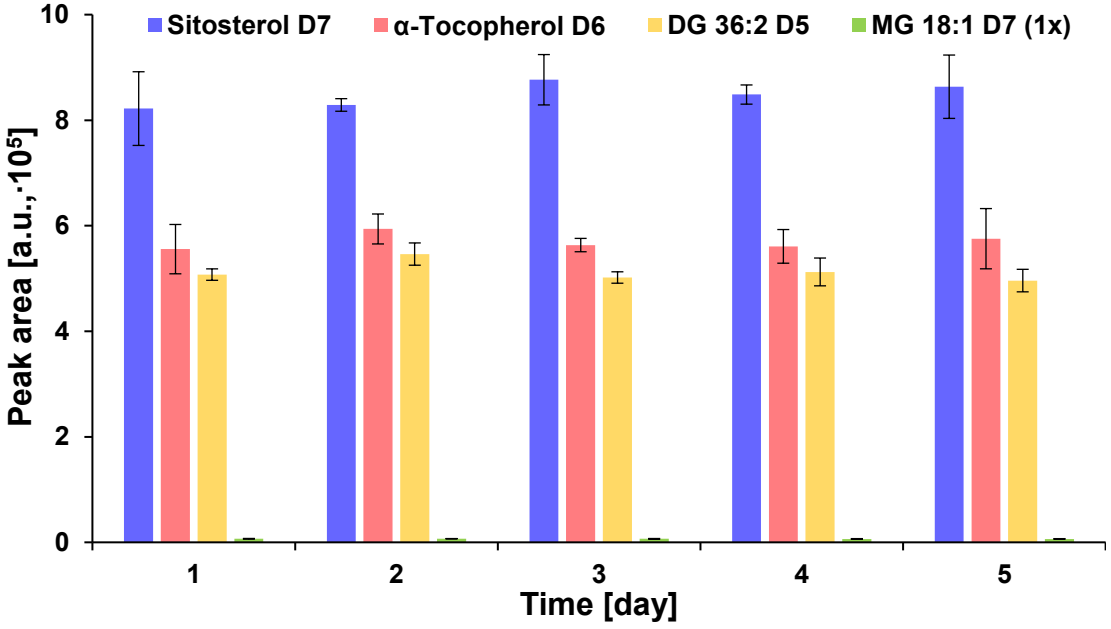
Short-term stability was performed in the autosampler (4 °C) for 24 hours. The representative sample (5 independently derivatized samples mixed in one vial) was analyzed every two hours and each analysis was measured twice. RSD < 10% for every result except for "12 hours" values (RSD < 20%). Certainly, after 18 hours the concentration of lipids and solvents in the sample was increased, and eventually, the error in the data was higher. Therefore, the prepared sample was stable within 18 hours in the autosampler. Stability decreased after 18 hours due to solvent evaporation leading to sample concentration.




*Graph 13. Stability during the day*

### 4.5.3.2. Long-term stability

Long-term stability was tested during the working days (5 days). Three out of fifteen freshly prepared and derivatized samples were analyzed immediately and other aliquots were stored at −80 °C. The next day from the freezer 3 samples were taken, which were measured shortly after they were brought up to ambient temperature. The day after that was taken another 3. The process was repeated continuously for a period of 5 days and the freeze/thaw cycles was only one. The data showed that the lipid extracts were indeed stable for 5 days.




*Graph 14. Stability during working week*

### 4.5.4. Investigation of degradation

I prepared two different STD Mix (**Supplement K** and **Supplement L**) and measured them in MRM mode using the same settings for both mixtures, where was submitted fragments for DG 18:1/18:1, MG 18:1 (for both MG 1x and MG 2x) and cholesterol. STD $Mix_2$ contained lipid classes that could during degradation disintegrate to lipid classes presented in STD $Mix_1$. After evaluating data in Microsoft Excel, the relative percentage was calculated using the equation: $\frac{STD\ Mix_1}{STD\ Mix_2} \cdot 100$ [%]. The peak of monoacylglycerol was not detected, cholesterol showed degradation possibility percentage at 0.3 %, and diacylglycerol had 17.5 % (*Table 4*), meaning that DG have small chances to disintegrate.

*Table 4. Comparison between the peak areas of STD Mix1 and STD Mix2*

| **Lipid species** | **DG 18:1/18:1** | **MG 18:1 (2x)** | **MG 18:1 (1x)** | **Cholesterol** |
|---|---|---|---|---|
| M-H | 803.513 | 723.250 | 539.260 | 569.330 |
| Fragments | 521.256 | 200.986 | 257.009 | 200.986 |
| CE | 75.420 | 62.670 | 50.060 | 59.300 |
| DP | 270.090 | 20.100 | 246.000 | 229.240 |
| **STD Mix1** | **Area** | | | |
| STD1 a | n.d. | n.d. | n.d. | 75069 |
| STD1 b | 915 | n.d. | n.d. | 195619 |
| STD1 c | 1034 | n.d. | n.d. | 81746 |
| Average | 974.500 | 0 | 0 | 117478 |

| **Lipid species** | **DG 18:1/18:1** | **MG 18:1 (2x)** | **MG 18:1 (1x)** | **Cholesterol** |
|---|---|---|---|---|
| **STD Mix2** | **Area** | | | |
| STD2 a | 5244 | 270606 | 4897 | 39254244 |
| STD2 b | 6512 | 239517 | 5381 | 32122688 |
| STD2 c | 4964 | 236628 | 5239 | 38550180 |
| Average | 5573.330 | 248917 | 5172.330 | 36642370.670 |

### 4.6. Identification of lipid classes

The pooled human plasma sample was derivatized and analyzed to identify lipid classes using the optimized method. The identification was performed based on the retention behavior and unique MRM spectra obtained in the negative ion mode. To identify lipids, $[M\text{-}H]^-$ *m/z* values were required in the precursor ion scan (PIS), followed by observation of one or two characteristic fragments (for MG and DG) in the corresponding MRM spectra. Based on lipids structural information, fragment ions can be classified into three levels: lipid class, lipid species,

and molecular species. The derivatization of functional groups (like -OH) leads to specific fragment ions that differ from those observed in the original form. The derivatization method, which was performed in this thesis work, using Cl-SBA showed the common fragment *m/z* 200.986 ($C_7H_5O_5S^-$) that was observed in every lipid species. Other class-selective fragment ions were *m/z* 257.009 for MG (1x) and specific *m/z* for all DG based on loss of fatty acids.

The retention times were predicted using the equivalent carbon number (ECN) rule, which served as an additional parameter for identifying lipid species. Prediction was based on the retention behavior of the standards. The identification was confirmed based on retention dependencies retention time on the length of the fatty acyl chain/number of carbon (**Supplement M**) and the double bond(s) number (**Supplement N**). In total, 58 derivatized lipid species from 4 lipid classes were identified (**Supplement O**). Compared to other works that required analyses in the positive ion mode, this derivatization method allowed the detection of sterols, prenols, monoacylglycerols, and diacylglycerols in the negative ion mode, which minimize ion source fragmentation (common in positive ion mode) leading to misidentification.

# 5. Conclusion

In this thesis, a novel chemical derivatization technique was introduced using 3-(chlorosulfonyl)benzoic acid for the determination of sterols, prenols, and neutral lipids, measured by RP-UHPLC/MS/MS in the negative ion mode.

During the work, the mass spectrometry (*QTRAP® 6500*) and the liquid chromatography (*Agilent 1260*) conditions were optimized based on 30 derivatized standards from 4 lipid classes (sterols, prenols, monoacylglycerols, and diacylglycerols). The derivatization reaction was thoroughly optimized using the spiked pooled human plasma (9 internal standards from 4 lipid classes), and the molar ratio 6:1 of pyridine with the derivatization agent reacting for 1 hour at 60 °C provided the best yield. The Folch extraction using pure water as water phase was used to eliminate the excess of pyridine and derivatization agent. The RP-UHPL/MS/MS method combined with the derivatization enabled the separation of isomers and isobars of sterols, such as desmosterol/cholecalciferol/7-dehydrocholesterol or cholesterol/lathosterol, which was confirmed by standards. Repeatability and reproducibility were investigated by one operator or two operators, reporting RSD lower than 15 %. The short-term stability in the autosampler (4 °C) for 18 hours and the long-term stability in the freezer at -80 ° C for one week was determined with RSD less than 10 %.

The fully optimized method was applied for the identification of sterols, prenols, monoacylglycerols, and diacylglycerols in human plasma. In order to ensure reliable lipid identification, retention time behavior and fragmentation behavior using MRM were considered. Based on these parameters, 58 lipid species from 4 lipid classes were detected.

It is important to mention that this work primarily aimed to optimize the derivatization method and instruments, while identification was the final application to assess the effectiveness of the method using 3-(chlorosulfonyl)benzoic acid. Future studies will involve the identification of additional sterols and prenols, as well as the use of the method not only for identification but also for quantitation analysis including full validation evaluating the accuracy of the method.

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**Supplement A.** Table of the list of STD Mix

| Lipid class | Lipid species | Stock conc. (µg/µL) | V (µL) | µg/mL STD-Mix | µg/mL plasma | nnmol/ mL plasma |
|---|---|---|---|---|---|---|
| DG | DG 16:0/16:0 | 2 | 8.9 | 36 | 28 | 50 |
| | DG 17:0/17:0 | 2 | 9.3 | 37 | 30 | 50 |
| | DG 18:0/18:0 | 2 | 9.8 | 39 | 31 | 50 |
| | DG 18:1/18:1 | 2 | 9.7 | 39 | 31 | 50 |
| | DG 18:2/18:2 | 2 | 9.6 | 39 | 31 | 50 |
| | DG 18:3/18:3 | 2 | 9.6 | 38 | 31 | 50 |
| | DG 19:0/19:0 | 2 | 10.2 | 41 | 33 | 50 |
| | DG 20:0/20:0 | 2 | 10.6 | 43 | 34 | 50 |
| | DG 14:0/14:0 | 2 | 8.0 | 32 | 26 | 50 |
| MG | MG 16:0 | 2 | 5.2 | 21 | 17 | 50 |
| | MG 18:0 | 2 | 5.6 | 22 | 18 | 50 |
| | MG 18:1 | 2 | 5.6 | 22 | 18 | 50 |
| | MG 18:2 | 2 | 5.5 | 22 | 18 | 50 |
| | MG 19:1 | 2 | 5.8 | 23 | 19 | 50 |
| ST | Desmosterol | 2 | 6.0 | 24 | 19 | 50 |
| | Cholesterol D7 | 1 | 12.3 | 25 | 20 | 50 |
| | Lathosterol | 1 | 12.1 | 24 | 19 | 50 |
| | Cholestanol | 1 | 12.1 | 24 | 19 | 50 |
| | Campesterol | 1 | 12.5 | 25 | 20 | 50 |
| | Stigmasterol | 1 | 12.9 | 26 | 21 | 50 |
| | Sitosterol | 1 | 13.0 | 26 | 21 | 50 |
| | Lanosterol | 1 | 13.3 | 27 | 21 | 50 |
| | Dihydrolanosterol | 1 | 13.4 | 27 | 21 | 50 |
| | Ergocalciferol | 1 | 12.4 | 25 | 20 | 50 |
| | Cholecalciferol | 1 | 12.0 | 24 | 19 | 50 |
| PR | Retinol | 2 | 4.5 | 18 | 14 | 50 |
| | α-Tocopherol | 1 | 13.5 | 27 | 22 | 50 |
| | γ-Tocopherol | 2 | 6.5 | 26 | 21 | 50 |
| | δ-Tocopherol | 2 | 6.3 | 25 | 20 | 50 |
| | α-Tocotrienol | 2 | 6.6 | 27 | 21 | 50 |
| | **$CHCl_3$/MeOH** | | **217.2** | | | |

**Supplement B.** Table of the list of IS Mix

| Lipid class | Lipid species | Stock conc. (µg/µL) | V (µL) | µg/mL IS-Mix | µg/mL plasma | nnmol/mL plasma |
|---|---|---|---|---|---|---|
| DG | DG 14:0/14:0 | 2 | 2.6 | 3 | 5 | 10 |
| | DG 15:0/18:1 D7 | 1 | 5.9 | 3 | 6 | 10 |
| | DG 18:1/18:1 D5 | 1 | 6.3 | 3 | 6 | 10 |
| MG | MG 18:1 D7 | 1 | 3.6 | 2 | 4 | 10 |
| | MG 19:1 | 0.4 | 9.3 | 2 | 4 | 10 |
| ST | Cholesterol D7 | 6 | 19.7 | 59 | 118 | 300 |
| | Desmosterol D6 | 1 | 3.9 | 2 | 4 | 10 |
| | Sitosterol D7 | 1 | 4.2 | 2 | 4 | 10 |
| PR | α-Tocopherol D6 | 2 | 2.2 | 2 | 4 | 10 |
| | **CHCl3/MeOH** | | **1942.4** | | | |

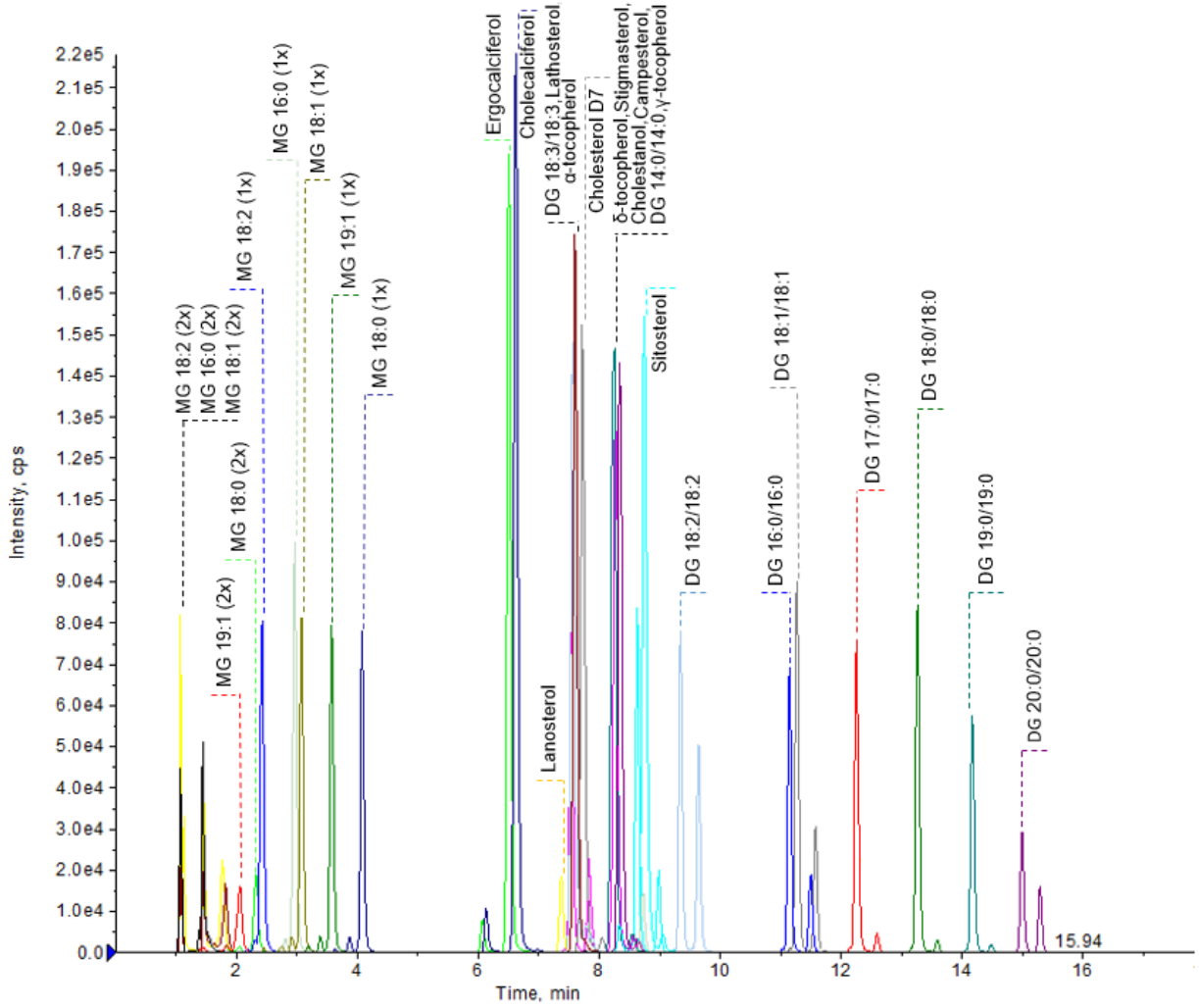
**Supplement C.** Table of final MS/MS conditions

| Lipid class | Lipid species | Fragments | CE | DP |
|---|---|---|---|---|
| DG | DG 16:0/16:0 | 200.9858 | 54.4 | 171.6 |
| | | 495.2422 | 75.42 | 270.09 |
| | DG 17:0/17:0 | 200.9858 | 54.4 | 171.6 |
| | | 509.2558 | 75.42 | 270.09 |
| | DG 18:0/18:0 | 200.9858 | 54.4 | 171.6 |
| | | 523.2729 | 75.42 | 270.09 |
| | DG 18:1/18:1 | 200.9858 | 54.4 | 171.6 |
| | | 521.2555 | 75.42 | 270.09 |
| | DG 18:2/18:2 | 200.9858 | 54.4 | 171.6 |
| | | 519.2372 | 75.42 | 270.09 |
| | DG 18:3/18:3 | 200.9858 | 54.4 | 171.6 |
| | | 517.223 | 75.42 | 270.09 |
| | DG 19:0/19:0 | 200.9858 | 54.4 | 171.6 |
| | | 537.2856 | 75.42 | 270.09 |

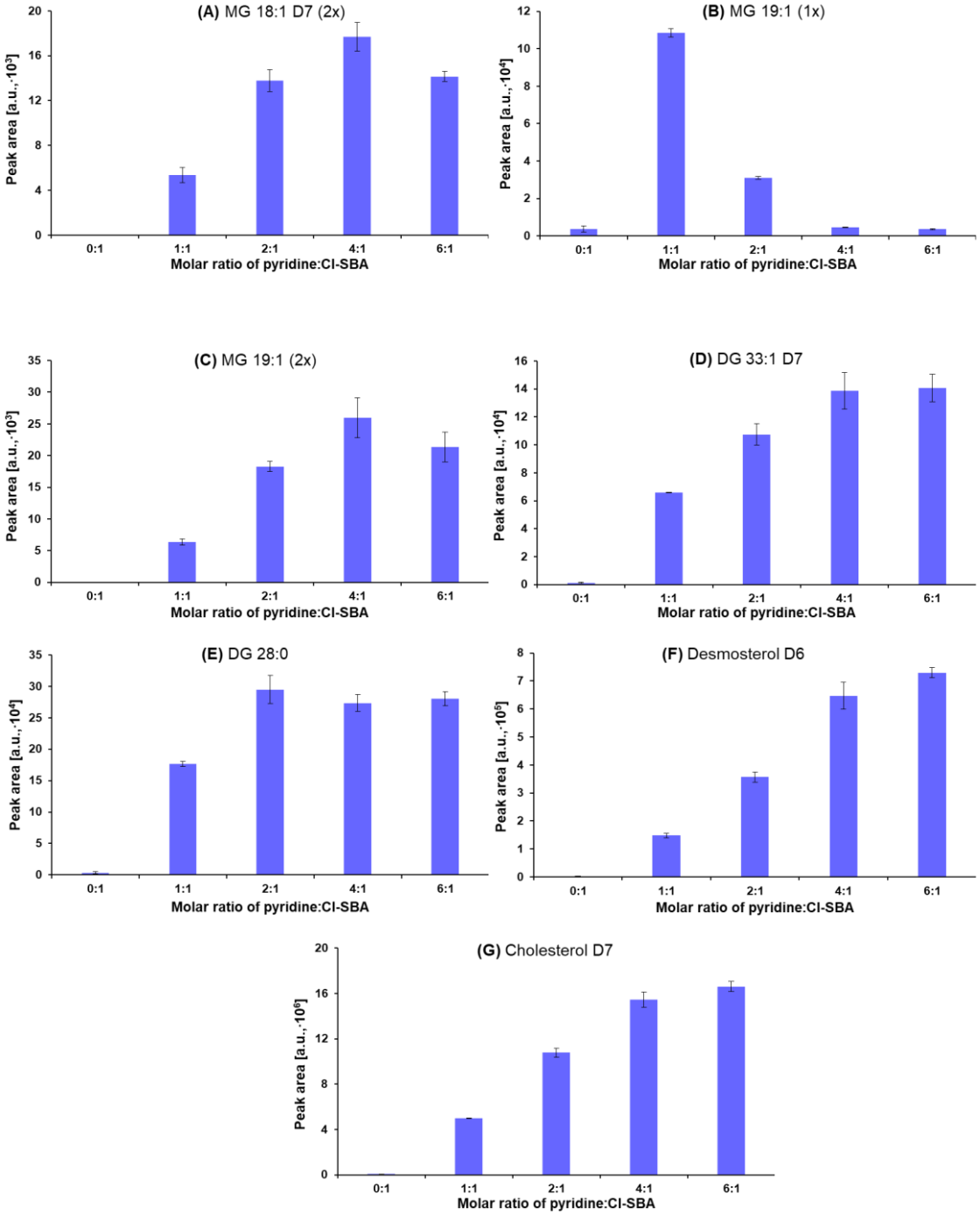
| Lipid class | Lipid species | Fragments | CE | DP |
|---|---|---|---|---|
| DG | DG 20:0/20:0 | 200.9858 | 54.4 | 171.6 |
| | | 551.3055 | 75.42 | 270.09 |
| | DG 14:0/14:0 | 200.9858 | 54.4 | 171.6 |
| | | 467.2079 | 75.42 | 270.09 |
| MG | MG 16:0 (2x) | 200.9858 | 62.67 | 20.1 |
| | MG 16:0 (1x) | 257.009 | 50.06 | 246 |
| | MG 18:0 (2x) | 200.9858 | 62.67 | 20.1 |
| | MG 18:0 (1x) | 257.009 | 50.06 | 246 |
| | MG 18:1 (2x) | 200.9858 | 62.67 | 20.1 |
| | MG 18:1 (1x) | 257.009 | 50.06 | 246 |
| | MG 18:2 (2x) | 200.9858 | 62.67 | 20.1 |
| | MG 18:2 (1x) | 257.009 | 50.06 | 246 |
| | MG 19:1 (2x) | 200.9858 | 62.67 | 20.1 |
| | MG 19:1 (1x) | 257.009 | 50.06 | 246 |
| ST | Desmosterol | 200.9858 | 59.3 | 229.24 |
| | Cholesterol D7 | 200.9858 | 59.3 | 229.24 |
| | Cholesterol | 200.9858 | 59.3 | 229.24 |
| | Lathosterol | 200.9858 | 59.3 | 229.24 |
| | Cholestanol | 200.9858 | 59.3 | 229.24 |
| | Campesterol | 200.9858 | 59.3 | 229.24 |
| | Stigmasterol | 200.9858 | 59.3 | 229.24 |
| | Sitosterol | 200.9858 | 59.3 | 229.24 |
| | Lanosterol | 200.9858 | 59.3 | 229.24 |
| | Dihydrolanosterol | 200.9858 | 59.3 | 229.24 |

| Lipid class | Lipid species | Fragments | CE | DP |
|---|---|---|---|---|
| ST | Ergocalciferol | 200.9858 | 53.02 | 233.77 |
| | Cholecalciferol | 200.9858 | 53.02 | 233.77 |
| PR | α-tocopherol | 347.0585 | 62.47 | 251.31 |
| | γ-tocopherol | 333.04285 | 56.31 | 193.02 |
| | δ-tocopherol | 319.0267 | 54.89 | 189.22 |
| | α-tocotrienol | 200.98 | 25.9 | 31.76 |

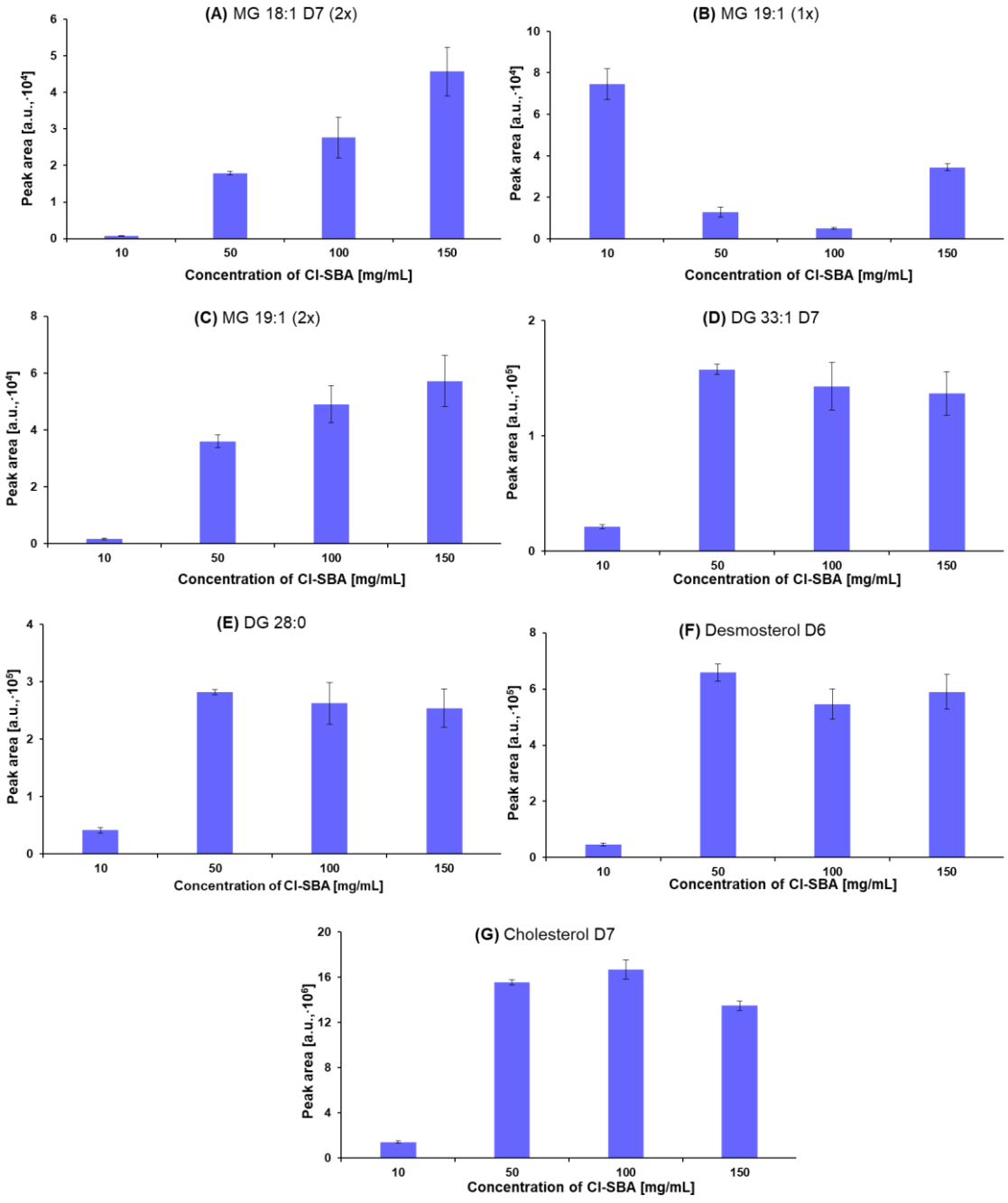
**Supplement D.** Chromatogram of STD Mix with the optimized gradient



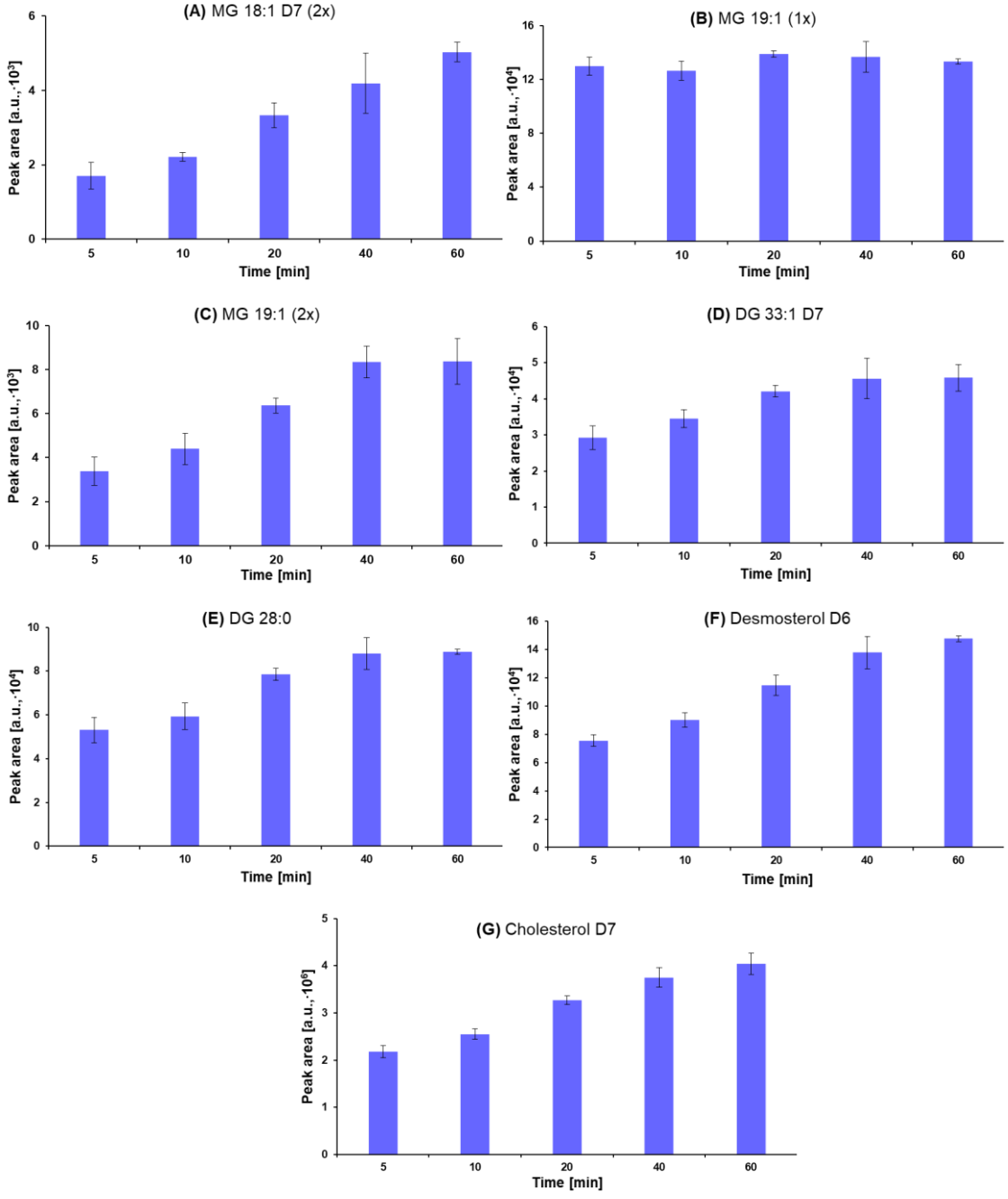
**Supplement E**. Optimalization of the molar ratio of reagents, where (**A**) MG 18:1 D7 (2x), (**B**) MG 19:1 (1x), (**C**) MG 19:1 (2x), (**D**) DG 33:1 D7, (**E**) DG 28:0, (**F**) Desmosterol D6, (**G**) Cholesterol D7



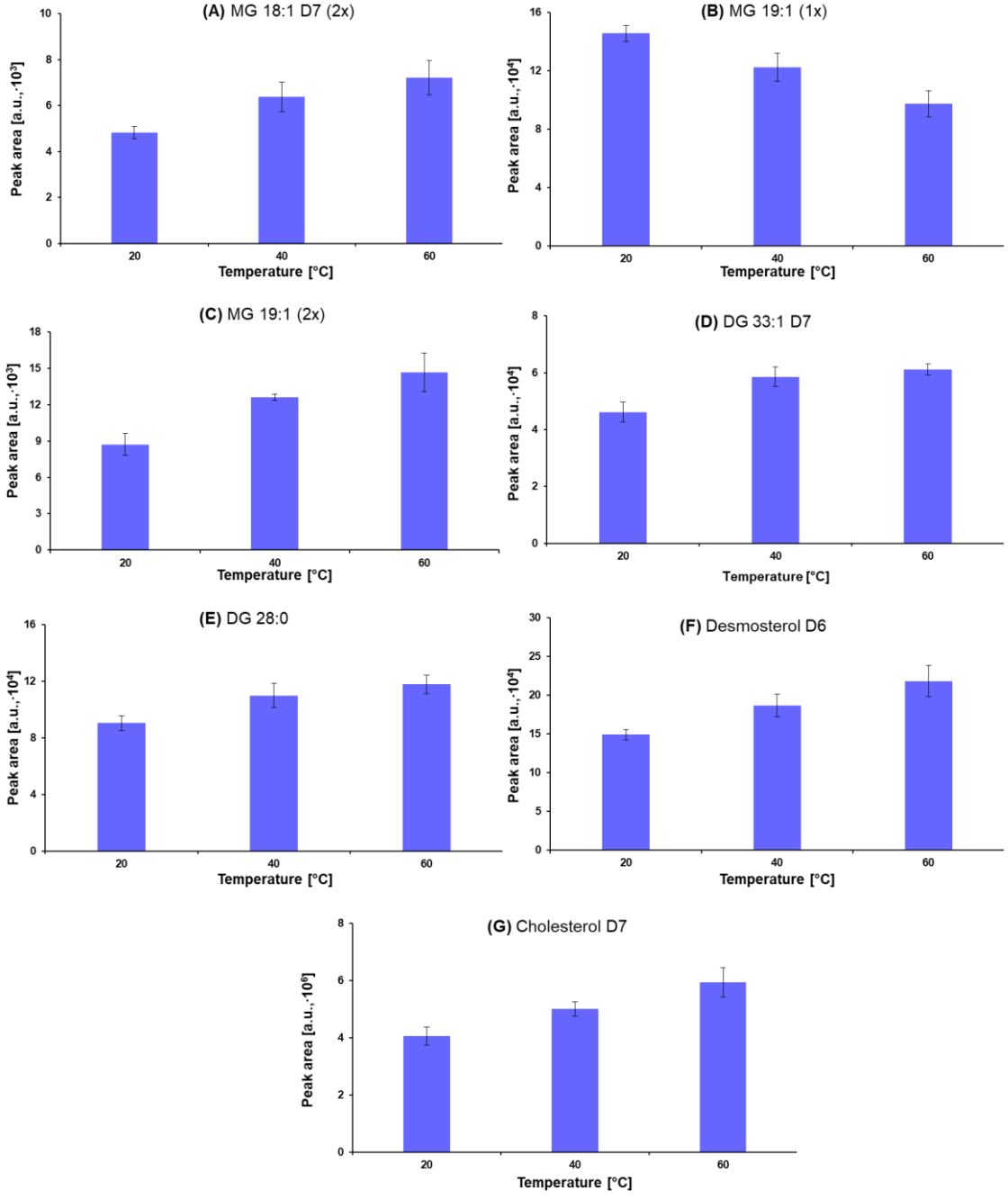
**Supplement F.** Optimalization of concentration of derivatization agent, where (**A**) MG 18:1 D7 (2x), (**B**) MG 19:1 (1x), (**C**) MG 19:1 (2x), (**D**) DG 33:1 D7, (**E**) DG 28:0, (**F**) Desmosterol D6, (**G**) Cholesterol D7



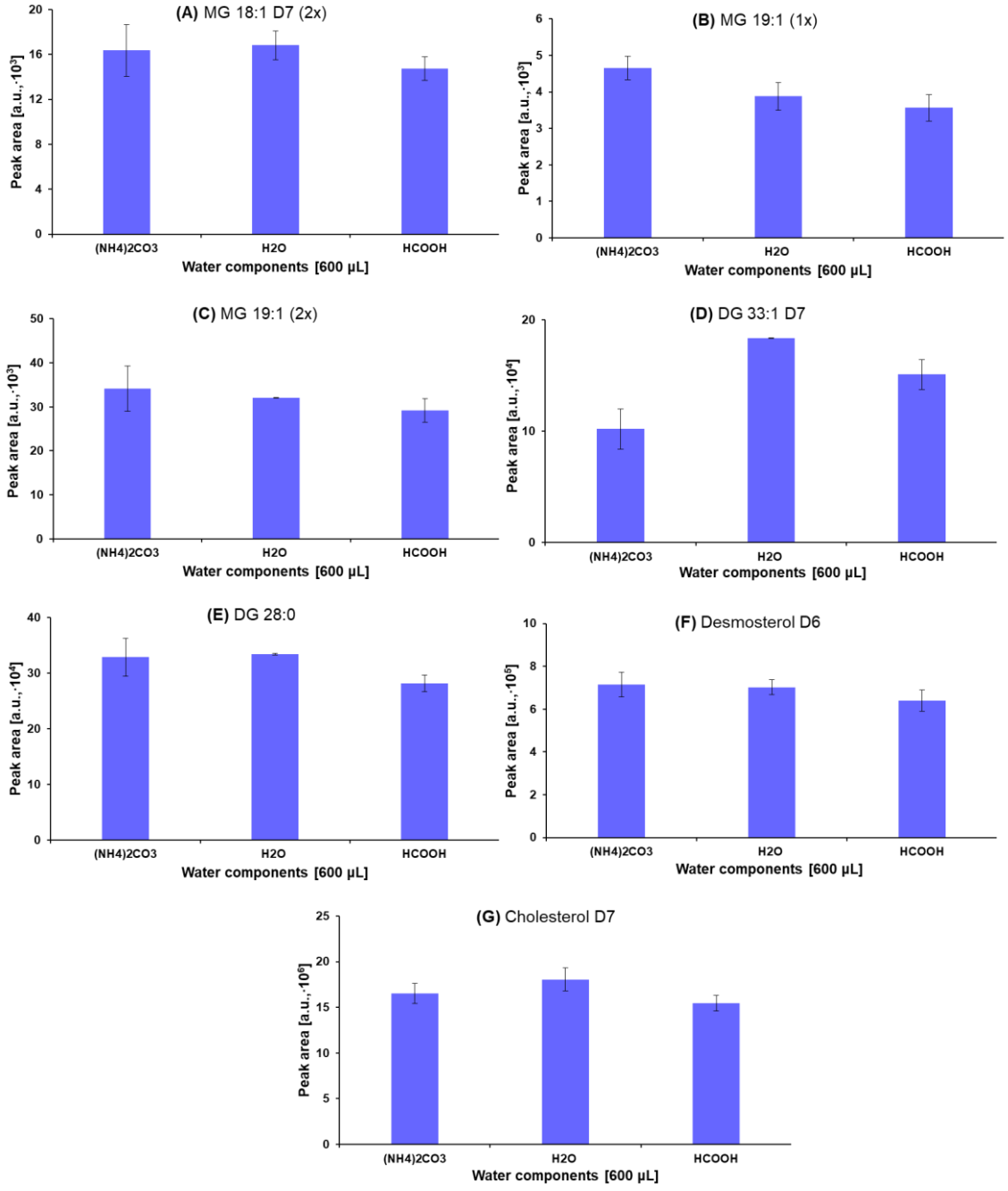
**Supplement G.** Optimalization of the reaction time, where (**A**) MG 18:1 D7 (2x), (**B**) MG 19:1 (1x), (**C**) MG 19:1 (2x), (**D**) DG 33:1 D7, (**E**) DG 28:0, (**F**) Desmosterol D6, (**G**) Cholesterol D7



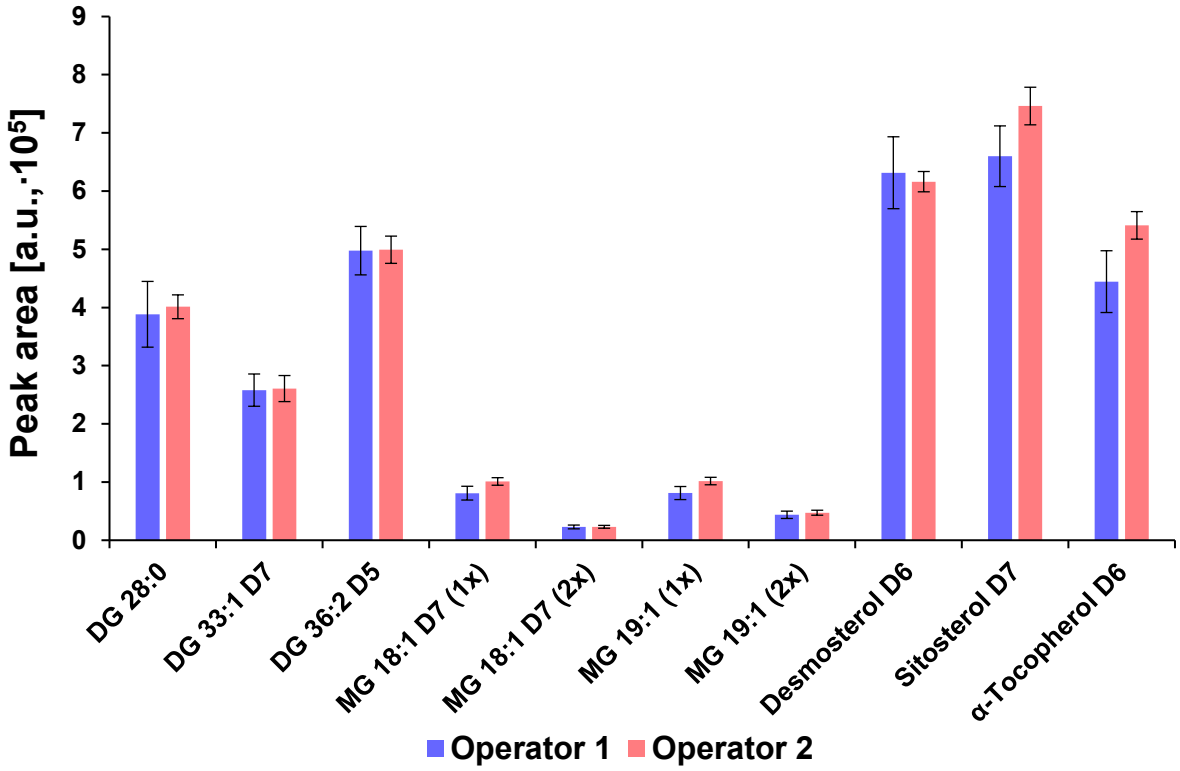
**Supplement H.** Optimalization of the reaction temperature, where (**A**) MG 18:1 D7 (2x), (**B**) MG 19:1 (1x), (**C**) MG 19:1 (2x), (**D**) DG 33:1 D7, (**E**) DG 28:0, (**F**) Desmosterol D6, (**G**) Cholesterol D7



**Supplement I.** Optimalization of the extraction, where (**A**) MG 18:1 D7 (2x), (**B**) MG 19:1 (1x), (**C**) MG 19:1 (2x), (**D**) DG 33:1 D7, (**E**) DG 28:0, (**F**) Desmosterol D6, (**G**) Cholesterol D7



**Supplement J.** Reproducibility test of all IS



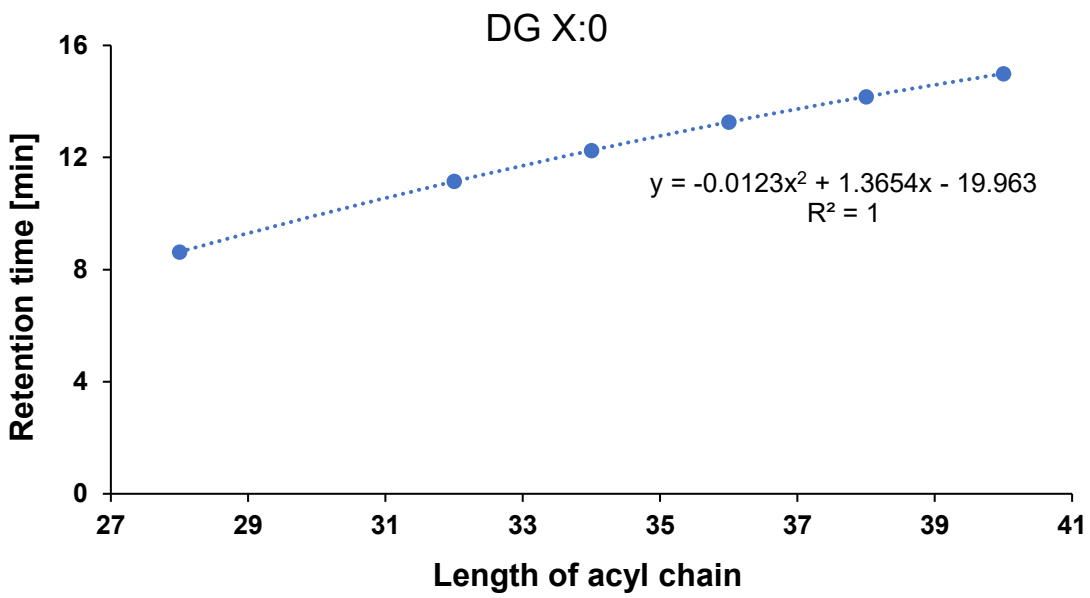

**Supplement K.** Table of STD Mix$_1$

| Lipid species | Stock conc. (µg/µL) | Volume (µL) | ug/mL IS-Mix | ug/mL plasma | nnmol/mL plasma |
|---|---|---|---|---|---|
| Cer 18:1/18:1 | 0.2 | 7.0 | 1.41 | 1.13 | 2 |
| TG 18:1/18:1/18:1 | 2 | 33.2 | 66.36 | 53.09 | 60 |
| PC 18:1/18:1 | 2 | 36.8 | 73.55 | 58.84 | 74.9 |
| LPC 18:1 | 2 | 9.8 | 19.62 | 15.69 | 30.1 |
| PE 18:1/18:1 | 2 | 2.8 | 5.58 | 4.46 | 6 |
| LPE 18:1 | 0.2 | 6.0 | 1.20 | 0.96 | 2 |
| SM 18:1;O2/18:1 | 2 | 13.7 | 27.41 | 21.93 | 30.1 |
| GlcCer 18:1;O2/16:0 | 0.25 | 7.3 | 1.84 | 1.47 | 2.1 |
| CE 18:1 | 2.5 | 65.0 | 162.57 | 130.05 | 199.9 |
| **$CHCl_3$/MeOH** | | **818.3** | | | |

**Supplement L.** Table of STD Mix$_2$

| Lipid species | Stock conc. (µg/µL) | Volume (µL) | ug/mL IS-Mix | ug/mL plasma | nnmol/mL plasma |
|---|---|---|---|---|---|
| DG 18:1/18:1 | 2 | 3.9 | 7.83 | 6.27 | 10.1 |
| MG 18:1 | 2 | 2.3 | 4.59 | 3.67 | 10.3 |
| Cholesterol | 2 | 48.3 | 96.59 | 77.27 | 200 |
| **$CHCl_3$/MeOH** | | **945.5** | | | |

**Supplement M.** Influence of the retention time on the length of acyl chain, where X is the carbon number and zero means no double bond(s)

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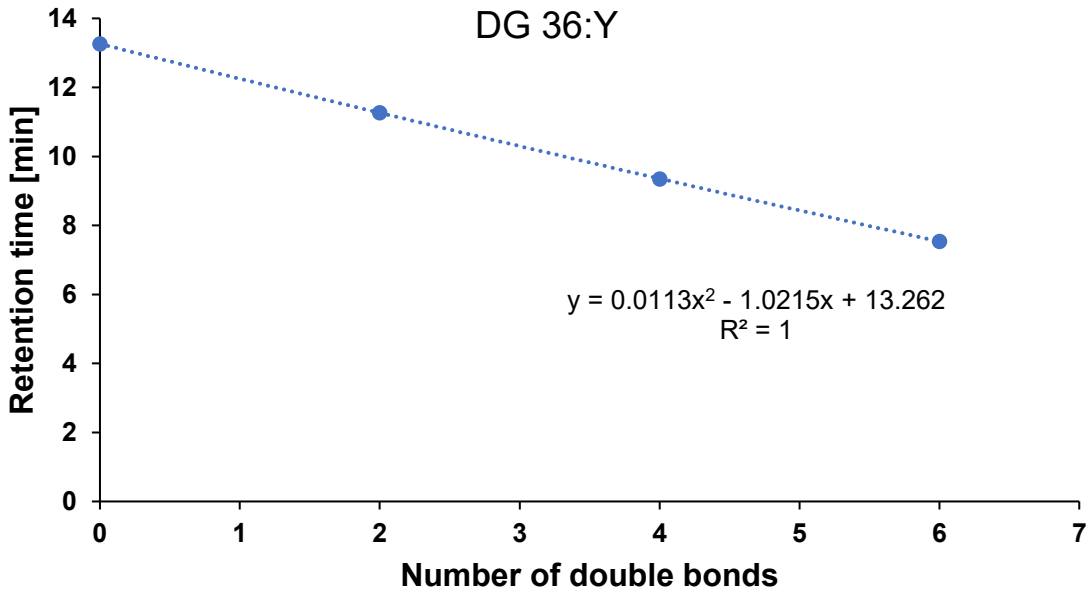
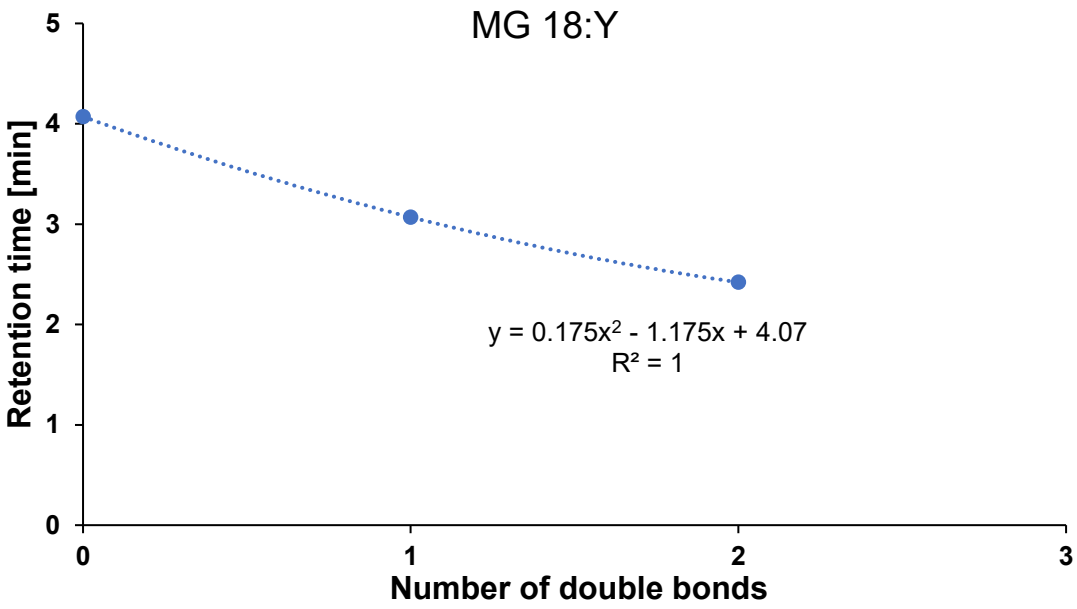
**Supplement N.** Influence of retention time on the double bond number, where Y is the number of double bond(s) and DG 36 and MG 18 is the number of carbons






**Supplement O.** Identified lipid species in pooled human plasma

| **Sterols (ST)** | | | | |
|---|---|---|---|---|
| **Lipid species** | **Level Fatty alkyl/acyl level** | **RT [min]** | **Precursor scan [M-H]⁻** | **MS/MS [M-C₇H₅O₅S]⁻** |
| Desmosterol | | 6.07 | 567.314 | 200.986 |
| Cholesterol | | 7.41 | 569.330 | 200.986 |
| Lathosterol | | 7.20 | 569.330 | 200.986 |
| Cholestanol | | 7.88 | 571.345 | 200.986 |
| Campesterol | | 7.87 | 583.346 | 200.986 |
| Stigmasterol | | 7.95 | 595.345 | 200.986 |
| Sitosterol | | 8.34 | 597.361 | 200.986 |
| Lanosterol | | 6.82 | 609.361 | 200.986 |
| Cholecalciferol | | 6.40 | 567.314 | 200.986 |
| 7-dehydrocholesterol | | 6.70 | 567.314 | 200.986 |
| **Prenols (PR)** | | | | |
| **Lipid species** | **Level Fatty alkyl/acyl level** | **RT [min]** | **Precursor scan [M-H]⁻** | **MS/MS [M-C₁₉H₄₀-H]⁻** |
| α-tocopherol | | 7.25 | 613.356 | 347.059 |
| **Monoacylglycerols (MG)** | | | | |
| **Lipid species** | **Level Fatty alkyl/acyl level** | **RT [min]** | **Precursor scan [M-H]⁻** | **MS/MS [M-RᵢCOOH-H]⁻** |
| MG 16:0 | | 2.83 | 513.756 | 257.009 |
| MG 18:2 | | 2.34 | 537.756 | 257.009 |
| MG 18:1 | | 2.94 | 539.772 | 257.009 |
| MG 18:0 | | 3.88 | 541.787 | 257.009 |

| Diacylglycerols (DG) | | | | |
|---|---|---|---|---|
| **Lipid species** | **Level Fatty alkyl/acyl level** | **RT [min]** | **Precursor scan $[M-H]^-$** | **MS/MS $[M-R_iCOOH-H]^-$** |
| DG 30:1 | DG 16:1_14:0 | 8.66 | 721.435 | 493.242/467.208 |
| DG 30:0 | DG 16:0_14:0 | 9.85 | 723.451 | 495.242/467.208 |
| DG 31:0 | DG 16:0_15:0 | 10.44 | 737.466 | 495.242/481.242 |
| DG 32:2 | DG 18:2_14:0 | 8.92 | 747.451 | 519.237/467.208 |
| DG 32:1 | DG 16:0_16:1 | 9.93 | 749.466 | 495.242/493.242 |
| | DG 18:1_14:0 | | | 521.256/467.208 |
| DG 32:0 | DG 16:0_16:0 | 11.02 | 751.482 | 495.242 |
| | DG 18:0_14:0 | | | 523.273/467.208 |
| DG 33:1 | DG 17:1_16:0 | 10.51 | 763.482 | 507.242/495.242 |
| | DG 18:1_15:0 | | | 521.256/481.242 |
| DG 34:3 | DG 18:3_16:0 | 9.26 | 773.466 | 517.223/495.242 |
| | DG 18:2_16:1 | | | 519.237/493.242 |
| DG 34:2 | DG 18:2_16:0 | 10.12 | 775.482 | 519.237/495.242 |
| | DG 18:1_16:1 | | | 521.256/493.242 |
| DG 34:1 | DG 18:1_16:0 | 11.03 | 777.497 | 521.256/495.242 |
| DG 34:0 | DG 17:0_17:0 | 11.73 | 779.513 | 509.256 |
| | DG 18:0_16:0 | | | 523.273/495.242 |
| DG 36:6 | DG 14:1_22:5 | 7.39 | 795.466 | 465.208/569.242 |
| DG 36:5 | DG 16:1_20:4 | 8.69 | 797.466 | 493.242/543.244 |
| | DG 18:2_18:3 | | | 519.237/517.223 |
| | DG 16:0_20:5 | | | 495.242/541.244 |
| DG 36:4 | DG 18:2_18:2 | 9.26 | 799.482 | 519.237 |
| | DG 16:1_20:3 | | | 493.242/537.244 |
| | DG 18:1_18:3 | | | 521.255/517.223 |
| DG 36:3 | DG 18:1_18:2 | 10.16 | 801.497 | 521.256/519.237 |
| | DG 18:0_18:3 | | | 523.273/517.223 |
| DG 36:2 | DG 18:1_18:1 | 11.14 | 803.513 | 521.256 |
| | DG 16:0_20:2 | | | 495.242/535.242 |
| | DG 16:1_20:1 | | | 493.242/533.242 |
| DG 36:1 | DG 16:0_20:0 | 12.05 | 805.529 | 495.242/551.242 |
| | DG 18:0_18:1 | | | 523.273/521.256 |
| DG 36:0 | DG 18:0_18:0 | 12.96 | 807.544 | 523.2729 |
| | DG 16:0_20:0 | | | 495.242/551.242 |
| DG 38:6 | DG 18:1_20:5 | 8.75 | 823.482 | 521.256/541.244 |
| | DG 18:2_20:4 | | | 519.237/543.244 |
| DG 38:5 | DG 18:1_20:4 | 9.55 | 825.497 | 521.256/543.244 |
| DG 38:4 | DG 16:0_22:4 | 10.66 | 827.513 | 495.242/567.242 |
| | DG 18:1_20:3 | | | 521.256/537.244 |
| DG 38:3 | DG 18:1_20:2 | 11.19 | 829.529 | 521.256/535.242 |
| | DG 18:2_20:1 | | | 519.237/533.242 |
| | DG 18:0_20:3 | | | 523.273/537.244 |

| Lipid species | Level Fatty alkyl/acyl level | RT [min] | Precursor scan $[M-H]^-$ | MS/MS $[M-R_iCOOH-H]^-$ |
|---|---|---|---|---|
| DG 38:2 | DG 18:1_20:1 | 12.02 | 831.544 | 521.256/533.242 |
| DG 38:1 | DG 18:1_20:0 | 12.98 | 833.560 | 521.256/551.242 |
| | DG 18:0_20:1 | | | 523.273/533.242 |